INTRODUCTION

Hyperandrogenism is a key diagnostic feature of polycystic ovary syndrome (PCOS) and is a heritable PCOS trait. Sisters of women with PCOS have increased androgen levels suggesting that hyperandrogenism may be partly genetically determined (1). Furthermore, experimental animal research and clinical observations have led to the developmental origin hypothesis of PCOS, whereby exposure of the female fetus to androgen excess may program in utero the development of the PCOS phenotype in adult life (2, 3).

The potential origin of androgen excess during intrauterine life in humans, to account for fetal programming of PCOS, is not known. Normally, the female fetus is protected in utero from maternal androgens, or androgens from other source, by the increased placental sex hormone-binding globulin (SHBG) that binds the androgens and the aromatase activity that converts them to estrogens. However, this buffering effect may be overcome if either the production of SHBG or the aromatase activity is lower than normal due to genetic or other influences.

Based on these observations, polymorphisms of SHBG and aromatase cytochrome P450, family 19 (CYP19) genes may have a synergistic role in the development of PCOS.

With regard to SHBG, we have previously shown a significant association between a functional (TAAAA)n repeat polymorphism of the SHBG gene with serum SHBG levels and PCOS phenotype, providing evidence for a possible genetic contribution for prenatal androgen excess (4). Similar findings were reported in other populations of women with hyperandrogenism (5, 6) and in a more recent study in young men (7).

On the other hand, regarding the CYP19 gene, women with congenital aromatase deficiency, caused by loss-of-function mutations of CYP19 gene, develop the features of PCOS phenotype (8, 9). Although earlier linkage and association studies failed to find an association between CYP19 and PCOS, probably due to low statistical power (10–12), a recent association study showed a strong association between a common
genetic variant (the single nucleotide polymorphism (SNP)50) in CYP19 and androgen excess in girls with precocious puberty and functional hyperandrogenism (13). Furthermore, a recent study indicated that a short microsatellite (TTTA)n repeat allele in the fourth intron of the CYP19 gene is associated with elevated androgens, perturbed regulation of the hypothalamic–pituitary–adrenal axis, and abdominal obesity among premenopausal women from the general population (14).

In the light of these observations, we hypothesized that the SHBG and the CYP19 genes may interact in providing the androgenic stimulus for the developmental programming of PCOS. For this purpose, we examined the distribution of combined SHBG(TAAAA)n and CYP19(TTTA)n repeat variants between patients and controls and their association with the hyperandrogenic phenotype of PCOS.

Subjects and methods

Subjects

The study population consisted of 180 cases of PCOS and 160 age-matched healthy controls. The diagnosis of PCOS was based on the criteria proposed by the 1990 National Institutes of Health-National Institute of Child Health and Human Development conference on PCOS. These criteria are ovulatory dysfunction, clinical evidence of hyperandrogenism, and/or hyperandrogenemia and exclusion of related disorders such as congenital adrenal hyperplasia, hyperprolactinemia, or Cushing’s syndrome (15). Hyperandrogenism was defined by the clinical presence of hirsutism (Ferriman–Gallwey score >8) acne or alopecia and/or elevated androgen levels. Menstrual dysfunction was defined by the presence of oligomenorrhea or amenorrhea. In those patients who were on medication, treatment was discontinued at least 6 months prior to their inclusion in the study. The control group consisted of women with normal menstrual cycles (28–30 days) and no signs of hyperandrogenism.

All patients were studied on days 3–5 of either a normal or progesterone-induced withdrawal period. The body mass index (BMI) of each patient, calculated as weight (kg)/height2 (m), was recorded. Blood samples were drawn after overnight fasting for the measurement of fasting serum glucose and insulin, serum gonadotropins (luteinizing hormone, LH, follicle-stimulating hormone, FSH), total testosterone, estradiol, SHBG, and DHEAS. The free androgen index (FAI) was calculated using the formula: (total testosterone (nmol/l)/SHBG (nmol/l)) × 100 (16). Insulin resistance was assessed by the fasting glucose-to-insulin ratio (17). From both patients and controls, whole blood samples were used for isolation of peripheral blood leucocytes for the genetic analysis. The study protocol was approved by the Hospital Ethics Committee, and all subjects studied gave their informed consent.

Hormonal assays

Serum glucose was determined by the hexokinase method using a analyzer (Olympus 600, Clinical Chemistry Analyser, Olympus Diagnostica GmbH, Co Clare, Ireland). The coefficient of variation (CV) of this method was <3%. Insulin was measured by microparticle enzyme immunoassay on an AXXSYM Immunoanalyser (Abbott Laboratory). The CV of this method was 5%. Total testosterone and serum gonadotrophins (LH, FSH) were determined by chemiluminescent microparticle immunoassay on an Abbott-ARCHITECT Immunoanalyser (Abbott Laboratory). The CV values were 4% for total testosterone, 3.5% for LH, and 4% for FSH. DHEAS and SHBG were measured by chemiluminescent immunometric method (IMMULITE 2000 Immunoanalyser, DPC, Los Angeles, CA, USA), and the CV values were 9 and 5.5% respectively.

Genotype analysis

Genomic DNA was isolated from peripheral blood leucocytes of women with PCOS and the controls. Amplification of the SHBG(TAAAA)n and CYP19(TTTA)n repeat region was accomplished using PCR with a forward primer (5′-GCTTGAACCTGAGGCG-3′) and a reverse primer (5′-CAACTCGACCCTTCTTTATG-3′) and a reverse primer (5′-GTTTGACTCCGTGAGTTTGA-3′) for the SHBG(TAAAA)n polymorphism and a forward primer (5′-CAACTCGACCCTTCTTTATG-3′) for the CYP19(TTTA)n polymorphism. Amplified products were separated by 12% PAGE followed by silver staining and the number of repeats of each allele was determined. The number of TAAAA and TTTA repeats in every particular allele was analyzed by sequencing the appropriate PCR products. A quality control assessment of our PCR method was done by random sampling and sequencing of the PCR products and duplication of the PCR assays.

Statistical analysis

Statistical analysis of differences in genotype frequencies between PCOS and controls was performed using the χ2 test. Normal distribution of continuous parameters was tested by Kolmogorov–Smirnov test. Differences in continuous parameters between genotypes were assessed with ANOVA test and confirmed with the non-parametric Kruskal–Wallis test. Stepwise multiple regression analysis was performed to identify independent predictors of androgen levels. P < 0.05 was considered statistically significant. All results are reported as mean ± S.D. All analyses used the SPSS statistical package (version 14.0, SPSS Inc., Chicago, IL, USA).
Results

Distribution of SHBG(TAAAA)n and CYP19(TTTA)n alleles

The characteristics of women with PCOS and the control group are presented in Table 1. As expected, PCOS women had higher mean BMI and insulin resistance indexes as well as lower SHBG and higher androgen levels compared with control women.

In both patients and controls, 6 SHBG(TAAAA)n alleles with 6–11 repeats and 6 CYP19(TTTA)n alleles with 7–12 repeats were identified. Comparing the frequencies of SHBG alleles between the two groups, long (TAAAA)n alleles (>8 repeats) were more frequent in PCOS women than in controls, while shorter SHBG alleles (<8 repeats) were more frequent among control women (P=0.001), as reported previously (4).

With regard to CYP19(TTTA)n alleles, women with PCOS tended to have more frequently short CYP19 alleles (≤9 repeats) than controls, although the difference did not reach statistical significance (33.1 vs 29.5%).

The study population was further subdivided into subgroups according to different combinations of the two gene polymorphic variants, using as cut-offs the (TAAAA)8 allele of the SHBG gene and the (TTTA)9 allele of the CYP19 gene respectively. According to this categorization, four subgroups were identified: women with short SHBG alleles (≤8 TAAAA repeats) and short CYP19 alleles (≤9 TTTA repeats), women with short SHBG–long CYP19 alleles, women with long SHBG–short CYP19 alleles, and women with long SHBG and CYP19 alleles. We found that in both PCOS women and controls, the combination of long SHBG–short CYP19 alleles was at high prevalence, compared with other genotype combinations. However, this combined genotype tended to be more frequent among PCOS women than healthy (57.3 vs 42.4%, P=0.07, Fig. 1). This difference in grouped genotype distribution was also observed when we compared lean PCOS women with lean controls (BMI < 25 kg/m², P=0.06) and overweight–obese PCOS women with overweight–obese controls (BMI ≥ 25 kg/m², P=0.09). Subjects heterozygous for short and long alleles for both genes were not included in this analysis.

Association of combined SHBG–CYP19 polymorphisms with phenotypic variables in women with PCOS

Among the four genotype subgroups, patients with the combination of long SHBG–short CYP19 alleles (subgroup 3) had higher levels of total testosterone (P=0.02), FAI (P=0.006), DHEAS (P=0.04), and tended to have lower serum SHBG levels (P=0.06), compared with other genotype subgroups, while no difference was found in BMI and insulin resistance indexes between the different patient subgroups. Subjects heterozygous for short and long alleles for both genes were not included in this analysis.

When patients with long SHBG–short CYP19 alleles were compared with the total population of PCOS women (including heterozygotes for short and long alleles), it was found that this subgroup had the lowest serum SHBG levels (P=0.02) and the highest levels of total testosterone (P=0.008), FAI (P=0.002), DHEAS (P=0.02), and testosterone/estradiol ratio (P=0.03, Table 2). This association between genotype and androgen levels was independent of age, BMI, and insulin resistance indexes. Similarly, among the healthy women, the subgroup with long SHBG–short CYP19 alleles tended to have a higher androgen profile compared with other subgroups, although these differences were not statistically significant (results not shown).

Discussion

In the present study, we investigated whether the SHBG and CYP19 genes may exert a synergetic effect in the development of PCOS. In particular, we examined the combined effect of two functional polymorphisms, the SHBG(TAAAA)n and the CYP19(TTTA)n repeat polymorphisms, on the phenotypic expression of PCOS.

By subdividing the study population into subgroups according to the number of repeats of both SHBG and CYP19 polymorphisms, we found that the combination of long SHBG(TAAAA)n–short CYP19(TTTA)n alleles was at high prevalence in both PCOS women and controls, compared with other genotype combinations. However, this genotype combination was more frequent among PCOS women compared with control women, a difference that was close to being significant. More importantly, PCOS women who were carriers of the above genotype combination had a higher androgenic profile compared with patients with other genotype combinations.

Previous studies were consistent in showing that long SHBG(TAAAA)n alleles are associated with low SHBG and increased androgens in women with PCOS.
or hyperandrogenic women (4–6). On the other hand, the CYP19 (TTTA)n polymorphism has been shown to influence aromatase activity. In particular, lower aromatase activity was reported in fibroblasts obtained from subjects homozygous for the low (TTTA)n genotype of CYP19 than in those from subjects homozygous for a high TTTA repeat genotype (18, 19). In this context, a recent study reported an association between short CYP19 (TTTA)n repeat alleles with increased androgens and abdominal obesity among premenopausal women in the general population (14).

The present study is the first to show an interactive effect of SHBG and CYP19 gene polymorphisms on the phenotype of PCOS. In particular, the co-existence of long SHBG(TAAAA)n–short CYP19(TTTA)n alleles appears to contribute in a synergistic manner to the hyperandrogenism of PCOS, which is the fundamental manifestation of the syndrome.

The findings of this study have two potential implications. First, regarding the CYP19 gene, the findings revive an old hypothesis that PCOS results from partial aromatase deficiency (20). Based on the earlier reports for an increased ratio of androstenedione to estradiol in the follicular fluid (21), Gabrilove suggested, in a classification of virilizing and feminizing syndromes, that PCOS be categorized as resulting from decreased conversion of androgens to estrogens (22). This concept was also hinted at later on by Grumbach & Auchus (23) and Bulun (24) but was dismissed by others (25), mainly because of failure to find linkage of PCOS with the CYP19 gene (11). The present study offers a possible explanation of this hypothesis.

Secondly, perhaps the more important but related implication is that the combined long SHBG–short CYP19 genotype may contribute to the developmental programming of PCOS. Since increased SHBG and aromatase activity constitute the main fetoplacental barrier protecting the female fetus from maternal androgens, the presence of this particular genotype may confer a less efficient barrier function, allowing the exposure of the developing fetus to androgen excess. This in turn may exert a ‘programming’ effect for the development of PCOS phenotype in adult life. Interestingly, in this regard, the ovaries of aromatase knockout mice exhibit an increased interstitium with the presence of theca cells that morphologically resemble Leydig cells (26).

Supporting evidence for this hypothesis comes from the findings in our study that PCOS women with the

![Figure 1](https://example.com/fig1.png)

**Figure 1** Distribution of grouped SHBG(TAAAA)n and CYP19(TTTA)n alleles in PCOS women compared with controls. Short SHBG alleles: ≤ 8 TAAAA repeats, long SHBG alleles: > 8 TAAAA repeats, short CYP19 alleles: ≤ 9 TTTA repeats, long CYP19 alleles: > TTTA repeats. Heterozygotes for short and long alleles were not included in the analysis.

<table>
<thead>
<tr>
<th>Patients with long SHBG and short CYP19 alleles</th>
<th>Other patient subgroups</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>43</td>
<td>137</td>
</tr>
<tr>
<td>Age (years)</td>
<td>21.0±5.2</td>
<td>22.4±6.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.6±5.8</td>
<td>28.1±7.9</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.3±0.8</td>
<td>1.4±1.1</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>31.5±20.4</td>
<td>39.5±27.6</td>
</tr>
<tr>
<td>FAI</td>
<td>16.5±10.5</td>
<td>12.5±12.1</td>
</tr>
<tr>
<td>Total testosterone (ng/ml)</td>
<td>1.1±0.4</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>DHEAS (ng/ml)</td>
<td>3292.8±1279.2</td>
<td>2691.3±1077.5</td>
</tr>
<tr>
<td>Total testosterone (ng/ml)/E₂</td>
<td>0.024±0.002</td>
<td>0.016±0.015</td>
</tr>
<tr>
<td>Fasting glucose/insulin</td>
<td>10.1±10</td>
<td>10.4±8.0</td>
</tr>
</tbody>
</table>

NS, non statistical significant; values presented as means ± s.d.

Table 2 Differences in biochemical parameters between patients with long sex hormone-binding globulin (SHBG) alleles (> 8 TAAAA repeats) and short CYP19 alleles (≤ 9 TTTA repeats) and patients with other genotype subgroups.
combined long \textit{SHBG}–short \textit{CYP19} genotype exhibit not only increased \textit{FAL} and testosterone/estradiol ratio, as would be expected from the low \textit{SHBG} levels and reduced aromatase activity respectively, but also increased total testosterone and DHEAS levels. These findings indicate the presence of functional ovarian and adrenal hyperandrogenism that was probably programmed in utero by androgen excess. In conclusion, the findings of the present study support the hypothesis that \textit{SHBG} and \textit{CYP19} gene variants play a synergistic role in the hyperandrogenic phenotype of PCOS, by affecting androgen bioavailability and aromatization respectively. Whether this combined genotype may contribute to the development of PCOS, by affecting androgen bioavailability and aromatization respectively, but also increased total testosterone and DHEAS levels. These findings indicate the presence of functional ovarian and adrenal hyperandrogenism that was probably programmed in utero by androgen excess. In conclusion, the findings of the present study support the hypothesis that \textit{SHBG} and \textit{CYP19} gene variants play a synergistic role in the hyperandrogenic phenotype of PCOS, by affecting androgen bioavailability and aromatization respectively. Whether this combined genotype may contribute to the development of PCOS, by affecting androgen bioavailability and aromatization respectively, but also increased total testosterone and DHEAS levels. These findings indicate the presence of functional ovarian and adrenal hyperandrogenism that was probably programmed in utero by androgen excess. In conclusion, the findings of the present study support the hypothesis that \textit{SHBG} and \textit{CYP19} gene variants play a synergistic role in the hyperandrogenic phenotype of PCOS, by affecting androgen bioavailability and aromatization respectively.

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