The role of sex hormone-binding globulin and androgen receptor gene variants in the development of polycystic ovary syndrome

N. Xita¹, I. Georgiou², L. Lazaros², V. Psofaki³, G Kolios³ and A. Tsatsoulis¹,⁴

¹Department of Endocrinology, University of Ioannina, Ioannina 45110, Greece; ²Laboratory of Human Reproductive Genetics, University of Ioannina, Ioannina 45110, Greece; ³Laboratory of Biochemistry, University of Ioannina, Ioannina 45110, Greece
⁴Correspondence address. Tel: +3026510-99625; Fax: +3026510-46617; E-mail: atsatsou@uoi.gr

BACKGROUND: Polycystic ovary syndrome (PCOS) may be programmed in utero by androgen excess. Our aim was to examine the role of the sex hormone-binding globulin (SHBG) and androgen receptor (AR) gene polymorphisms, in the phenotypic expression of PCOS. METHODS: A cohort of 180 women with PCOS and 168 healthy women of reproductive age were investigated. BMI was recorded and the hormonal profile was determined on Day 3–5 of menstrual cycle. DNA was extracted from peripheral blood leucocytes and the SHBG(TAAA)n and AR(CAG)n polymorphisms were genotyped by PCR. RESULTS: Genotype analysis revealed six SHBG(TAAA)n alleles with 6–11 repeats and 19 AR(CAG)n alleles with 6–32 repeats, present in both PCOS and control women. Long SHBG(TAAA)n alleles (>8 repeats) were at greater frequency in PCOS than normal women (P = 0.001), whereas short AR(CAG)n alleles (≤20 repeats) tended to be more frequent in PCOS women than controls. When categorized into subgroups, PCOS women also tended to have at greater frequency the combination of long SHBG–short AR alleles (8.3%) than normal women (6.5%). Furthermore, PCOS women with combined long SHBG–short AR alleles had significantly lower serum SHBG levels (P = 0.001) and higher serum androgens (P = 0.03) compared with those with other genotype combinations. This difference was independent of BMI or insulin resistance. CONCLUSIONS: The presence of long SHBG(TAAA)n alleles is associated with increased risk for PCOS and in combination with short AR(CAG)n alleles may influence the hyperandrogenic phenotype of PCOS. This combined genotype may contribute to ‘fetal programming’ of PCOS.

Keywords: sex hormone-binding globulin; androgen receptor gene; polycystic ovary syndrome; fetal programming; polymorphism

Introduction

Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism, chronic anovulation and/or polycystic ovaries, and is frequently associated with central adiposity and hyperinsulinemia, which further exaggerate the hyperandrogenic state (Ehrmann, 2005).

The pathogenesis of PCOS remains elusive, but there are indications for a strong genetic basis (Xita et al., 2002). In addition, increasing evidence based on experimental animal research supports the developmental origin hypothesis of PCOS, whereby fetal exposure to androgen excess may programme in utero the development of PCOS in adult life (Abbott et al., 2002, 2005; Xita and Tsatsoulis, 2006).

The potential origin of excess androgens during intrauterine life to account for the fetal programming of PCOS in humans is not fully understood. However, recent genetic association studies have indicated that a number of genes may be implicated (Xita et al., 2002). These are genes involved in the availability of androgens to target tissues or genes determining androgenic activity at the molecular level. In this regard, the (TAAA)n polymorphism of the sex hormone-binding globulin (SHBG) (SHBG) gene has been associated with low SHBG levels and increased risk for PCOS or hyperandrogenism (Xita et al., 2003, Cousin et al., 2004, Ferk et al., 2007).

Furthermore, the effect of androgens at the molecular level is mediated through activation of the androgen receptor (AR). A CAG repeat polymorphism in exon 1 of the AR gene on chromosome X was shown to influence the transactivation function of the receptor (Chamberlain et al., 1994). In vitro studies showed an inverse relation between number of repeats in the AR and AR sensitivity (Chamberlain et al., 1994), and the resulting variation in androgen activity may be related to a number of clinical conditions (Ellis et al., 2001; Westberg et al., 2001). Thus, variation in the length of the AR(CAG)n repeat may provide another genetic risk factor for the development of PCOS. In this respect, a number of studies have examined the association of AR(CAG)n polymorphism with PCOS with controversial results (Legro et al.,...
1994; Sawaya and Shalita, 1998; Vottero et al., 1999; Calvo et al., 2000; Misfud et al., 2000; Hickey et al., 2002; Jaaskelainen et al., 2005; Mohlig et al., 2006). However, a recent study showed that lower AR(CAG) repeat number increases the risk for precocious puberty and subsequent ovarian hyperandrogenism in adolescent Spanish girls (Ibanez et al., 2003). Another population study showed an association between short CAG alleles and central obesity indices among adult men and postmenopausal women (Gustafson et al., 2003).

The aim of the present study was to investigate the possible interactive role of the SHBG and AR genes in the phenotypic expression of PCOS, since polymorphic variants of both genes may enhance androgen bioactivity by influencing androgen availability and action, respectively.

Subjects and methods

Subjects

The study population consisted of 180 women with PCOS aged 23.7 ± 6.4 (mean ± SD) years. The cohort was published in part previously (Xita et al., 2003). The diagnosis 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 ovarioly dysfunction, clinical evidence of hyperandrogenism, and/or hyperandrogenemia and exclusion of related disorders such as congenital adrenal hyperplasia, hyperprolactinemia or Cushing’s syndrome (Zawadzki and Dunaif, 1992). 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 six months prior to their inclusion in the study. The control group consisted of 168 healthy women with normal menstrual cycles (28–30 days) and no signs of hyperandrogenism.

All patients were studied in the early follicular phase (Day 3–5) of menstrual cycle. The 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 (LH, FSH), total testosterone, SHBG, dehydroepiandrosterone sulphate (DHEAS) and 17-hydroxyprogesterone. The free androgen index (FAI) was calculated using the formula: [total testosterone (nmol/l)/SHBG (nmol/l)] × 100. Insulin resistance was assessed by the fasting glucose to insulin ratio and the homeostasis model assessment (HOMA) using the formula: [fasting glucose (mmol/l) × fasting insulin (μU/ml)]/22.5. 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.

Hormone assays

Serum glucose was determined by the hexokinase method using a glucose analyzer (Olympus 600, Clinical Chemistry Analyser, Olympus Diagnostica GmbH, Ireland). The coefficient of variation (CV) of this method was <3%. Insulin was measured by Microparticle Enzyme Immunoassay on an AXSYM Immunoanalyser (Abbott Laboratory, Abbott Park, IL, USA). The CV of this method was 5%. Total testosterone and serum LH and FSH were determined by Chemiluminescent Microparticle Immunoassay on an Abbott-ARCHITECT Immunoanalyser (Abbott Laboratory). The CVs 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, CA, USA) and the CVs were 9 and 5.5%, respectively.

Genotype analysis

Genomic DNA was isolated from peripheral blood leukocytes of women with PCOS and the controls. Amplification of the TAAA repeat region within the Alu sequence in the SHBG promoter and CAG region within the AR was accomplished by PCR using 1 IU of recombinant TaqDNA polymerase (Gibco/BRL Life Technologies Inc., Gaithersburg, MD, USA) with a forward primer (5’-GCTTGAACCTCGAGG-CAG-3’) and a reverse primer (5’-CAGGGCTAAACAGTC-TAGCAGT-3’) for the SHBG(TAAA)n polymorphism and a forward primer (5’-TCCAGAATCTGTTCAGAGCGTGC-3’) and a reverse primer (5’-GCTTGAAGGTGCTGTT CCTCAT-3’) for the AR(CAG)n polymorphism. PCR was carried out for 30 cycles in a DNA thermal cycler PTC-100 (Peltier-Effect Cycling, MJ Research, Waltham, MA, USA) using a thermal profile of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and primer extension at 72°C for 90 s (for SHBG(TAAA)n polymorphism), or a thermal profile of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and primer extension at 72°C for 90 s (for AR(CAG)n polymorphism). DNA fragments amplified with PCR were 159–184 bp in size for SHBG(TAAA)n polymorphism and 240–318 bp in size for AR(CAG)n polymorphism. Amplified products were separated by 10% polyacrylamide gel electrophoresis followed by silver staining and the number of repeats of each allele was determined (Radojkovic and Kusic, 2000). The number of TAAAAs repeats and CAG repeats in every particular allele was analysed 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 PCR assays.

Statistical analysis

Statistical analysis of differences in genotype frequencies between PCOS and controls was performed using the chi-square test. Normal distribution of continuous parameters was tested by Kolmogorov–Smirnov test. Differences in continuous parameters between genotypes were assessed with analysis of variance test (ANOVA) and were confirmed with the non-parametric Kruskal–Wallis test. Analysis of covariance was performed including BMI and insulin resistance indexes as covariates to find the independent impact of genotypes on hormonal profile. P-value of <0.05 was set as statistically significant. All results are reported as the mean ± SD.
All analyses used the Statistical Package for the Social Sciences (SPSS) (version 11.0, SPSS Inc, Chicago, IL, USA).

Results

Distribution of SHBG (TAAAA)n and AR (CAG)n alleles

The characteristics of women with PCOS and the control group are presented in Table I. 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, six (TAAAA)n alleles with 6–11 repeats in the SHBG gene and 19 (CAG)n alleles with 6–32 repeats within the AR gene were identified. Distribution of the frequencies of the different SHBG(TAAAA)n and AR(CAG)n alleles in PCOS women and controls is presented in Fig. 1. Comparing the frequencies of SHBG alleles between the two groups (Fig. 2), long (TAAAA)n alleles (>8 repeats) were more frequent in PCOS women than in controls ($P = 0.001$), whereas shorter SHBG alleles ($\leq 8$ repeats) were more frequent among control women ($P = 0.001$), as was previously reported (Xita et al., 2003).

With regard to AR(CAG)n alleles, PCOS women tended to have more frequently short AR alleles than controls, although the difference did not reach statistical significance ($P = 0.1$) (Fig 3). In these analyses, subjects heterozygotes for short and long alleles for both genes were not included.

Table I: Anthropometric and biochemical characteristics of PCOS women and controls (mean ± SD, ns, non significant).

<table>
<thead>
<tr>
<th></th>
<th>PCOS women ($n = 180$)</th>
<th>Controls ($n = 168$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>180</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.7 ± 6.4</td>
<td>25.1 ± 5.95</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 ± 6.9</td>
<td>22.99 ± 3.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.4 ± 1.0</td>
<td>1.1 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>37.9 ± 26.4</td>
<td>60.8 ± 26.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FAI</td>
<td>13.7 ± 10.4</td>
<td>3.4 ± 1.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total testosterone (ng/ml)</td>
<td>0.98 ± 0.44</td>
<td>0.4 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHEAS (ng/ml)</td>
<td>2838 ± 1155</td>
<td>2001 ± 903</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting glucose/insulin</td>
<td>10.3 ± 7.4</td>
<td>14.4 ± 5.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA</td>
<td>3.8 ± 3.2</td>
<td>1.8 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

DHEAS, dehydroepiandrosterone sulphate; FAI, free androgen index, HOMA, homeostasis model assessment.

Figure 1: Distribution of the frequencies of the different SHBG SHBG(TAAAA)n and AR AR(CAG)n alleles in PCOS women ($n = 180$) and controls ($n = 168$)

Figure 2: Shift in the distribution of grouped SHBG(TAAAA)n alleles in PCOS women compared with controls

Short SHBG alleles: $\leq 8$ TAAAA repeats, long SHBG alleles: $>8$ TAAAA repeats. Heterozygotes for short and long alleles were not included in the analysis

Figure 3: Distribution of grouped AR(CAG)n alleles in PCOS women compared with controls

Short AR alleles: $\leq 20$ CAG repeats, long AR alleles: $>20$ CAG repeats. (ns, non significant). Heterozygotes for short and long alleles were not included in the analysis.
The study population was further subdivided into subgroups according to different combinations of the two gene polymorphic variants using as thresholds the alleles with the highest frequency: the (TAAAA)_8 allele of the SHBG gene and the (CAG)_{20} allele of the AR gene, respectively. According to this categorization, four subgroups were identified: women with short SHBG alleles (≤8 TAAAA repeats) and short AR alleles (≤20 CAG repeats), women with short SHBG–long AR alleles, women with long SHBG–short AR alleles and women with long SHBG and AR alleles. Women with PCOS had in greater frequency the combination of long SHBG alleles with short AR alleles compared with healthy controls (8.3 versus 6.5%, \(P = 0.09\)), although the difference was not statistically significant. Conversely, PCOS women tended to have less frequently the combination of short SHBG–long AR alleles than control group (50% versus 57.7%, \(P = 0.09\)).

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

Patients with the combination of long SHBG–short AR alleles (subgroup 3) had lower serum SHBG levels (\(P = 0.001\)) and higher levels of total testosterone (\(P = 0.03\), FAI (\(P = 0.001\)) and DHEAS (\(P = 0.001\)) compared with other patient subgroups and this association was independent of BMI and insulin resistance indexes. After controlling for BMI and insulin resistance indexes, patients with long SHBG–short AR alleles had the highest androgen levels and the lowest SHBG levels compared with other patient subgroups. No hormonal difference was observed between the other subgroups (Fig. 4). No difference was observed in fasting glucose to insulin ratio or HOMA index between the different patient subgroups. Similarly, among the healthy women, the subgroup with long SHBG–short AR alleles tended to have lower SHBG levels and greater FAI compared with other subgroups, although these differences were not statistically significant (results not shown). Subjects heterozygotes for short and long alleles for both genes were not included in this analysis.

**Discussion**

In the present study, we investigated the possible interaction of SHBG and AR genes in the pathogenesis of PCOS. In particular, we examined the combined effect of two functional polymorphisms, the \(SHBG(TAAAA)n\) and the \(AR(CAG)n\) repeat polymorphisms on the phenotypic expression of PCOS. These polymorphic variants are known to influence androgen availability (through variation of SHBG levels) and AR sensitivity in target tissues, respectively (Chamberlain et al., 1994; Xita et al., 2003; Cousin et al., 2004; Ferk et al., 2007).

Regarding the \(SHBG\) gene, women with PCOS were more frequently carriers of longer TAAAA repeat alleles than normal women and these alleles were associated with lower SHBG levels, as was previously reported (Xita et al., 2003). In addition, PCOS women tended to have in higher frequency shorter \(AR(CAG)n\) repeat alleles than controls, although the difference was not statistically significant. Focusing on the distribution of the combined polymorphic variants of the two genes, women with PCOS tended to have more frequently the combination of long \(SHBG(TAAAA)n\) alleles with short \(AR(CAG)n\) alleles and less frequently the combination of short \(SHBG(TAAAA)n\) and long \(AR(CAG)n\) alleles compared with healthy controls.

The novel finding of this study was the synergistic effect of the combined genotypes on the phenotype of PCOS. After adjusting for BMI and insulin resistance indexes, PCOS women with the combination of long \(SHBG(TAAAA)n\) and short \(AR(CAG)n\) alleles had the lowest SHBG and the highest androgen levels compared with other patient subgroups. A similar but not statistically significant trend was also seen among the healthy women. Conversely, women with short \(SHBG(TAAAA)n\) and long \(AR(CAG)n\) alleles were found to have the lesser androgenic profile. Taken together, the above findings indicate that PCOS women tend to have more frequently the combination of \(SHBG\) and \(AR\) polymorphic variants, that is associated with increased ‘androgenic activity’ and less frequently the ‘protective’ genotype combination than normal women.

Previous studies have examined the association of \(SHBG\) and \(AR\) gene polymorphism with PCOS in isolation. With regard to \(SHBG\) gene, there is agreement that long \(SHBG(TAAAA)n\) alleles are more frequent in PCOS women and women with hyperandrogenism and are associated with low SHBG levels, indicating the \(SHBG\) gene as a major determinant of PCOS (Xita et al., 2003; Cousin et al., 2004; Ferk et al., 2007). Regarding the role of the \(AR(CAG)n\) repeat polymorphism, results of association studies in different populations are rather controversial (Legro et al., 1994; Sawaya and Shalita, 1998; Vottero et al., 1999; Calvo et al., 2000; Misfud et al., 2000; Ellis et al., 2001; Westberg et al., 2001; Hickey et al., 2002; Jaakselainen et al., 2005; Mohlig et al., 2006). One study provides convincing evidence for a role of the \(AR(CAG)n\) repeat length in ovarian hyperandrogenism among Spanish adolescents (Ibanez et al., 2003). This study followed up girls with premature pubarche and found that the \(AR(CAG)n\) repeat length is shorter in these girls compared with healthy controls. It was also shown that the girls that developed ovarian hyperandrogenism after menarche had shorter mean repeat length than those with normal ovarian function (Ibanez et al., 2003). Other studies, however, showed that the \(AR(CAG)n\) polymorphism may not be a significant determinant of PCOS, but it may be a modulator of androgen-mediated phenotypes in some individuals (Calvo et al., 2000; Jaakselainen et al., 2005). Furthermore, there is also a significant variation in the number of \(AR\) gene CAG repeats in different populations and this may account for the inconsistent findings of association studies in different populations.

Another point of note regarding the \(AR\) gene in women is the phenomenon of X-inactivation, whereby one X-chromosome becomes inactive through methylation in every somatic cell, further complicating interpretation (Lyon, 1988). Previous studies that have investigated X-inactivation patterns of \(AR(CAG)n\) repeat alleles in peripheral lymphocytes of women with PCOS or hirsutism reported inconsistent findings. Thus, preferential methylation of longer \(AR(CAG)n\) alleles was reported by some but not other studies (Vottero et al., 1999, Calvo et al., 2000, Hickey et al., 2002). Furthermore,
the possibility exists that X-chromosome inactivation pattern found in peripheral lymphocytes may be different from that in androgen target tissues (Ibanez et al., 2003). In the present study, we did not perform X-chromosome inactivation analysis and in order to avoid pitfalls in the interpretation of the data, we did not include in the analysis heterozygotes for short and long AR alleles.

The present study is the first to examine the interactive effect of SHBG and AR gene polymorphisms on PCOS phenotype. Although the main determinant for the development of PCOS appears to be the presence of long SHBG(TAAAA)n alleles associated with low SHBG levels, the coexistence of short AR(CAG)n alleles that are related to increased AR sensitivity may contribute to the hyperandrogenic phenotype of PCOS.
Since SHBG provides the main feto–placental barrier protecting the female fetus from maternal androgens, and the AR gene is involved in prenatal sexual differentiation (Patterson et al., 1994), we can hypothesize that the combined long SHBG–short AR genotype may create an hyperandrogenic environment even in intrauterine life, 'programming' the adult PCOS phenotype (Abbott et al., 2002, 2005).

Supporting evidence for this hypothesis comes from the finding in our study that PCOS women with the combined long SHBG–short AR genotype combination exhibit not only increased FAI and total testosterone, but also DHEAS levels indicative of functional ovarian and adrenal hyperandrogenism, respectively. The adrenal hyperandrogenism may reflect the adult phenotypic expression of fetal programming by androgen excess.

In conclusion, the findings of the present study support a synergistic role of SHBG and AR gene variants in the hyperandrogenic phenotype of PCOS. This combined genotype may, in part, provide the genetic link to the developmental origin of PCOS. However, further research is needed to substantiate this hypothesis and the possible mechanisms involved.

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Submitted on July 6, 2007; resubmitted on October 23, 2007; accepted on November 2, 2007