Clomiphene citrate resistance in relation to follicle-stimulating hormone receptor Ser680Ser-polymorphism in polycystic ovary syndrome

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BACKGROUND: Clomiphene citrate (CC) response in anovulatory women is difficult to predict and patient-tailored treatment would benefit patient care and time-management. The objective of this study was to evaluate the role of the follicle-stimulating hormone receptor (FSHR) Ser680Ser-polymorphism as a predictor for CC response.

METHODS: In this retrospective study, 193 patients, diagnosed with polycystic ovary syndrome (PCOS) according to Rotterdam criteria and treated with ovulation induction, were included over a 5-year period in a university hospital in the Netherlands. Data on demographics, BMI, menstrual cycle, laboratory screening (including FSHR genotyping), transvaginal ultrasonography of ovaries and ovulation parameters were collected. Main outcome measures were response to CC and FSHR genotype.

RESULTS: The frequency distribution of the 680-polymorphism was 26% (Asn/Asn), 50% (Asn/Ser) and 24% (Ser/Ser). No significant differences in basal characteristics were found. Significantly more patients with Ser/Ser-polymorphism were resistant to CC (28%) compared with Asn/Ser (14%) and Asn/Asn group (15%), with an odds ratio for ovulation of 0.44 (95% CI, 0.21–0.97). Patients with higher FSH levels, higher age and lower BMI were significantly more likely to ovulate in univariate analysis. In a multivariate logistic regression model, corrected for age, BMI, mean ovarian, volume, hyperandrogenism, and amenorrhoea, only FSHR and basal FSH levels were predictive for ovulation.

CONCLUSIONS: Chance of resistance to CC is almost double in women with PCOS harbouring the Ser/Ser genotype.

Key words: polycystic ovary syndrome / anovulation / FSH receptor / polymorphism / clomiphene citrate

Introduction

Polycystic ovary syndrome (PCOS) is the most common form of anovulatory infertility. Its prevalence is difficult to establish due to the heterogeneous nature of this disorder. As agreed in the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group in 2003, the diagnosis is based on the presence of two of three possible features: clinical or biochemical hyperandrogenism, oligo- or anovulation, and on the presence of polycystic ovaries on ultrasound examination (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). The syndrome is furthermore often accompanied by obesity, high levels of LH (Hendriks et al., 2008) and insulin resistance. The first-choice treatment of infertility caused by PCOS in infertility centers is clomiphene citrate (CC). CC acts as a competitive antagonist of 17β-estradiol at the level of the cytoplasmic nuclear receptor complex in the hypothalamus, and possibly elsewhere. Due to this blockade of the estrogen receptor, GnRH release is not restrained and increased amounts of LH and FSH are released, which enhances follicle growth and ovulation. Ovulation is restored in ~80% of treated patients, and CC resistance (no ovulation up to 150 mg) is seen in the other 20% (Homburg, 2003). Whether or not a patient will ovulate in response to CC treatment is difficult to predict. Imani et al. (1998, 2000) developed a nomogram in which several factors [such as free androgen index (FAI), BMI, cycle
Materials and Methods

Patients

Two hundred and eighty-three female patients, diagnosed with PCOS according to Rotterdam criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004), who attended the Reproductive Medicine Unit of the Obstetrics and Gynecology Department of the VU University Medical Center (VUMc) between January 2003 and December 2007 were screened. According to local protocol, all patients underwent a standardized evaluation that included clinical data on age, ethnicity, cycle history, BMI and a physical examination for signs of hyperandrogenism, laboratory screening and transvaginal ultrasonography of the ovaries. FSHR genotyping was done routinely in every patient visiting the Unit of Reproductive Medicine in our center, allowing for optimal interpretation of individual patient’s serum FSH levels according to the FSHR genotype (Perez et al., 2000). Exclusion criteria were patients without a wish to conceive, those who were not eligible for ovulation induction with CC (couples with an additional male or cervical factor and a patient who had previously been treated with laparoscopic electrocoagulation of the ovaries (LEO) and CC treatment data previous to the LEO was not available) and those who did not complete the treatment until the end-point of either ovulation or resistance (Fig. 1). For this retrospective, anonymous evaluation of routinely acquired patient material, informed consent was waived by the Medical Ethics Board.

Ethnicity

Four groups were distinguished to record ethnicity: Caucasian, Asian, Black and unknown (U.S. Census Bureau, 2000).

Transvaginal ultrasonography

Polycystic ovary morphology on transvaginal ultrasonography was defined according to the Rotterdam criteria, by the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter, and/or increased ovarian volume (>10 ml). Calculation of ovarian volume was performed using the simplified formula for a prolate ellipsoid (0.523 × length × width × thickness), calculated for both ovaries and divided by two. The transvaginal ultrasound was done within one month of endocrinological evaluation.

Hyperandrogenism

Hirsutism and acne were considered signs of clinical hyperandrogenism. Hirsutism was assessed by the Ferriman–Gallwey score. A score of ≥8 was regarded as clinical hyperandrogenism.Biochemical hyperandrogenism was diagnosed when testosterone >2.5 nmol/l and/or androstenedione >9.0 nmol/l. The normal ranges were obtained from the manufacturers, re-evaluated and confirmed in our local laboratory. Both clinical as well as biochemical hyperandrogenism were evaluated as dichotomous variables. Hyperandrogenism in general (as used in the Rotterdam criteria) was computed if either clinical or biochemical hyperandrogenism was present.

Endocrinological evaluation

Laboratory screening was performed by the Endocrine Laboratory of the VU University Medical Center. Reference values are given in Table I. Plasma FSH levels were analyzed by immunometric assay (Delfia, Wallac, Turku, Finland), with a lower detection limit of 0.5 IU/l and an intra-assay coefficient of variation of 5% at a concentration of 2 IU/l and of 3% at >4 IU/l. Plasma LH levels were determined by immunometric assay (Delfia), with a lower detection limit of 0.3 IU/l. Androstenedione was measured using radioimmunoassay (DSL, Webster, TX, USA), with a lower detection limit of 0.5 nmol/l. Dehydroepiandrosterone sulfate was analyzed with a radioimmunoassay (Coat-A-Count, Siemens Medical Solutions Diagnostics, USA), with a lower detection limit of 0.2 μmol/l. Testosterone was analyzed with a radio immunooassay (Coat-A-Count, DPC, Los Angeles, CA, USA), with a lower detection limit of 1.0 nmol/l. Estradiol was measured by radioimmunoassay (Sorin Biomedical, Sallugia, Italy), with a lower limit of quantification of 18 pmol/l. Progesterone was analyzed using competitive immunoassay (Architect, Abbott Laboratories Diagnostic Division, Abbott Park, IL, USA), with a lower detection limit of 2 nmol/l. For patients with a regular cycle, blood samples were taken on cycle Day 3. For oligomenorrheic patients, blood samples were drawn during the specific oligomenorrheic phase (described in more detail in a previous report (Hendriks et al., 2008), meaning that in patients with oligomenorrhea blood was sampled on cycle Days 14 and 21 and in patients with amenorrhea once at a random day).

Detection of FSHR genotypes

For genotyping the Asn680Ser variant in exon 10 of the FSHR gene, genomic DNA was isolated from buffy coats using automated isolation on the BioRobot MDX according to the manufacturer’s instructions.
A DNA fragment of exon 10 of the FSHR gene, restricted by primers (Oligold, Eurogentec, San Diego, CA, USA), was amplified by PCR. The fragment was purified and digested by BsrI (BioLabs, Schwalbach, Germany). Gel electrophoresis was performed on a 2% agarose gel at 80 V for 75 min. The uncleaved 307 base pairs (bp) fragment indicates homozygosity for asparagine (Asn/Asn), whereas the cleaved fragment of 189 and 118 bp indicates homozygosity for serine (Ser/Ser). The presence of all three fragments indicates heterozygosity (Asn/Ser). This procedure is described in more detail in a previous report (Kuijper et al., 2008).

Ovulation induction
PCOS patients with a wish to conceive were prescribed CC in a dose of 50 mg/day for 5 days from cycle Day 5 to 9, raising the dose in increments of 50 mg/day each cycle until an ovulatory cycle was achieved. Ovulation was confirmed by basal body temperature (BBT) charts when (i) BBT was biphasic followed by a menstruation or pregnancy and (ii) BBT was monophasic plus elevated midluteal progesterone (over 10 nmol/l), followed by a menstruation or pregnancy. A monophasic BBT and no rise in progesterone and no subsequent menses or pregnancy after 28 days of CC administration were deemed determinative for anovulation. Resistance to CC was diagnosed after subsequent non-ovulatory cycles with up to 150 mg of CC.

Statistical analysis
Statistical analysis was performed by SPSS software package version 15.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed for normal distribution and were log transformed if not normally distributed. Data are presented as the mean (+ SD) if normally distributed or as median and range if not normally distributed. In case of two independent groups, an independent Student’s t-test was used to compare the normally distributed data, and for the comparison of three or more normally distributed groups, analysis of variance analysis (ANOVA) was used. Mann–Whitney U-test was used to compare groups who were not normally distributed. Logistic regression analysis was performed to evaluate the effect of FSHR genotype on ovulation and corrected for confounders. A value of $P < 0.05$ was considered statistically significant.

Results
Of 283 PCOS patients, 193 (68%) were eligible for inclusion. No differences in basic characteristics were found between the excluded and the included patients. The frequency distribution of the 680-polymorphism of the FSHR was 26% for the Asn/Asn genotype, 50% for the Asn/Ser genotype and 24% for the Ser/Ser genotype. The total group composed of 193 PCOS patients of which 92% was of Caucasian, 3% of Asian, 4% of Black and 1% of unknown origin. One hundred patients presented with all three criteria of PCOS according to the 2003 ESHRE consensus and 93 patients fulfilled two criteria (Fig. 2). Demographic, hormonal and ultrasonographical characteristics of the different genotypes are shown in Table I. No significant differences in basal characteristics were found, including levels of FSH. Table II depicts the clinical and endocrine data of patients who did or did not ovulate after CC medication. Significantly more ($P < 0.05$) patients with the homozygous Ser/Ser-polymorphism were resistant to CC (28%) compared with the heterozygous Asn/Ser.

![Figure 1](enrollment-and-outcomes.png)

**Figure 1** Enrollment and outcomes.
The majority of the patients who were excluded had no wish to conceive or did not finish the treatment with CC to either end-points; ovulation or resistance to CC.
between patients who responded and those resistant to CC. FSH levels were significantly higher in the group who ovulated on any dose of CC, independent of levels of estradiol (E2). Clinical and biochemical hyperandrogenism, ovarian volume and previous amenorrhea were not significantly different between responders and resistant patients. Patients who ovulated also were significantly older and had lower BMI. Logistic regression analyses were conducted with ovulation as a dependent variable and FSHR variant as the predictive variable (Model 1). This model was also tested with age, BMI, amenorrhea, hyperandrogenism (either biochemical or clinical), mean ovarian volume and FSH levels as possible confounders (Model 2). Results are shown in Table III. Corrected for the above-mentioned confounders, the OR for ovulation is 0.26 in the Ser/Ser-variant (95% CI, 0.09–0.85). These results are comparable in a subgroup of only Caucasian patients. In univariate analysis, OR for ovulation in the Ser/Ser group of only Caucasian patients is 0.40 (95% CI, 0.18–0.87), and in the multivariate model, the OR for ovulation is 0.25 (95% CI, 0.07–0.81). A relationship between the specific dose of CC used and FSHR genotype was not found.

**Discussion**

This study was designed to evaluate a possible role for the FSHR polymorphism on position 680 in response to CC treatment for ovulation induction in patients with PCOS. Our data show that FSHR
polymorphism is a factor of some importance in predicting response to CC. FAI, insulin resistance and leptin have previously been recognized as promising predictors for CC response (Imani et al., 1998, 2000; Kurabayashi et al., 2006). Since our routine endocrinological evaluation of PCOS patients does not include FAI, insulin resistance or leptin, it was not possible to analyze these factors.

Table II  Characteristics of clomiphene responders and non-responders

<table>
<thead>
<tr>
<th></th>
<th>Ovulatory (n = 158)</th>
<th>Clomiphene resistant (n = 35)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>29.1 ± 4.2</td>
<td>27.0 ± 4.6</td>
<td>0.009*</td>
</tr>
<tr>
<td>Range</td>
<td>19–40</td>
<td>20–36</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.6</td>
<td>26.3</td>
<td>0.039**</td>
</tr>
<tr>
<td>Range</td>
<td>14.5–47.4</td>
<td>17.6–41.6</td>
<td></td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.82 ± 1.1</td>
<td>2.08 ± 1.1</td>
<td>0.222</td>
</tr>
<tr>
<td>Range</td>
<td>0.5–8.2</td>
<td>0.5–5.9</td>
<td></td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>8.88 ± 3.5</td>
<td>9.77 ± 3.6</td>
<td>0.208</td>
</tr>
<tr>
<td>Range</td>
<td>3.1–23.0</td>
<td>3.7–17.9</td>
<td></td>
</tr>
<tr>
<td>Luteinizing hormone (IU/l)</td>
<td>11.0 ± 5.6</td>
<td>11.0 ± 4.5</td>
<td>0.981</td>
</tr>
<tr>
<td>Range</td>
<td>2.1–30.0</td>
<td>1.9–26.0</td>
<td></td>
</tr>
<tr>
<td>Follicle-stimulating hormone (IU/l)</td>
<td>5.85 ± 1.4</td>
<td>5.05 ± 1.6</td>
<td>0.003*</td>
</tr>
<tr>
<td>Range</td>
<td>1.8–10.0</td>
<td>2.5–10.0</td>
<td></td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>150.6 ± 67.6</td>
<td>151.4 ± 68.7</td>
<td>0.952</td>
</tr>
<tr>
<td>Range</td>
<td>47–499</td>
<td>43–376</td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone (µmol/l)</td>
<td>5.14 ± 2.3</td>
<td>5.87 ± 2.9</td>
<td>0.142</td>
</tr>
<tr>
<td>Range</td>
<td>1.4–12.0</td>
<td>0.6–16.0</td>
<td></td>
</tr>
<tr>
<td>Mean ovarian volume (ml)</td>
<td>11.2 ± 5.5</td>
<td>12.0 ± 4.0</td>
<td>0.551</td>
</tr>
<tr>
<td>Range</td>
<td>3.3–32.4</td>
<td>5.6–18.5</td>
<td></td>
</tr>
<tr>
<td>Amenorrhea (n)</td>
<td>33 (20.9%)</td>
<td>6 (17.1%)</td>
<td>0.618</td>
</tr>
<tr>
<td>Polycystic ovaries on ultrasound (n)</td>
<td>142 (89.9%)</td>
<td>32 (91.4%)</td>
<td>0.832</td>
</tr>
<tr>
<td>Clinical hyperandrogenism (n)</td>
<td>51 (32.3%)</td>
<td>8 (22.9%)</td>
<td>0.274</td>
</tr>
<tr>
<td>Biochemical hyperandrogenism (n)</td>
<td>74 (49.7%)</td>
<td>18 (54.5%)</td>
<td>0.612</td>
</tr>
</tbody>
</table>

Values are mean ± SD or median (range) except for last four characteristics where values are number of patients (percentages). Data analyzed using ANOVA for normally distributed variables and Mann–Whitney U-test for not normally distributed variables.

*P < 0.01.

**P < 0.05.

Table III  Logistic regression model

<table>
<thead>
<tr>
<th>Model</th>
<th>OR</th>
<th>P-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>0.44</td>
<td>0.04*</td>
<td>0.205–0.965</td>
</tr>
<tr>
<td>Dependent variable: ovulation Predictive variable: FSHR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>0.26</td>
<td>0.03*</td>
<td>0.085–0.845</td>
</tr>
<tr>
<td>Dependent variable: ovulation Predictive variables: FSHR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent variables: FSH, BMI, age, amenorrhea, mean ovarian volume and hyperandrogenism</td>
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</tr>
</tbody>
</table>

*P < 0.05.

Figure 3 Percentage of patients with resistance to CC per group. In the Ser/Ser group, chance to ovulate on CC was half (OR, 0.44; 95% CI, 0.21–0.97) that of the combined Asn/Asn + Asn/Ser group. *P < 0.05.
To the best of our knowledge, the only other study that has analyzed CC resistance to the FSHR polymorphism is that of Laven et al. (2003). In their study, they report a frequency distribution of 9%, 40% and 51% (Asn/Asn, Asn/Ser and Ser/Ser, respectively) in a group of CC-resistant patients. It was concluded that there was no difference between CC-resistant and ovulatory patients, yet these data were not shown. In our data set, this distribution in a subgroup of Caucasian CC-resistant patients was 22%, 53% and 25% (Asn/Asn, Asn/Ser and Ser/Ser, respectively). Two methodological differences between our study and that of Laven et al. make it difficult to compare the aforementioned results. First, it is not clear from the report of Laven et al. whether the patients discussed are true CC-resistant patients (i.e. no ovulation) or whether they failed to conceive on CC. Second, patients included in the study of Laven et al. were normogonadotrophic anovulatory women, whereas in our study, only PCOS patients were included.

Unlike Imani et al., we were not able to find a relationship between CC resistance and hyperandrogenism, amenorrhea and/or ovarian volume. Possibly, these differences can be explained by the inclusion criteria of the patient population. Our group consisted solely of PCOS patients (diagnosed conform Rotterdam criteria), whereas the group of Imani et al. consists of all oligoamenorrheic or amenorrheic patients, thereby creating a heterogeneous group. As it is still not clear whether the pathogenesis of PCOS is the same as that of an anovulatory patient of unexplained cause (i.e. no PCOS), we hypothesized that including all anovulatory patients would possibly influence the outcome. Since our group and that of Imani et al. are so different (lower androgen levels, lower rates of amenorrhea and higher levels of LH), this can be seen as an indication that there is in fact a difference between the subgroup of PCOS patients and the anovulatory group in general.

In univariate analysis, increasing age is significantly associated with a better response to CC. As already indicated by Elting et al. (2000), women with PCOS gain regular menstrual cycles when aging. Similar to results of Elting et al. (2003), in our data, there is a linear non-significant trend for increasing FSH levels with increasing age (data not shown). It is very likely that those patients of increased age have a much better response to CC, as they are on the brink of reaching their FSH threshold for natural follicle maturation. A higher BMI reduces the chance of becoming ovulatory with CC. This can be due to several factors. Insulin resistance could play a pivotal role in this association. Additionally, obesity can alter pharmacokinetic characteristics of CC, for example, by increasing volume of distribution or a different accumulation in fat tissue. Possibly, the pathogenesis of PCOS in obese patients is different from that of lean PCOS patients. Although age and BMI have a significant effect on response to CC, this effect disappears after correcting for FSH level and receptor polymorphism.

We believe that this is caused by faulty feedback in the pituitary. In healthy women, the pituitary is able to overcome the relative insensitivity of the FSHR by increasing the level of FSH. In women with PCOS, FSH levels, although within normal values, are too low to induce follicle maturation. In women harboring the SS variant of the FSHR, it is even harder to overcome this high FSH threshold with CC. This is supported by the fact that more CC resistance is seen in this group. Basal FSH levels were significantly higher in both univariate and multivariate analyses with respect to response to CC, indicating that women with a higher basal FSH are more likely to ovulate. We believe that this is caused by the same mechanism as described above; namely that a higher basal FSH may facilitate reaching the threshold for raising the FSH levels to a level that ovulation can occur.

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