Steroid profiles in ovarian follicular fluid in women with and without polycystic ovary syndrome, analyzed by liquid chromatography-tandem mass spectrometry

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Objective: To compare steroid concentrations and steroid product-to-precursor ratios in ovarian follicular fluid (FF) from women with polycystic ovary syndrome (PCOS) and from regularly menstruating women in their early follicular phase, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Polycystic ovary syndrome involves abnormal regulation of the steroidogenic enzymes, leading to arrest of follicle development.

Design: Case-control study.

Setting: University hospital clinic.

Patient(s): Follicular fluid from size-matched ovarian follicles (5–8 mm) in 27 nonstimulated women with PCOS and in 21 women without PCOS was sampled. Thirteen steroids were quantitated from 40 μL of FF, using LC-MS/MS.

Intervention(s): None.

Main Outcome Measure(s): Concentrations of steroids in the FF and product-to-precursor ratios (enzyme activity) were compared between the groups.

Result(s): In women with PCOS, ovarian FF contained higher concentrations of individual and total androgens, lower individual and total estrogens (E), and a lower total E-to-androgen ratio, compared with regularly menstruating women. The product-to-precursor concentration ratios indicated higher CYP17-linked and lower CYP19-linked (aromatase) enzyme activity. Receiver operating characteristic plots indicated the early CYP17 step (17-OHSP/SP) being highly important for the prevalence of PCOS (c = 0.95).

Conclusion(s): The women with PCOS had higher ovarian CYP17-linked and lower CYP19-linked (aromatase) enzyme activity, confirming previous data. Multiple steroid assessments from minute volumes including FF from nonstimulated ovaries, using LC-MS/MS, might be useful in research, clinical endocrinology, and in IVF.

Key Words: Polycystic ovary syndrome, PCOS, follicular fluid, LC-MS/MS, mass spectrometry, steroid hormones, androgens, estrogens, CYP17, CYP19

Polycystic ovary syndrome (PCOS) is a common endocrine disorder characterized by hyperandrogenism and anovulatory infertility (1). In PCOS, follicular development arrests at the stage of selection of the dominant follicle when aromatase activity in the granulosa cells (GC) and production of E2 normally increase (2). The amount of estrogen (E) produced by the dominant follicle indicates the “vitality” of the follicle and successful ovulation (3, 4). In PCOS, the follicular fluid (FF) concentrations of androgens are higher and E2 lower than in women without PCOS (5).

We recently developed high sensitivity liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods, enabling identification and quantification of a large number of steroids in small sample volumes (6–9), including ovarian FF from nonstimulated ovaries (Fig. 1) (10). In the present study we used LC-MS/MS to compare the steroid pattern in FF from similar-sized ovarian follicles in nonstimulated women diagnosed with PCOS and from regularly menstruating women in their early follicular phase.

MATERIALS AND METHODS

Clinical Material

The study subjects were recruited by advertising in local media and investigated at the Donetsk Regional Center of Mother and Child Care, Donetsk, Ukraine, as described in detail elsewhere (11). Available FF from 27 women with PCOS and 21 regularly cycling women without PCOS were included. All participants were unstimulated and follicles were aspirated without hCG priming. The diagnostic criteria for PCOS were according to the Rotterdam...
FIGURE 1

Biosynthesis of steroid hormones of the cholesterol pathway. Steroids with underlined names were analyzed using liquid chromatography-tandem mass spectrometry methods.

Cholesterol

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Enzyme Activity</th>
<th>Enzyme Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
<td>CYP17</td>
<td>3β-HSD</td>
</tr>
<tr>
<td>17-OH-pregnenolone</td>
<td>CYP17</td>
<td>3β-HSD</td>
</tr>
<tr>
<td>Progesterone</td>
<td>CYP17</td>
<td>3β-HSD</td>
</tr>
<tr>
<td>17-OH-progesterone</td>
<td>CYP17</td>
<td>3β-HSD</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>CYP11B2</td>
<td>11β-HSD</td>
</tr>
<tr>
<td>11-deoxycortisol</td>
<td>CYP11B2</td>
<td>11β-HSD</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>CYP11B2</td>
<td>11β-HSD</td>
</tr>
<tr>
<td>Cortisol</td>
<td>CYP11B2</td>
<td>11β-HSD</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>CYP11B2</td>
<td>11β-HSD</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>CYP17</td>
<td>3β-HSD</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>CYP19</td>
<td>17α-HSD</td>
</tr>
<tr>
<td>Estrone</td>
<td>CYP19</td>
<td>17α-HSD</td>
</tr>
<tr>
<td>Testosterone</td>
<td>CYP19</td>
<td>17α-HSD</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>CYP19</td>
<td>17α-HSD</td>
</tr>
<tr>
<td>Estradiol</td>
<td>CYP19</td>
<td>17α-HSD</td>
</tr>
<tr>
<td>Dihydroprogesterone</td>
<td>CYP19</td>
<td>17α-HSD</td>
</tr>
</tbody>
</table>


criteria: amenorrhea or oligomenorrhea (<10 cycles per year), a characteristic ovarian image on ultrasonography (≥ 10 small follicles per plane, in association with a marked ovarian stroma) (12). Hirsutism was assessed by a modified Ferriman–Gallwey protocol (13) and women with a score of >8 were considered clinically hirsute. The body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. For diagnosis, all ultrasound examinations were performed transabdominally or transvaginally using a Kranzbühler GmbH, Germany, fitted with a 3.5- and 5-MHz sector probe, respectively. The patients with PCOS were treated for infertility by ovarian wedge resection and ovarian FF was collected during the operation.

Control subjects were women with infertility, assumed to be caused by pelvic adhesions. All control women were healthy, had regular menstrual cycles, and normal ovaries on ultrasound. All participants, PCOS and controls, were not taking oral contraceptives (OC), insulin sensitizers, and other medications that might affect study outcome, during the previous 3 months of study start. Ultrasound images from both study groups were blindly evaluated by two independent ultrasound experts. Control data used were previously reported (10) and used here with the permission of the publisher.

Sample Collection

Sampling of FF was performed between days 4 and 7 in the follicular phase in regularly menstruating women and in women with PCOS, if possible, after a menstrual period in the early part of follicular phase. The FF from ovarian follicles (5–8 mm), in 27 women with PCOS and in 21 women without PCOS was sampled, pooled within each subject, and centrifuged. In both study groups, the size of the follicles was measured by transvaginal ultrasonography (TVUS) during the laparoscopic procedure, with the needle outside the follicle to ensure that FF from correctly sized follicles was sampled. The samples were kept frozen at -20°C until analysis.

Venous blood samples were drawn after fasting between 7:30 and 9:00 AM on the day before surgery. Serum was separated and kept at -20°C. Frozen samples of serum and FF were packed in dry ice during transportation between centers.

Serum Hormone Analysis

Sex hormone-binding globulin (SHBG) was measured by chemiluminescence immunoassay (Immullite 2000) and of T by RIA, both obtained from Diagnostic Products Corporation, Los Angeles, CA. Serum 17α-hydroxyprogesterone (17-OHP) by a commercial RIA kit was obtained from Bio-Rad Laboratories, Hercules, CA. Analyses were performed at the Department of Clinical Chemistry, University Hospital, Uppsala, Sweden. The T-to-SHBG ratio (free androgen index) was used as a marker and a useful index of biologically active T (14, 15). The upper normal clinical reference limit for the ratio T-to-SHBG (based on data from 260 healthy women) was 0.05.

Reagents and Standards for FF Analysis

The reagents and the internal standards used were extensively described in our recent report (10).

LC-MS/MS Methods

Five classes of steroids of the steroid biosynthesis pathway were measured in the FF using LC-MS/MS methods: pregnenolones, progestines, androgens, Est, and glucocorticoids. Thirteen steroids were analyzed, underlined in Figure 1 and see detailed descriptions of the LC-MS/MS methods in our recent report (10).

Statistical Analysis

Values are expressed as median and range. Baseline comparisons between the study groups were evaluated using nonparametric Wilcoxon two-group tests for continuous variables. Associations between variables were accessed using Spearman rank correlation test. Adjustment for differences in BMI was analyzed using analysis of variance (ANOVA). Multiple logistic regression analysis was used to explore the putative independent effects of the measured steroids and product-to-precursor ratios (enzyme activities) with regard to prevalence of PCOS. Receiver operating characteristic (ROC) curves were plotted to illustrate the discriminative abilities of FF steroids and steroid product-to-precursor ratios with regard to prevalence of PCOS (16). In ROC plots, area under the curve (AUC or c value) can maximally be 1.0. An AUC of 0.5, means not useful, like tossing a coin; AUC of 0.7 is considered useful; and above 0.75 is very good. A P value of less than .05 was considered statistically
**RESULTS**

Clinical characteristics are given in Table 1. Women with PCOS were slightly younger and had significantly higher values for BMI, serum T, T-to-SHBG ratio, 17-OHP, hirsutism index, and lower serum SHBG, compared with regularly menstruating women. Based on the results of serum 17-OHP, most likely, none of the participating women had 21-hydroxylase deficiency and participating women, including those with oligomenorrheic PCOS, were not close to ovulation or in the luteal phase.

**Comparison of Steroid Median Values**

The 13 steroids analyzed in FF are underlined in Figure 1 and their concentrations are given in Table 2. Of androgen precursors, only 17-OHPpregnenolone (17-OH5P) had significantly higher levels (double) in FF from patients with PCOS compared with those from controls. Levels of the four individual androgens and of total androgens were significantly higher, whereas individual Es and total Es were significantly lower in FF from women with PCOS. Differences in total androgens, total Es, and E-to-androgen ratios remained statistically significant after adjustment for differences in BMI (Table 2). Analysis in women with PCOS who had severe oligomenorrhea (<6 cycles/year) revealed similar differences and significance levels, except stronger significance levels for the 17-OHPpregnenolone/pregnenedione (17-OH5P/5P) and E2-to-T, compared with values in Table 2.

**Associations Between Concentrations of Steroids and Baseline Characteristics**

In women with PCOS, BMI was negatively associated with FF concentrations of total Es (r = −0.53; P = .006), 17-OHP (r = −0.40; P = .04), E2 (r = −0.57; P = .003), and with E2-to-T ratio (r = −0.40; P = .04). Hirsutism index was positively associated with FF concentrations of T (r = 0.51; P = .006). In regularly menstruating women, BMI was negatively associated with concentration of Pregnenolone (5P) (r = −0.51; P = .018).

**Comparison of the Ratios of Concentrations of Steroids Products/Precurors in the Pathway**

Steroid product-to-precursor ratios in FF showing significant differences between patients with PCOS and controls are given in Table 2. The initial CYP17 (cytochrome P450, subfamily XVII) enzyme step (17-OH5P/5P) (Figure 1) was significantly higher in PCOS FF, increasing levels of androgen precursors and androgens. The ratio (total androgens-to-5P), somewhat reflecting the side chain cleaving enzyme activity, was significantly higher in FF from women with PCOS. One marker for 3β-hydroxysteroid dehydrogenase (3βHSD) and two markers for CYP19 (cytochrome P450, subfamily IXX) enzyme were significantly (5 and 3 times) lower, the latter reducing E synthesis in PCOS ovarian follicles. For other markers of 3βHSD and 17βHSD, no significant differences were found (data not shown).

**Logistic Regression Analysis**

Among the Es tested, estrone (E1) had a strong independent impact with regard to prevalence of PCOS (P = .02). Among the pregnenolones tested, 17-OH5P had the strongest, significant and independent impact (P = .049) followed by 5P (P = .061). Included in the same model, both E2 and 17-OH5P had significant and independent effects, but it was slightly stronger for 17-OH5P (P = .003 vs. P = .031 for E2). Among six significant product-to-precursor ratios

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**TABLE 1**

Characteristics of women with PCOS and regularly menstruating women during the follicular phase of the menstrual cycle.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women with PCOS (n = 27)</th>
<th>Control women* (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>25 ± 3.5c</td>
<td>28 ± 3.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164 ± 6.4</td>
<td>165 ± 6.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73.5 ± 14.9</td>
<td>64.8 ± 10.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.2 ± 5.2c</td>
<td>23.9 ± 3.8</td>
</tr>
<tr>
<td>Parity (n)</td>
<td>1.4 ± 0.9</td>
<td>2.1 ± 1.7</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>9/27</td>
<td>9/21</td>
</tr>
<tr>
<td>Average number of menstrual cycles during the past 12 mo</td>
<td>6/12 (0–9)</td>
<td>12/12</td>
</tr>
<tr>
<td>Menstrual cycle day of follicular fluid sampling</td>
<td>NA</td>
<td>6 (4–7)</td>
</tr>
<tr>
<td>Menstrual cycle length (d)</td>
<td>NA</td>
<td>28 (21–32)</td>
</tr>
<tr>
<td>Hirsutism index*</td>
<td>9 (6–24)d</td>
<td>3 (1–8)</td>
</tr>
<tr>
<td>Serum T (nmol/L)</td>
<td>2.7 ± 1.2c</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Serum SHBG (nmol/L)</td>
<td>42.8 ± 31c</td>
<td>67.0 ± 27</td>
</tr>
<tr>
<td>Serum T-to-SHBG ratio</td>
<td>0.11 ± 0.2d</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Serum 17-OHP (nmol/L)</td>
<td>4.1 ± 2.4c (1.1–12.2)</td>
<td>2 ± 1.5 (0.8–6)</td>
</tr>
</tbody>
</table>

*Values represented as either mean ± SD or median (range). NA = not applicable; SHBG = sex hormone-binding globulin; 17-OHP = 17α-hydroxyprogesterone; BMI = body mass index; PCOS = polycystic ovary syndrome.

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tested, the strongest single \( P \) values were found for the earliest CYP17 step (17-OH5P/5P) and the earliest CYP19 step (E1/androstenedione [A]) in the steroid biosynthesis pathway (\( e \) and \( f \) in Table 2; Fig. 1). In a model, including both the earliest CYP17 (17-OH5P/5P) and the earliest CYP19 (E1/A) ratios, only the 17-OH5P/5P ratio had an independent and significant effect (\( P = .019 \)), suggesting that in patients with PCOS the increased activity of CYP17 might be as important as the reduced activity of CYP19. Furthermore, when the 17-OH5P/5P ratio and age were evaluated in the same model, the ratio remained significant (\( P = .006 \)), but age had also a significant negative association (\( P = .039 \)), indicating that with increasing age the association with the 17-OH5P/5P ratio becomes weaker.

**DISCUSSION**

To our knowledge this is the first study comparing multiple steroid profiles in FF from similar-sized follicles in women with and without PCOS, using LC-MS/MS, enabling comparison of multiple steroids in FF from unstimulated ovaries. Thirteen steroids were quantitated, requiring only 40 \( \mu \)L of FF. The concentrations of total and individual androgens and Es, product-to-precursor ratios, and ROC curve plottings all indicated a higher activity of CYP17-linked enzymes increasing FF androgen levels combined with lower ovarian CYP19-linked enzyme (aromatase) activity lowering FF Es in women with PCOS, confirming previous data (5, 11, 17, 18). The ratio (total androgens/5P), somewhat reflecting the C17-20 side chain cleaving enzyme activity, was significantly higher in FF from PCOS.

Among the evaluated product-to-precursor ratios, the earliest CYP17 step (17-OH5P/5P) was the only independent and significant predictor with regard to prevalence of PCOS (Fig. 1). The strongly increased first CYP17 step, combined with reduced 3\( \beta \)HSD (17-OH5P/17-OHP4), strongly contribute to the significantly higher values for 17-OH5P (double), and the following higher DHEA, A, and T in women with PCOS. More important, this most likely explains why the following C17-20lyase step (DHEA/17-OH5P)
(Figure 1 and Table 2) was slightly lower in PCOS and the A/DHEA and T/A ratios were similar to those in healthy women, despite the higher levels of DHEA, A, and T. In addition, reduced CYP19 activity (aromatase) contributes to piling up of A and T.

A lower capacity in women with PCOS, compared to regularly menstruating women, to transform (aromatize) androgens to E2 in the ovarian follicle might be associated with the classic arrest in follicular growth at 7–8 mm diameter in the PCOS ovary and anovulation (2). These findings agree with our report on GC aromatase enzyme activity after exposure to FF from women with PCOS, showing reduced GC aromatase conversion activity (E biosynthesis), partly by blocking the active site of the enzyme and partly by decreased substrate affinity (11). The lack of differences (although numerically lower) in FF E levels in two previous studies (19, 20) might be explained by the low number of patients with PCOS (five) or the RIA methods used. The ratio 17-OHSP/5P was significantly higher in PCOS, an effect that significantly decreased with increasing age. This last finding is in line with the previously reported phenomenon of “self cure” with advancing age (i.e., normalization of symptoms of hyperandrogenism and regain of regular menstrual periods) (21, 22).

The finding of higher androgen and lower E concentrations in PCOS FF is in agreement with earlier findings by Eden et al. (5). Furthermore, increased CYP17 and reduced CYP19-linked enzyme activity are in line with previous findings for CYP17 (17, 18) and for CYP19 (11) activities in women with PCOS. In women diagnosed with PCOS, BMI was negatively associated with concentrations of total Es, E2T, and 17-OHP. In clinical practice, weight reduction and reduced BMI in patients with PCOS are often associated with return of ovulation, menstrual bleedings, and normalization of other metabolic parameters (23, 24).

There are some limitations to the present study. This was a clinical study, not a laboratory dissection of ovarian follicles, therefore we sampled FF from similar-sized follicles (5–8 mm), in both women with PCOS and control women during the operation. Ultrasound during the sampling procedure assured sampling from follicles of correct size. The steroid profiles in FF from regularly menstruating women were sampled in the early part of the follicular phase. We believe that this is the best time for comparison because it is closer to the time of ovulation and the E biosynthesis (aromatase activity) is dramatically increased. Furthermore, restricting analysis to women with PCOS having more severe oligomenorrhea (<6 cycles/year), revealed similar differences and significance levels, as shown in Table 2. The PCOS and regularly menstruating women in this study were subjects from our previous study on ovarian aromatase enzyme regulation (11). Women in the regularly menstruating group were also included in our recent report, illustrating the methodology and feasibility of quantitating multiple steroids in FF using LC-MS/MS methods (10).

The highly sensitive and specific LC-MS/MS methods (6–9, 25, 26) allow simultaneous measurement of multiple steroids from minute sample volumes. The LC-MS/MS methods are therefore suitable for future research to better understand underlying mechanisms/processes involved in the regulation of the menstrual cycle, including follicular development, ovulation, and anovulation. Such knowledge and the use of LC-MS/MS might also help to improve the tailoring and fine-tuning of IVF treatment regimens and in selecting the most suitable eggs for IVF to reach the goal of a successful pregnancy.

In conclusion, compared with FF from similar-sized follicles in healthy regularly menstruating women, FF from women with PCOS has higher concentrations of 17-OHSP, individual and total androgens, and lower concentrations of individual and total Es. In line with previous results, this finding and the steroid product-to-precursor ratios indicated increased CYP17-linked and reduced CYP19-linked enzyme activity in women with PCOS. Furthermore, data indicated an importance for the earliest CYP17 step (17-OHSP/5P) in women with PCOS. Simultaneous and accurate measurement of multiple steroids in minute sample volumes, including from non-stimulated ovaries, using LC-MS/MS methods, might be useful for research in gynecological and clinical endocrinology and of potential use in improvement of IVF treatment regimens.

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