Steroid Profiles in Ovarian Follicular Fluid from Regularly Menstruating Women and Women after Ovarian Stimulation

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BACKGROUND: Information on the concentrations of steroids in ovarian follicular fluid (FF) from regularly menstruating (RM) women has been limited because of the absence of methods for the simultaneous quantification of multiple steroids in small volumes of FF. We studied steroid profiles in FF during the early follicular phase of the menstrual cycle and after ovarian stimulation for in vitro fertilization (IVF), and compared concentrations with published values obtained by immunoassay (IA).

METHODS: We used liquid chromatography–tandem mass spectrometry (LC-MS/MS) to measure 13 steroids in 40-µL aliquots of FF samples from 21 RM women and from 5 women after ovarian stimulation for IVF. Relationships between concentrations of steroids and their ratios (representations of the enzyme activities) were evaluated within and between subgroups.

RESULTS: The concentrations of testosterone (Te), androstenedione (A4), and estradiol (E2) measured by LC-MS/MS were lower than those previously reported in studies with IAs. In RM women, androgens were the most abundant class of steroids, with A4 being the major constituent. The concentrations of 17-hydroxyprogesterone (17OHP), total androgens, and estrogens were 200- to 1000-fold greater in FF than in serum. Compared with RM women, FF samples from women undergoing ovarian stimulation had significantly higher concentrations of E2 (P = 0.021), pregnenolone (P = 0.0022), 17OHP (P = 0.0007), and cortisol (F) (P = 0.0016), and significantly higher ratios of F to cortisone (P = 0.0006), E2 to estrone (P = 0.0008), and E2 to Te (P = 0.0013).

CONCLUSIONS: The data provide the first MS-based concentration values for 13 steroids in ovarian FF from RM women, from estrogen- and androgen-dominant follicles, and from women after ovarian stimulation for IVF.

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In women of fertile age, ovarian follicles are the main source for a major fraction of the estrogens present in the circulation. Although ovarian follicles also contribute circulating androgens, the adrenal cortex is the major source of the androgens in the circulation. Follicular steroids are secreted by granulosa, theca, and hilus cells under the control of gonadotropins, and the hormonal microenvironment affects follicle development and oocyte viability (1). Higher estradiol (E2)7 concentrations in follicular fluid (FF) are associated with healthy mature follicles containing oocytes capable of meiosis, whereas higher androgen concentrations are indicative of atretic changes (1–5). With the introduction of in vitro fertilization (IVF), a number of studies have analyzed FF from women undergoing ovarian stimulation. The majority of these studies were undertaken to identify prognostic variables for the likelihood of a successful implantation (6–8). Relatively few publications, however, have focused on the steroid hormones present in the FF of regularly menstruating (RM) women and on the relationship between steroid concentrations and follicular development (3–5, 9–11).

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7 Nonstandard abbreviations: E2, estradiol; FF, follicular fluid; IVF, in vitro fertilization; RM, regularly menstruating; IA, immunoassay; MS, mass spectrometry; LC-MS/MS, liquid chromatography–tandem MS; EDF, estrogen-dominant follicle; ADF, androgen-dominant follicle; Te, testosterone; E1, estrone; E3, estriol; Preg, pregnenolone; 17OHPreg, 17-hydroxyprogrenenolone; 17OHP, 17-hydroxyprogesterone; 11DC, 11-deoxycortisol; F, cortisol; E, cortisone; A4, androstenedione; DHEA, dehydroepiandrosterone.
The information available on the steroids in FF and their concentrations is conflicting (5, 9, 10). This situation is due in part to the very limited volume of FF that can be obtained from follicles of RM women and the absence of methods for simultaneous quantitative analysis of multiple analytes in small samples. Previous studies have measured steroids in FF with immunosays (IAs) (12–15), which are known to have a high potential for cross-reactivity (16–18), or with GC-MS methods, which are more specific but require larger sample aliquots (19–21). Recent advances in biological mass spectrometry (MS) have overcome the problems associated with poor specificity in earlier methods and have enabled simultaneous quantification of multiple steroids. To our knowledge, no comprehensive published study has used liquid chromatography–tandem MS (LC-MS/MS) methods for quantitative analysis of multiple steroids in FF from RM women.

Our aims were to use high-sensitivity LC-MS/MS methods to evaluate steroid profiles in FF samples from RM women during the early follicular phase of menstrual cycle and from women after ovarian stimulation for IVF, to compare the patterns of steroid distribution in FF samples from estrogen-dominant follicles (EDFs) and androgen-dominant follicles (ADFs), to compare the distribution patterns of steroid concentrations in serum and FF in RM women during the early follicular phase of the menstrual cycle, and to compare the steroid concentrations in FF obtained in this study with published values obtained by other methods.

Materials and Methods

STUDY PARTICIPANTS
Twenty-one RM women who responded to advertising in the media were recruited at the Donetsk Regional Center of Mother and Child Care, Donetsk, Ukraine. The women entered the hospital for laparoscopic treatment of infertility, presumably caused by pelvic adhesions. All women had regular cycles, and pelvic ultrasound examinations revealed typical healthy ovaries. The women were in good general health and had not taken hormonal medications or oral contraceptives during the 3 months before inclusion in the study. The RM women had periods with intervals of 21–32 days (Table 1). The ultrasound examinations showed typical ovarian images, with no signs of polycystic ovaries or increased stromal density. Table 1 summarizes the clinical and anthropometric characteristics of the participants.

We also sampled FF from 5 Swedish patients undergoing IVF treatment because of male-factor, tubal-factor, nonovarian endometriosis–related, or unexplained infertility. These patients underwent infertility treatment at the Uppsala University Hospital, Uppsala, Sweden. The treatment protocol for the IVF patients consisted of pituitary down-regulation by means of the gonadotropin-releasing hormone analog Buserelin (Hoechst) and the “long” protocol initiated at the midluteal phase (900 µg/day, intranasal administration). Recombinant follicle-stimulating hormone (Gonal-f®; Serono) was injected subcutaneously (225 IU/day), starting on cycle day 3. The dose was adjusted from cycle day 7 as necessary. Human chorionic gonadotropin (10 000 IU Profasi® HP; Serono) was administered when one or more follicles had reached a diameter >23 mm. Oocytes were retrieved transvaginally under ultrasound guidance 36 h after human chorionic gonadotropin administration. All women participating in the study were Caucasian. Informed consent was obtained from all of the women, and the ethics committees of Donetsk State Medical University and Uppsala University approved the study protocol.

COLLECTION AND HANDLING OF FF SAMPLES
FF samples were obtained from the RM women during laparoscopic adhesiolysis performed between day 4 and day 7 of the follicular phase of a cycle. FF samples were aspirated from ovarian follicles (5–8 mm in diameter), pooled for each study participant, and centrifuged. The samples were transferred and stored in Cryovial tubes (SciMart) below −20 °C. Follicle size was measured by transvaginal ultrasonography during laparoscopic adhesiolysis. For the women undergoing ovarian stimulation for IVF, FF was aspirated from single follicles >15 mm in diameter into Falcon tubes (BD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>28 (3.2)b</td>
</tr>
<tr>
<td>Height, cm</td>
<td>165 (6.2)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>64.8 (10.4)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.9 (3.8)</td>
</tr>
<tr>
<td>Parity</td>
<td>2.1 (1.7)c</td>
</tr>
<tr>
<td>Menstrual cycles during previous 12 months, n</td>
<td>12</td>
</tr>
<tr>
<td>Menstrual cycle day at FF sampling</td>
<td>6 (4–7)</td>
</tr>
<tr>
<td>Menstrual cycle length, days</td>
<td>28 (21–32)</td>
</tr>
<tr>
<td>Hirsutism indexd</td>
<td>3 (1–8)</td>
</tr>
<tr>
<td>Current smokers, n</td>
<td>9</td>
</tr>
</tbody>
</table>

a Data are presented as the mean (SD) or as the median (range), as indicated.
b Age range, 21–34 years.
c Parity range, 1–8.
d Modified Ferriman–Gallwey scale.
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Biosciences). The samples were transferred into Sci- Mart Cryovial tubes and stored at −70 °C until analysis. FF samples were packed in dry ice during transport between the participating centers.

REAGENTS AND CALIBRATORS
Testosterone (Te), estrone (E1), 17β-E2, 17α-E2, estriol (E3), pregnenolone (Pregn), 17-hydroxyprogrenolonol (17OHPPregn), 17-hydroxyprogesterone (17OHHP), 11-deoxy cortisol (11DC), cortisol (F), cortisone (E), hydroxyamine, formic acid, trifluoroacetic acid, dansyl chloride, and sodium carbonate were purchased from Sigma–Aldrich. Androstenedione (A4), dehydroepiandrosterone (DHEA), dihydrotestosterone, and androstenedione were purchased from Steraloids. The internal standards were deuterium-labeled analogs of the steroids: d3-Te, d3-Pregn, d2-11DC, d8-17OHP, d3-androstenedione (DHEA), dihydrotestosterone, and androstenedione were purchased from Steraloids. The internal standards were deuterium-labeled analogs of the steroids: d3-Te, d3-Pregn, d2-11DC, d8-17OHP, d3-17OHPPregn, d4-F, and d3-E were purchased from Cambridge Isotope Laboratories; d4-E1, d3-E2, d3-E3 were purchased from C/D/N Isotopes. HPLC-grade methanol, acetonitrile, and methyl-tet-butyl ether were all obtained from VWR. All other chemicals were of the highest purity commercially available.

LC-MS/MS METHODS
We used LC-MS/MS methods to measure steroid concentrations in FF. Estrogens were analyzed as dansyl derivatives (22, 23), ketosteroids were analyzed as oxime derivatives (24, 25), and F and E were analyzed nonderivatized (26). The HPLC system consisted of 1200 Series HPLC pumps (Agilent Technologies), a 10-port switching valve, a vacuum degasser, and an HTC PAL autosampler (LEAP Technologies) equipped with a fast-wash station. An API 4000 tandem mass spectrometer (Applied Biosystems/MDS Sciex) was used in the positive-ion mode with a TurbolonSpray™ ion source. Sample preparation, chromatographic-separation conditions, and mass transitions used in the methods are outlined in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue3. The quadrupoles Q1 and Q3 were tuned to unit resolution, and the MS conditions were optimized for the maximum signal intensity of each steroid. Two mass transitions were monitored for each steroid and its internal standard. The primary mass transitions were used to measure the concentration of each steroid, and the specificity of the analysis for each steroid in every sample was evaluated by comparing the concentrations measured with the primary and secondary mass transitions of each steroid and its internal standard (27, 28). Data were analyzed quantitatively with Analyst™ 1.4.2 software (Applied Biosystems). We used 6 calibrators to generate calibration curves for every set of samples and included 3 QC samples with every set of samples. Assay imprecision was <15% (see Table 1 in the online Data Supplement). No ion suppression was observed in the methods (22–26) during analyses of FF samples. Steroid recoveries from FF samples were between 94% and 125%, compared with serum samples. The methods showed the same lower limits of quantification and upper limits of linearity for FF and serum samples.

STATISTICAL ANALYSIS
Unless otherwise stated, results were expressed as the median and the range. The Mann–Whitney U-test was used to compare groups, and P values <0.05 were considered statistically significant. Statistical analyses were performed with the JMP software package (SAS Institute).

Results
The steroids measured in FF samples belong to 5 classes: pregnenolones, progestins, androgens, estrogens, and glucocorticoids (see Fig. 1 in the online Data Supplement). Fig. 1 shows pie diagrams of the distributions of steroid concentrations in FF samples from RM women and from women after ovarian stimulation for IVF. A4 was the predominant steroid (46.8%) in RM women, followed by 17OHPPregn and pregn, whereas 17OHHPregn was the predominant steroid (54.0%) in women undergoing ovarian stimulation, followed by E2 and Pregn (Fig. 1). Compared with RM women, FF samples from women undergoing ovarian stimulation had significantly higher concentrations of E2, Pregn, 17OHHPregn, and F; significantly higher E2/Te ratios (indicative of 17β-hydroxysteroid oxidoreductase activity), E2/Te ratios [indicative of CYP19 (aromatase) activity], and F/E ratios (indicative of 11β-hydroxysteroid oxidoreductase activity); and significantly lower concentrations of 17OHPPregn, 11DC, E, DHEA, A4, and Te (Table 2).

We also evaluated the distribution patterns of steroid concentrations in ADFs (n = 13) and EDFs (n = 8) in RM women. ADFs were defined as follicles with E2/Te ratios <4, and EDFs were defined as those with E2/Te ratios >4 (4). The medians and central 90% intervals (i.e., 5th–95th percentile) for the E2/Te ratio were 0.63 (0.1–2.5) and 14.1 (4.0–73.0) in the ADF and EDF groups, respectively. Fig. 2 shows pie diagrams of the distributions of median steroid concentrations in the ADF and EDF groups. Table 3 presents results for steroids with significant differences between ADFs and EDFs. A4 was the predominant steroid (56.4%) in ADFs, followed by 17OHHPregn and DHEA. A4 was also the predominant steroid in EDFs (30.8%), followed by 17OHHPregn and E2 (Fig. 2). Compared with ADFs, EDFs had significantly higher E2 concentrations, signifi-
cantly higher E2/E1 ratios, and significantly lower A4 and Te concentrations (Table 3).

We evaluated the relationships between the concentrations of the steroids involved in the pathway (see Fig. 1 in the online Data Supplement) and the associations between the concentrations of the steroids within the ADF and EDF subgroups. In ADFs, the 17OHPregn concentration was associated with the DHEA concentration ($r = 0.95; P = 0.001$), the concentration of 17OHP was associated with concentrations of E1 ($r = 0.88; P = 0.001$), Te ($r = 0.86; P = 0.001$), and androstanedione ($r = 0.98; P = 0.002$), and the A4 concentration was significantly associated with the Te concentration ($r = 0.85; P = 0.001$). In EDFs, the 17OHP concentration was positively associated with the concentrations of 11DC ($r = 0.96; P = 0.002$) and E3 ($r = 0.88; P = 0.003$) and was negatively associated with the F/11DC ratio (indicative of CYP11 activity; $r = -0.87; P = 0.004$). The 17OHPregn concentration was positively associated with the DHEA concentration ($r = 0.95; P = 0.001$), whereas the E2 concentration was positively associated with the E3 concentration ($r = 0.92; P = 0.001$) and negatively associated with the F/11DC ratio (CYP11 activity; $r = -0.93; P = 0.001$). The A4/17OHP ratio (CYP17 activity) was negatively associated with the E2/Te ratio (CYP19 activity; $r = -0.89; P = 0.002$); the F/11DC ratio (CYP11 activity) was negatively associated with the 17OHPregn concentration.

The concentrations of steroids in FF samples from women after ovarian stimulation measured in this study with LC-MS/MS methods were compared (Table 4) with values reported in 3 previous studies that used...
IA methods (13–15) and 1 study that used liquid chromatography followed by spectrophotometric detection (12). Lower concentrations for DHEA, A4, E2, and F and considerably lower Te concentrations were observed in the present study than were obtained with these other techniques (Table 4).

**Discussion**

During last decade, tandem MS has become the method of choice for analyzing endogenous steroids (18–27). To our knowledge, there have been no published LC-MS/MS–based measurements of steroids in ovarian FF samples from RM women and from women after ovarian stimulation for IVF. In our study, LC-MS/MS methods produced lower concentrations for all androgens, especially for Te, than those obtained with other techniques (Table 4) (12–15). E2, A4, and 17OHP concentrations in FF samples from RM women were comparable to previously published values obtained with IAs (29–31). In FF samples from women undergoing ovarian stimulation for IVF, the concentrations of DHEA, E1, and E2 obtained with LC-MS/MS and IA methods were comparable (Table 4). The good agreement might be due to the relatively low concentrations of possible cross-reacting compounds, i.e., other 3β-hydroxy-5-ene steroids and estrogens (Table 2). On the other hand, large differences were found for the 3-oxo-4-ene steroids (A4, Te, and F), with IA values being much higher than those obtained with LC-MS/MS. The large differences were likely due to cross-reactivity with other 3-oxo-4-ene steroids in IAs, notably 17OHP and progesterone, which are present at very high concentrations in the FF of women undergoing ovarian stimulation (Table 2). The comparable concentrations of 17OHP and E obtained with LC-MS/MS and liquid chromatography–spectrophotometry (12) may be because both methods used chromatographic separation; however, A4 concentrations measured by liquid chromatography–spectrophotometry were affected by interference, likely caused by the similar chromatographic retentions of A4 and other androgens (25).

In previous reports, we described our LC-MS/MS–based measurements of steroid concentrations in serum samples from women of reproductive age (22–26). Compared with serum, the concentrations of 17OHP, androgens, and estrogens were 200- to 1000-
fold greater in FF during the early follicular phase of the menstrual cycle. This finding is consistent with the fact that ovarian follicles are the major site of biosynthesis for 17OHP and estrogens and an important site of androgen biosynthesis in women of reproductive age (32). The significant differences between RM women and women undergoing ovarian stimulation for IVF with respect to steroid concentrations in FF samples (Table 2) clearly reflect the effect of increased gonadotropin stimulation and androgen consumption for increased estrogen biosynthesis in women undergoing ovarian stimulation. Furthermore, the finding might reflect an increasingly reductive environment, as reflected by increased E2/E1 and F/E ratios. The occurrence of 17β-hydroxysteroid oxidoreductase in the ovaries is very well known, and the presence of 11β-hydroxysteroid oxidoreductase has also been demonstrated (33). The increased concentrations of Pregn and 17OHP in women undergoing ovarian stimulation for IVF may also reflect a generally increased stimulation of follicular steroidogenesis. The concentrations of F and E in FF samples were within the reference limits for serum (26). This result suggests that glucocorticoids are likely distributed to ovarian follicles by transport from the peripheral circulation, because 11β-hydroxylase has never been demonstrated in the ovaries; however, one cannot exclude ovarian production of 11DC by 21-hydroxylase from 17OHP, because 21-hydroxylase is present not only in the adrenal cortex but also in a number of other tissues, including the ovaries (34). The low lower limits of quantification for nonovarian steroids was important in this study because it allowed measurement of low concentrations of steroids that have not previously been reported for FF.

We observed substantial differences between ADFs and EDFs in Te, E2, E1, and A4 concentrations and in the E1/E2 ratio (Table 4). In addition, ADFs and EDFs differ in the associations between the concentrations of many of the steroid intermediates of the pathway; these associations are indicative of the relative enzyme activities in the ADF and EDF groups (35). Differentiation of follicles based on the prevalence of androgens or estrogens and evaluation of the corresponding enzyme activities could be useful for determining the mechanisms of selection of the dominant follicle.

The methods used in this study (22–27) allowed the quantification of 13 steroids from 40 μL of FF. To analyze these steroids with IA-based methods would require several milliliters of FF, which is an unrealistic sample size for follicles during the early follicular stage of the menstrual cycle. In addition, there are some pitfalls associated with use of IAs for analyzing FF samples. Compared with serum, FF has substantially higher concentrations of some steroids, and the differences in concentration may cause cross-reactivity that is not observed with serum samples (for which IAs are typically validated). Another problem is the need to dilute FF samples to reduce steroid concentrations to concentration ranges measurable by the IA. The characteristics of the diluents may alter the equilibrium with binding proteins and with conjugated forms of the steroids, thereby affecting the observed concentrations. These problems are not relevant to MS-based methods. For these reasons and because the tandem MS methods used in this study have been extensively validated (22–26) and shown to be free of interference [i.e., analysis specificity was evaluated in every sample by monitoring multiple fragments of each steroid}

### Table 4. Reported median concentrations of steroids in FF samples collected at oocyte retrieval from women undergoing ovarian stimulation.$^a$

<table>
<thead>
<tr>
<th>Follicular diameter, mm</th>
<th>Present study</th>
<th>De Sutter et al. (12)</th>
<th>Andersen (13)</th>
<th>Bergh et al. (14)</th>
<th>Smitz et al. (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>LC-MS/MS</td>
<td>LC-Spectro IA</td>
<td>IA</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>17OHP, μg/L</td>
<td>520</td>
<td>460</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DHEA, μg/L</td>
<td>2.7</td>
<td>—</td>
<td>—</td>
<td>4.8</td>
<td>—</td>
</tr>
<tr>
<td>A4, μg/L</td>
<td>6.8</td>
<td>19.3</td>
<td>14.1</td>
<td>18.6</td>
<td>14.6</td>
</tr>
<tr>
<td>Te, μg/L</td>
<td>0.3</td>
<td>—</td>
<td>2.9</td>
<td>5.5</td>
<td>4.4</td>
</tr>
<tr>
<td>E1, μg/L</td>
<td>24</td>
<td>—</td>
<td>—</td>
<td>29</td>
<td>—</td>
</tr>
<tr>
<td>E2, μg/L</td>
<td>240</td>
<td>390</td>
<td>594</td>
<td>373</td>
<td>431</td>
</tr>
<tr>
<td>F, μg/L</td>
<td>53</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>188</td>
</tr>
<tr>
<td>E, μg/L</td>
<td>12</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>18</td>
</tr>
</tbody>
</table>

$^a$ NA, data not available; LC-Spectro, liquid chromatography–spectrophotometry.
Although our study provides the first MS measurements of concentrations of steroids in FF during the early follicular phase and after ovarian stimulation for IVF, it has some limitations. The RM women, whose samples were used in the study, underwent laparoscopic operations for presumed pelvic adhesions. All individuals included in the study were women of European descent, so generalization to other populations should be done with caution.

In conclusion, our data provide the first MS-based concentrations for multiple steroids in ovarian FF samples from RM women and from women after ovarian stimulation. The ability to accurately analyze multiple steroids from minute samples of FF, together with calculated product–precursor ratios of steroids and their metabolites (indicative of enzyme activities), is crucial for a better understanding of typical ovarian physiology, the menstrual cycle, anovulation, and the effects of different ovarian stimulation regimens used in IVF programs.

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