Assessment of theca cell function prior to controlled ovarian stimulation: the predictive value of serum basal/stimulated steroid levels

Jean-Noël Hugues1,4, Lucie Theron-Gerard1, Christiane Coussieu2, Maud Pasquier1, Didier Dewailly3, and Isabelle Cedrin-Durnerin1

1Unit of Reproductive Medicine, Department of Obstetrics-Gynaecology, Hôpital Jean Verdier, Assistance Publique – Hôpitaux de Paris, Université Paris XIII, 93143 Bondy, France 2Laboratory of Biochemistry, Hôpital Pitie Salpêtrière, Assistance Publique – Hôpitaux de Paris, Université Paris VII, Paris, France 3Department of Endocrine, Gynaecology and Reproductive Medicine, Hôpital Jeanne de Flandre, Université Lille, Lille, France

4Correspondence address. Tel: +33-1-48-02-68-56; Fax: +33-1-48-02-68-60; E-mail: jean-noel.hugues@jvr.aphp.fr

BACKGROUND: Serum androgen levels correlate with ovarian sensitivity to follicle-stimulating hormone (FSH) but in practice, standard baseline serum testosterone (T) levels prior to in-vitro fertilization (IVF) may not be the most appropriate marker for determination.

METHODS: Infertile women enrolled in an IVF programme were included in this study. Serum T and Δ4-androstenedione (A), and the androgen precursor 17-hydroxyprogesterone (17-OHP) were measured before and 24 h after a gonadotropin-releasing hormone agonist stimulation test (GAST). An early follicular phase antral follicle count (AFC) was also performed. Patients were subsequently enrolled in a long gonadotrophin-releasing hormone agonist protocol with a standard FSH dose (150 IU) for 7 days to assess the association between androgen levels and ovarian responsiveness to FSH.

RESULTS: The GAST elicited a significant increase in serum androgen levels that was well correlated with AFC. 17-OHP showed the greatest response to GAST and strongest correlation with AFC. The 17-OHP response to GAST differentiated patients with high ovarian reserve (OR) from those with low or normal OR as assessed by AFC, whereas only the estradiol response could differentiate those with low AFC. GAST-stimulated serum levels of 17-OHP were also correlated with ovarian response to FSH. Using receiver operating characteristic curve analysis, stimulated 17-OHP levels were predictive of the ovarian response to controlled ovarian stimulation, with similar power to that observed with AFC but lower power than with anti-Müllerian hormone.

CONCLUSIONS: Serum androgen levels following GAST are correlated with AFC and ovarian response to FSH. Serum T is a less sensitive marker of theca cell function than 17-OHP.

Key words: theca cell function / antral follicle count / gonadotrophin-releasing hormone agonist / in-vitro fertilization

Introduction

Assessment of serum androgen levels prior to controlled ovarian stimulation might be useful to predict the ovarian response and, thus, to adjust the starting dose of exogenous gonadotrophins. Indeed, there is some evidence that ovarian sensitivity to follicle-stimulating hormone (FSH) is closely related to androgen production. For instance, in women with polycystic ovary syndrome (PCOS), high androgen levels are closely related to FSH hypersensitivity. This relationship likely results from the effect of androgens on follicle growth and number (Zawadzki and Dunaif, 1992; Jonard et al., 2003; Rotterdam Consensus, 2004; Azziz et al., 2009).

Similarly, serum androgen levels in normo-ovulatory women are correlated with the ovarian response to FSH as assessed by peak estradiol (E2) levels and the number of mature follicles, oocytes and embryos (Barbieri et al., 2005). It has also been suggested that basal serum androgen levels can predict the ovarian response to FSH (Frattarelli and Peterson, 2004; Frattarelli and Gerber, 2006).
Finally ovarian ageing is characterized by a reduced ovarian sensitivity to FSH, which is accompanied by a progressive decline in serum androgen levels (Davison et al., 2005). Indeed, even in women with early ovarian ageing and a preserved follicular pool, the ovarian capacity to secrete androgens is decreased under both basal and human chorionic gonadotrophin-stimulated conditions (Piltonen et al., 2003).

These data highlight the close association between androgen production and ovarian sensitivity to FSH. They are in line with experiments in monkeys which demonstrated that androgens act synergistically with FSH to promote granulosa cell proliferation and reduce the rate of cellular apoptosis (Vendola et al., 1998; Weil et al., 1999). They also indicate that evaluation of theca cell function should be performed in addition to measurement of granulosa cell markers. However, there is no consensual agreement on which serum androgen is able to best predict the ovarian response to FSH. Indeed, the results of serum androgen assays are both imprecise and inconsistent (Barth et al., 2007).

Measurement of serum total testosterone (T) is usually recommended during clinical work-up but this practice should be questioned (Barth 2007). The inaccuracy of the measurement of low serum T levels is particularly marked among older women (Miller et al., 2004). For greatest accuracy, T assays require extraction and purification from serum, but these steps are usually omitted with the use of commercial kits, which showed substantial variability in outcomes (Boots et al., 1998). Furthermore, the normal range of female serum hormone levels is usually designated by commercial kit manufacturers after hormonal measurement among a limited number of unselected women.

The described shortcomings of serum T assays dictate the need for consideration of other serum androgens and/or stimulation tests to better assess theca cell function, specifically for women whose serum T levels are at the lower end of the normal range.

Few data have been published on the ability of serum androgens to quantify theca function. The most documented situation is PCOS, in which stimulation of theca cells by endogenous luteinizing hormone (LH) during a gonadotrophin-releasing hormone agonist stimulation test (GAST) induces a more rapid increase in serum levels of Δ4-androstenedione (A) and the androgen precursor 17-hydroxyprogesterone (17-OHP) than in T (Barnes et al., 1989; Rosenfield et al., 1993, 1994; Pasquali et al., 2007). Thus, 17-OHP and A may represent better markers of theca cell function than T. With these assays, the GAST has been able to reveal occult biochemical ovarian hyperandrogenism in normo-ovulatory women (Chang et al., 2000). On the other hand, to our knowledge, there is no data showing that the GAST is able to detect theca cell insufficiency in women with low ovarian reserve (OR).

Therefore, the aim of this study was to explore the relationship between the theca cell function as assessed by serum steroid response to the GAST and the antral follicle count (AFC), which is a valid marker of OR (Broekmans et al., 2006). In addition, we compared the predictive value of these factors for the ovarian response to FSH.

Materials and Methods

Patients and treatment protocols

Ninety-one unselected women participating in an in-vitro fertilization (IVF) programme were included in this prospective, single centre study. Written informed consent was obtained from all participants.

On Day 3 of a spontaneous menstrual cycle, a blood sample to evaluate baseline hormone levels was taken in the morning, and a transvaginal ultrasound (TVUS) scan performed. A GAST was then performed, as part of the endocrinological work-up for IVF. This involved administration of a subcutaneous injection of 0.1 mg triptorelin (Decapeptyl®, Ipsen Biotech, France) and collection of a second blood sample after 24 h. Blood samples were frozen and stored until testing. All samples were tested using the same assay for each hormone (as described below).

Ultrasound scans

TVUS scans were performed using a vaginal probe. The ovarian volume and size of all follicles of 2–9 mm in diameter (the mean of two measurements) were recorded. All scans were performed by the same physician (CD). The total number of follicles of 2–9 mm observed in both ovaries on TVUS on Day 3 was used to classify patients into one of three groups based on recommendations issued by the Rotterdam Consensus (2004): Group 1: < 10 follicles (low AFC, n = 21); Group 2: 10–23 follicles (normal AFC, n = 55); Group 3: > 23 follicles (high AFC, n = 15). Patients in Group 1 had high plasma FSH levels on Day 3 (FSH > 10 mIU/l), whereas those in Group 2 had normal FSH levels. In addition, Group 3 consisted of patients with PCOS criteria according to the Rotterdam Consensus (2004).

Ovarian response to FSH

Later in the same cycle, 70 of the 91 women received a long luteal phase gonadotrophin-releasing hormone (GnRH) agonist protocol. From Day 22 to 25, a single daily dose of triptorelin 0.1 mg was administered by subcutaneous injection. Once pituitary desensitization was achieved, a fixed daily dose (150 IU) of recombinant human (r-h) FSH (GONAL®; Merck Serono S.A.—Geneva, Switzerland) was administered for 7 days.

Following this stimulation period with a fixed dose of FSH, the ovarian sensitivity to FSH was assessed on Day 8 by measurement of the serum E2 level and number of follicles of >10 and >14 mm in mean diameter.

Finally, patients were classified into two groups according to the number of follicles >17 mm on the day of human chorionic gonadotrophin (hCG) administration: patients with ≤2 mature follicles were considered to be low responders to FSH and those with ≥3 mature follicles were considered to be normal responders. An assessment of predictive factors was performed by receiver operating characteristic (ROC) curve analysis.

Hormonal assays

Serum androgens were measured using highly specific assays. Serum total T levels were measured using a radio-immunometric technique (Spectria Orion Diagnostics, Finland). The sensitivity of the assay was 0.1 ng/ml, with intra- and inter-assay coefficients of variation of 3.8–7.5 and 4.8–7%, respectively. Free T levels and the free androgen index were calculated after measurement of serum albumin and sex hormone binding globulin (Vermeulen et al., 1999). Measurement of serum extracted A levels were carried out by radio-immunooassay (Immunotech Beckman-Coulter, France): the sensitivity of the assay was 0.1 ng/ml with intra- and inter-assay coefficients of variation were below or equal to 8.1 and 11.9%, respectively. Serum levels of 17-OHP were measured using a radio-immunometric technique (17OH-RIA-CT KIP1409 BioSource Europe, Nivelles, Belgium). The detection limit of the assay was 0.02 ng/ml, with intra- and inter-assay coefficients of variation of 5.1–6.25 and 5.0–9.2%, respectively.

Anti-Müllerian hormone (AMH) concentrations were assessed using a highly specific enzyme-linked immunosorbent assay (ELISA DSL-10-14400, DSL, France). The sensitivity of this assay was 0.025 ng/ml, with intra- and inter-assay coefficients of variation of 5 and ≤8%, respectively. E2 measurements were performed using a...
radio-immunometric technique (Estradiol-2 Clinical Assays, DiaSorin). The detection limit of the assay was 2 pg/ml, with intra- and inter-assay coefficients of variation of 2.6–2.8 and 4.6%, respectively.

Statistical analysis

Statistical analysis was performed using StatView (Abacus Concepts, Berkeley, USA). Normal distribution was tested using the Kolmogorov–Smirnov test. Results are expressed as mean ± standard deviation for normally distributed variables and as median (10th–90th percentile) for those not normally distributed. Normal variables were compared using the Student t-test or ANOVA as appropriate. ANOVA was followed by post hoc analysis using the Bonferroni correction for comparison of three groups. Paired analyses were performed for repeated measures. Non-parametric tests (Mann–Whitney or Wilcoxon tests as appropriate) were used to analyse variables that were not normally distributed. Simple regression analyses were performed to correlate baseline/GAST-stimulated serum steroid levels to the AFC or ovarian response to FSH on Day 8 of ovarian stimulation. Every significant correlation was controlled by the others through stepwise multiple regression analyses when the independent variable was continuous and through ordinal logistic regression when it was ordinal (follicle number). P-values of <0.05 were considered significant.

ROC curves were constructed to examine the diagnostic test performance, i.e. its capacity to discriminate between normal and poor responders. Sensitivity against (1-specificity) was plotted at each performance, i.e. its capacity to discriminate between normal and poor responders. A value of 0.5 means that the test result is no better than chance.

Results

Ultrasound and hormonal assessment on Day 3–4

As shown in Table I, the GAST resulted in a significant increase in all mean serum steroid levels within the first 24 h. The largest increase was observed in E2 levels and the greatest increase in serum theca cell steroids was recorded for 17-OHP (Table I). In addition, significant positive correlations were found between the AFC and baseline or stimulated steroid values, except for baseline E2 (Table II). After stepwise multiple regression analysis, only stimulated E2, A and 17-OHP were retained in a significant predictive model for AFC (P < 0.0001).

Table I Serum steroid levels before and 24 h after a GAST (n = 91)

<table>
<thead>
<tr>
<th></th>
<th>T0 (n = 91)</th>
<th>T24 (n = 91)</th>
<th>Increase (%)</th>
<th>Significance (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP, ng/ml</td>
<td>0.87 ± 0.4</td>
<td>1.69 ± 0.81</td>
<td>94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T, ng/ml</td>
<td>0.42 ± 0.17</td>
<td>0.49 ± 0.2</td>
<td>14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A, ng/ml</td>
<td>1.6 (0.9–2.64)</td>
<td>1.73 (1–2.89)</td>
<td>08</td>
<td>0.0003</td>
</tr>
<tr>
<td>E2, pg/ml</td>
<td>33 (20–57.8)</td>
<td>116 (53–253)</td>
<td>251</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation or median (10th–90th percentile).

Table II Correlation between the AFC and serum steroid levels at baseline and after a GAST (n = 91)

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>r (correlation coefficient)</th>
<th>Significance (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP, ng/ml</td>
<td>T0</td>
<td>0.308</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>T24*</td>
<td>0.587</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T, ng/ml</td>
<td>T0</td>
<td>0.251</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>T24</td>
<td>0.426</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A, ng/ml</td>
<td>T0</td>
<td>0.271</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>T24*</td>
<td>0.482</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E2, pg/ml</td>
<td>T0</td>
<td>0.179</td>
<td>NS (P = 0.9)</td>
</tr>
<tr>
<td></td>
<td>T24*</td>
<td>0.604</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

17-OHP, 17-hydroxyprogesterone; A, Δ4-androstenedione; E2, estradiol; NS, not significant; T, testosterone.

Analysis of the hormone tests according to AFC

Analysis of baseline and stimulated steroid serum concentrations according to the AFC classification (see Materials and Methods section) is presented in Table III. By ANOVA, no baseline serum steroid mean level was significantly different between the three groups (Group 1: low AFC; Group 2: normal AFC and Group 3: excessive AFC). In contrast, all GAST-stimulated serum mean levels were significantly different. Post hoc analysis indicated, however, that these global differences were due to differences between Group 3 versus Group 1 or 2. Significance level was not reached in the comparisons between these Groups 1 and 2, except for GAST-stimulated E2 (Table III).

Analysis of the ovarian response to FSH according to the hormonal test

Ovarian sensitivity to FSH

Data on ovarian responses to FSH are presented in Table IV. By univariate analysis, no significant correlation was found between baseline and stimulated serum T levels and ovarian response on Day 8. In contrast, baseline and stimulated serum 17-OHP and A levels were significantly correlated with the ovarian response as assessed by plasma E2 and the number of follicles of >10 or >14 mm in diameter on Day 8. Similarly, AFC and serum AMH values correlated strongly
with these markers of ovarian response while baseline FSH levels correlated poorly (Table IV). After ordinal logistic regression analysis entering the theca steroids as dependent variables, only post-stimulated 17-OHP levels correlated significantly to the number of follicles >10 mm and no parameter correlated to the number of follicles >14 mm although there was a trend for post-stimulated 17-OHP levels ($P = 0.077$). After stepwise multiple regression analysis entering the theca steroids as dependent variables, only post-stimulated 17-OHP was included in a significant predictive model for E2 on Day 8 ($P < 0.0001$).

**Prediction of the ovarian response to FSH**

Of the 70 women who proceeded to triggering of ovulation, 50 had a normal response to FSH while 20 were considered as low responders according to the number of mature follicles at the time of hCG administration (see Materials and Methods section). A comparative analysis of clinical characteristics, hormonal and TVUS data between normal and low responders showed that they actually differed by age [median and (5th–95th percentiles): 30.0 (20–40) versus 35.0 (20–38) years, respectively, $P = 0.042$], AFC [17.0 (7–40) versus 12.0 (5–40), respectively, $P = 0.014$], serum AMH levels [1.98 (0.26–6.6) versus 0.83 (0–6.1) ng/ml, respectively, $P < 0.001$] and stimulated levels of 17-OHP [1.80 (0.51–3.7) versus 1.29 (0.20–6.6) ng/ml, respectively, $P < 0.001$] and stimulated levels of 17-OHP yielded an AUC very close to the one of AFC and slightly lower than the one of AMH. The best compromise between sensitivity (60%) and specificity (82%) was obtained with a threshold of stimulated 17-OHP at 1.55 ng/ml.

### Discussion

Our data show that serum theca steroid levels following stimulation by a GAST are well correlated with the AFC and with the ovarian sensitivity to FSH. This supports the concept of a close relationship between theca cell secretion and folliculogenesis. They indicate also that assessment of theca cell function under stimulated conditions is a valuable tool to identify patients at risk of a low response to FSH. For that purpose, measurement of serum 17-OHP and A are more informative than T.

As far as we know, the usefulness of androgen measurement in clinical work-up for assisted reproductive technology (ART) has never been documented while evaluation of conventional granulosa cell markers has been extensively studied (Broekmans et al., 2006). Besides, several methodological shortcomings related to the measurement of female serum androgens have been reported. Firstly, the use of commercial kits without serum extraction of androgens carries risks of a misdiagnosis of hyperandrogenism (Azziz, 2003) or androgen deficiency (Davison et al., 2005). Substantial variability in total serum T levels detected by commercially available kits has been reported, and this accounts largely for the limited utility of these assays in clinical practice (Boots et al., 1998). Furthermore, many variables may also alter serum T levels (Barbieri et al., 2005; Barth et al., 2007).
The use of stimulation tests has been suggested to avoid the problems related to the measurement of basal serum T and to improve the diagnostic value of androgen assessment. Stimulation with a GnRH agonist induces the release of endogenous LH and increases ovarian response to FSH related to strong pituitary desensitization (Barnes et al., 1989, 1994). Conversely, ovarian T production, which is primarily dependent on 17β-hydroxysteroid dehydrogenase activity type 5, is less dependent on LH control (Nelson et al., 2001).

Additionally, our data provide evidence that, in an unselected infertile population, the theca steroid response to GAST is highly correlated with the AFC, with the strongest correlation being observed for 17-OHP. This observation concurs with a previous report (Jonard et al., 2003), and supports the notion that androgens act positively on granulosa cell proliferation and follicular recruitment, as previously shown in monkeys (Vendola et al., 1998; Weil et al., 1998). Indeed, androgens exert paracrine effects via specific granulosa cell receptors (Horie et al., 1992; Hillier et al., 1997). Through this mechanism, they control the expression of FSH receptors as shown in humans (Suikkari et al., 1995) and in monkeys (Weil et al., 1999).

In addition, among theca steroids, stimulated serum 17-OHP levels correlated the best with the markers of ovarian responsiveness to exogenous FSH at Day 8 and, according to ROC analysis, they were able to predict a low response to FSH with a power very close to that observed with AFC and slightly lower than with AMH. Therefore, the GAST-stimulated 17-OHP may be more useful than baseline serum T in distinguishing between patients with a normal or a low OR. However, whether the GAST adds information to AFC and AMH for predicting the ovarian response to FSH needs further verification by prospective studies.

Finally, our results might explain why the addition of exogenous LH to stimulation protocols could be beneficial for women with reduced theca cell secretion. This population may include women aged over 35 years (Marrs et al., 2003; Humaidan et al., 2004) or those with a poor ovarian response to FSH related to strong pituitary desensitization

### Table IV

<table>
<thead>
<tr>
<th>Follies &gt;10 mm</th>
<th>Follies &gt;14 mm</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP, ng/ml</td>
<td>T0</td>
<td>$r = 0.351$</td>
</tr>
<tr>
<td></td>
<td>T24</td>
<td>$r = 0.449$</td>
</tr>
<tr>
<td>T, ng/ml</td>
<td>T0</td>
<td>$r = 0.102$</td>
</tr>
<tr>
<td></td>
<td>T24</td>
<td>$r = 0.164$</td>
</tr>
<tr>
<td>A, ng/ml</td>
<td>T0</td>
<td>$r = 0.428$</td>
</tr>
<tr>
<td></td>
<td>T24</td>
<td>$r = 0.492$</td>
</tr>
<tr>
<td>E2, pg/ml</td>
<td>T0</td>
<td>$r = 0.031$</td>
</tr>
<tr>
<td></td>
<td>T24</td>
<td>$r = 0.402$</td>
</tr>
<tr>
<td>FSH, ng/ml</td>
<td>T0</td>
<td>$r = 0.271$</td>
</tr>
<tr>
<td>AMH, pmol/l</td>
<td>T0</td>
<td>$r = 0.658$</td>
</tr>
<tr>
<td>AFC</td>
<td>T0</td>
<td>$r = 0.497$</td>
</tr>
</tbody>
</table>

The ovarian response was assessed by determination of the number of follicles of >10 mm and/or >14 mm and/or E2 levels on Day 8 of FSH stimulation. Significance was assumed if $P < 0.05$.  
17-OHP, 17-hydroxyprogesterone; A, Δ4-androstenedione; AFC, antral follicle count; AMH, anti-Müllerian hormone; E2, estradiol; NS, not significant; T, testosterone.

### Table V

| Areas under the ROC curves (AUC) for predicting the low response to COH in women with low or normal AFC ($n = 59$) |
|---------------------------------|------------------|------------------|
|                                  | AUC              | Significance     |
| AMH                             | 0.833            | 0.0001           | 0.703 | 0.963 |
| AFC                             | 0.719            | 0.009            | 0.557 | 0.882 |
| Basal 17-OHP                    | 0.624            | 0.139            | 0.459 | 0.789 |
| Stimulated 17-OHP               | 0.721            | 0.008            | 0.591 | 0.850 |
| Basal A                         | 0.598            | 0.241            | 0.416 | 0.780 |
| Stimulated A                    | 0.559            | 0.482            | 0.388 | 0.730 |
| Basal T                         | 0.468            | 0.7              | 0.276 | 0.660 |
| Stimulated T                    | 0.432            | 0.424            | 0.243 | 0.620 |
| Basal E2                        | 0.487            | 0.88             | 0.318 | 0.656 |
| Stimulated E2                   | 0.667            | 0.046            | 0.509 | 0.824 |

17-OHP, 17-hydroxyprogesterone; A, Δ4-androstenedione; AFC, antral follicle count; AMH, anti-Müllerian hormone; E2, estradiol; T, testosterone.
following administration of a depot formulation of GnRH agonist (Ferraretti et al., 2004; De Placido et al., 2005; Ruvolo et al., 2007). Indeed, it is feasible that intra-ovarian secretion of steroid by theca cells in response to LH contributes to follicular development. Therefore, one could speculate that, among women with a low AFC, only those with a positive GAST response would benefit from exogenous LH supplementation.

In conclusion, this study shows that measurement of 17-OHP following a GAST may be considered to be a valuable, albeit indirect, method for assessing ovarian function. A better understanding of the association between theca cell production and folliculogenesis could assist in identification of women who would benefit from LH supplementation of ART cycles.

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