

Variability between the follicular steroid hormone levels in different follicles of the same patient and between patients

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ABSTRACT

CONTEXT: There is disagreement as to whether the concentration of a substance in follicular fluid is related to the quality of the follicle as a possible reflection of the oocyte quality or whether this concentration is related to the clinical characteristics of the patient. **AIM:** To establish the variability of steroid hormone levels in follicular fluids from different follicles of the same patient and between patients. **SETTINGS AND DESIGN:** Prospective cohort study. **MATERIALS AND METHODS:** In 31 patients who underwent intracytoplasmic sperm injection it was performed an ultrasound guided aspiration of follicular fluid of the first two mature follicles from each ovary. Chemiluminescent microparticle immunoassays were performed to determine the levels of estradiol, progesterone, testosterone, and dehydroepiandrosterone sulfate (DHEA-S). **STATISTICAL ANALYSIS:** Unconditional mixed model. **RESULTS:** Variation in estradiol levels between follicles in the same patient was approximately twice the variation between subjects ($P = 0.05$). In the case of progesterone, the intra-subject variation was similar to the inter-subject variation ($P = 0.006$). The testosterone levels had a slightly smaller intra-subject variation than inter-subject variation ($P = 0.002$), and the intra-subject variation in DHEA-S levels was approximately one-fifth of the inter-subject variation ($P = 0.0003$). **CONCLUSIONS:** The variations in the levels of follicular progesterone and testosterone were similar between patients and between a given patient's follicles; however, the estradiol levels variability was higher among different follicles. The amount of follicular estradiol may be considered a selection tool between the follicles of a given patient.

KEY WORDS: Androgens, estradiol, follicular fluid, progesterone

INTRODUCTION

Follicular fluid is a key biochemical environment for proper oocyte and embryo development.^[1]

Several authors have shown that fertilized oocytes are derived from follicles with higher levels of estradiol and progesterone.^[2,3] In addition, follicular fluids with higher estradiol levels are associated with an increased chance of pregnancy.^[3,4] However, not all authors have succeeded in demonstrating these results.^[5,6] Regarding the androgen levels in follicular fluids, fertilized oocytes have been associated with higher levels of follicular testosterone.^[2,3] However, other studies have found no association between testosterone levels and cases of normal fertilization.^[6]

In this context, it may be questionable whether the concentration of a substance in follicular fluid is related to the quality of the follicle as a possible reflection of the oocyte quality or whether this concentration is related to the clinical characteristics of the patient (i.e. age and type of ovarian stimulation).^[1]

The objective of the present study was to determine the differences in steroid hormone levels in follicular fluids between different follicles of a given patient and between patients.

MATERIALS AND METHODS

Patient selection, ovarian stimulation cycle and analysis of hormones in follicular fluid was described in a previous study of this research group.^[3]

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Thirty-four patients aged ≤ 38 years, with a body mass index < 30 , tubal infertility, unknown origin infertility or male infertility who underwent a cycle of intracytoplasmic sperm injection (ICSI) were included in the study.

Causes of infertility with ovarian component of any kind (polycystic ovarian syndrome, endometriosis or diminished ovarian reserve) were excluded due to their possible interference in hormone levels.

Eligible patients were followed in the Reproduction Service from February 2011 to February 2013 and were included consecutively to avoid selection bias. All procedures were conducted under the ethical standards of the Helsinki Declaration of 1975, as revised in 2000.

All patients were subjected to a long protocol with hypothalamic gonadotropin-releasing hormone (GnRH) agonists and controlled ovarian stimulation with exclusive recombinant follicle-stimulating hormone (rFSH). Stimulation with rFSH was administered according to an individual base ovarian response, as measured by transvaginal ultrasound and serial measurements of serum estradiol. Three women were excluded from the study due to the requirement for human menopausal gonadotropin in the ovarian stimulation cycle. Follicular growth was monitored by transvaginal ultrasound, and the follicles were measured considering the mean diameter in two dimensions.

The criterion for the administration of 250 mcg of human chorionic gonadotropin was the presence of two or more follicles > 18 mm in diameter in association with consistent serum estradiol levels. Thirty-six hours after dosing, ultrasound-guided follicular aspiration of the oocyte-corona-cumulus and follicular fluid was performed.

The follicular puncture was performed with ultrasound guidance (HD3 Philips, Eindhoven, Holland). A puncture needle attached to the transducer by a guide, a continuously adjustable vacuum pump (Labotect Aspirator 4014, Gottingen, Germany), and a thermal block set at 37°C was required.

Each follicle was aspirated individually. The first and second follicle of each ovary with a suitable size (18–21 mm) and regular outline was aspirated, emptied slowly until complete collapse, and collected.

The volume of fluid aspirated for a single follicle was recorded and correlated with the corresponding size of the follicle, as reported by Wittmaack *et al.*^[7]

Follicular fluids with significant hematic content were excluded, ultimately yielding 73 follicular fluid samples from 31 patients. The follicular samples were centrifuged,

transferred to a 5 ml Falcon tube, and placed at -70°C for later analysis.

Chemiluminescent microparticle immunoassay technology was used for measuring and quantifying the concentration of various hormones. The intra-assay variation coefficient was $< 15\%$.

The following components were measured: Estradiol (ng/ml), progesterone (pg/ml), testosterone (ng/dl), and dehydroepiandrosterone sulfate (DHEA-S) (mg/dl). A linearity study was conducted to verify the dilution required, and a previous repeatability study validated the test used in the follicular fluid.

Estradiol, testosterone, and progesterone were measured in the Architect i2000 device (Abbott Diagnostics, Mandaluyong City, Philippines). DHEA-S was measured on an IMMULITE[®] 2000 immunoassay system (SIEMENS Healthcare, Erlangen, Germany).

Dilutions were conducted before the measurement of estradiol (1:1000) and progesterone (1:1000). The measurements of testosterone and DHEA-S did not require dilution due to its similarity to serum levels. The concentrations of 17-hydroxy-progesterone, total androgen, and estrogen can be 200–1000 times higher in follicular fluid than in plasma.^[8]

Statistical analysis

A mixed statistical study was undertaken. The change in hormone levels between subjects and within subjects with an unconditional mixed model was analyzed and compared. The percentage of variability due to differences between subjects (intraclass correlation coefficient [ICC]) was quantified, and the 95% confidence range for the different mean hormone levels among patients was obtained. The variation in hormone levels was evaluated with estimated covariance parameters. The distribution of the hormonal levels and of the residuals of each level (intra-subject and inter-subject) of the unconditional linear mixed model was represented.

Statistical significance was established for values of $P \leq 0.05$, considering all the bilateral statistics test.

SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) was used to process the data.

RESULTS

Intra-subject and inter-subject variability of the estradiol levels [Figure 1a-c]

The intra-subject variation was approximately twice the inter-subject variation ($P = 0.05$) [Table 1].

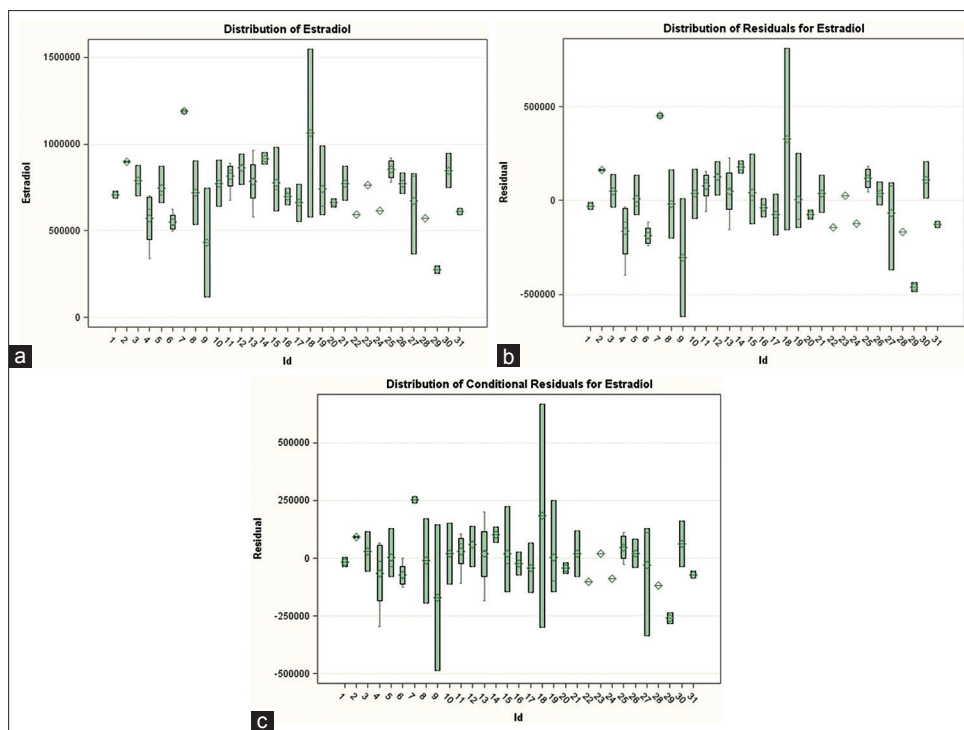


Figure 1: Intra-subject and inter-subject variability of the estradiol levels: (a) Distribution of the estradiol levels per patient, (b) distribution of the mean estradiol level variation per patient compared with the estradiol levels per patient, (c) distribution of the mean estradiol level variation per patient compared with the average global estradiol level, unconditional linear mixed model. Id: Identification

Table 1: Intra-subject and inter-subject variability of the estradiol levels

Estimate of covariance parameters	Estimate±SE
Inter-subject	1.31±8.18
Intra-subject	3.37±7.40

SE=Standard error

According to ICC, 28% of the total estradiol variability was due to the difference between subjects. Approximately, one-third of the variability corresponded to the difference between the average levels of estradiol. Thus, approximately 70% of the variability was explained by differences within each patient.

The 95% confidence interval representing the magnitude of change in the average estradiol level between subjects was (511794.57, 960801.43).

Intra-subject and inter-subject variability of the progesterone levels [Figure 2a-c]

The intra-subject variation was approximately the same as the inter-subject variation ($P = 0.006$) [Table 2].

In accordance to ICC: 51% of the total progesterone variability was due to the difference between patients.

The 95% confidence interval representing the progesterone variation between patients was (9599.63, 31820.37).

Intra-subject and inter-subject variability of the testosterone levels [Figure 3a-c]

The intra-subject variation was slightly less than the inter-subject variation ($P = 0.002$) [Table 3].

According to ICC: 54.78% of the total testosterone variability was due to the difference between subjects.

The 95% confidence interval representing the magnitude of change in the average testosterone level between subjects was (2.36, 7.55).

Intra-subject and inter-subject variability of the dehydroepiandrosterone sulfate levels [Figure 4a-c]

The intra-subject variation was approximately one-third the inter-subject variation ($P = 0.0003$) [Table 4].

In accordance to ICC: 78% of the total DHEA-S variability was due to a difference between patients.

The 95% confidence interval representing the DHEA-S variation between subjects was (30.94, 226.36).

DISCUSSION

The present study was designed to address the existing dilemma regarding whether the follicular concentration of a given hormone is predominantly an intra-individual

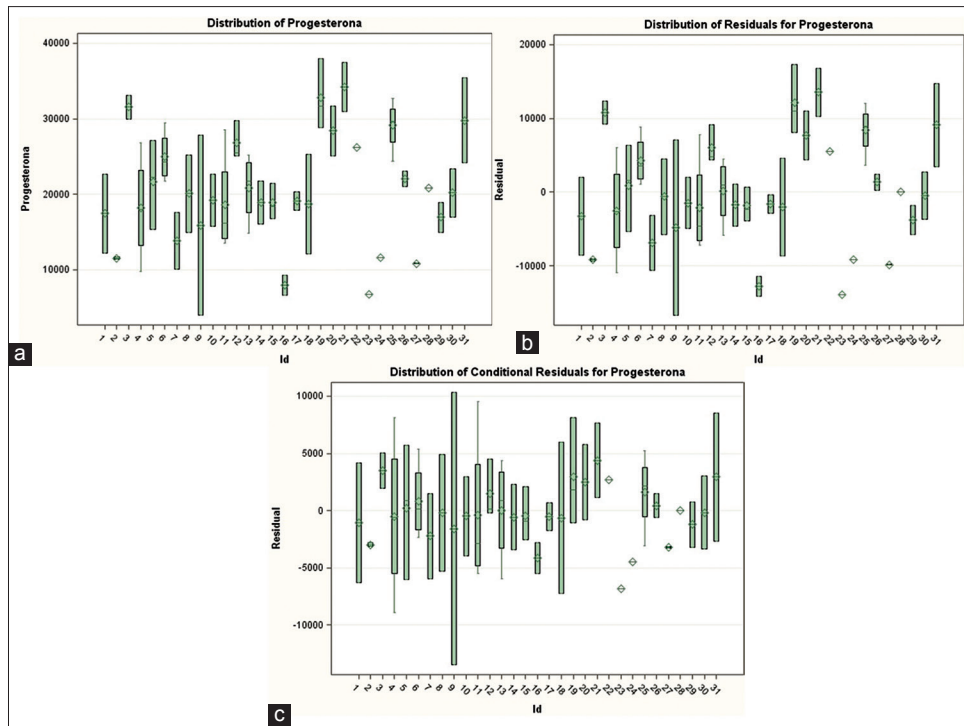


Figure 2: Intra-subject and inter-subject variability of the progesterone levels: (a) Distribution of the progesterone levels per patient, (b) distribution of the mean progesterone level variation per patient compared with the progesterone level per patient, (c) distribution of the average progesterone level variation per patient compared with the global average progesterone level, unconditional linear mixed model. Id: Identification

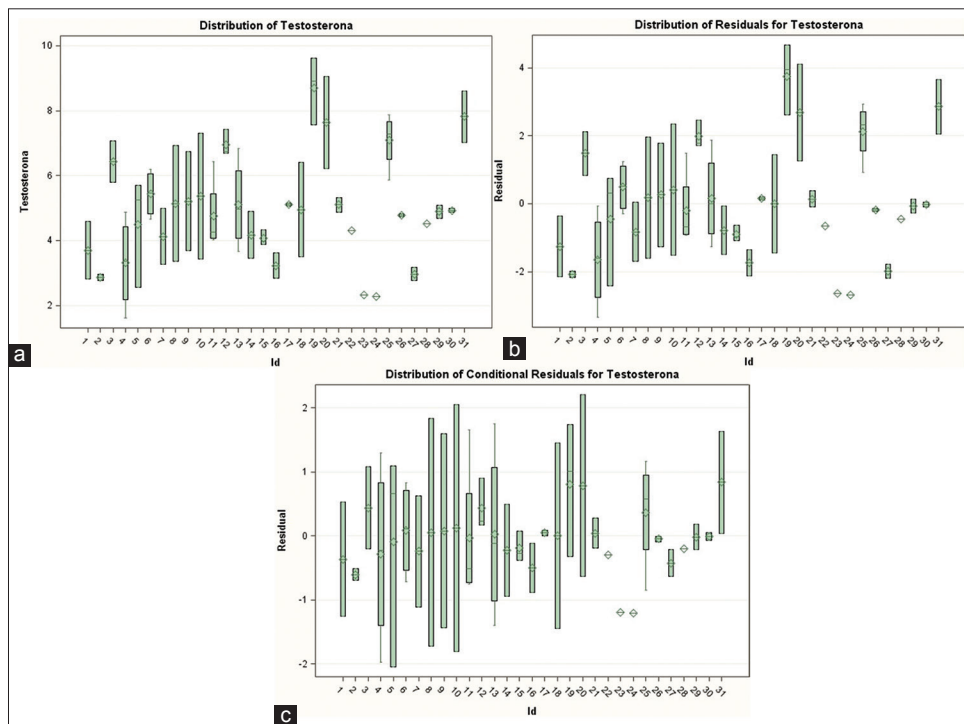


Figure 3: Intra-subject and inter-subject variability of the testosterone levels: (a) Distribution of the testosterone levels per patient, (b) distribution of the average testosterone level variation per patient compared with the testosterone levels per patient, (c) distribution of the average testosterone level variation per patient compared with the global average testosterone level, unconditional linear mixed model. Id: Identification

variable relative to the quality of each ovarian follicle of the patient during hormonal stimulation or whether this

concentration is an inter-individual variable potentially associated with the fertilization status of each woman.

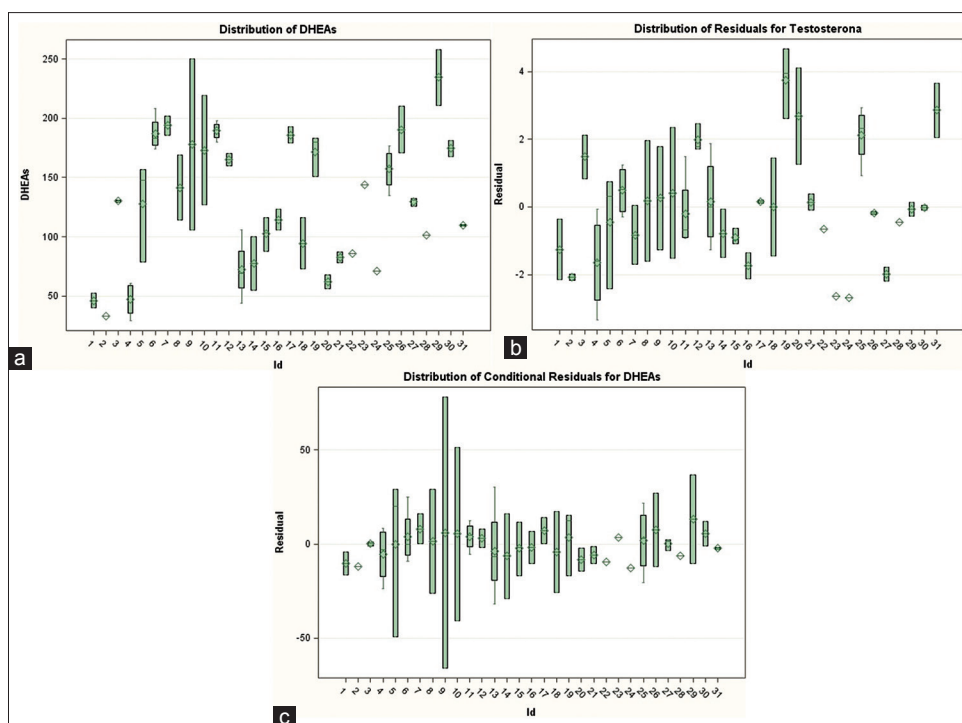


Figure 4: Intra-subject and inter-subject variability of the dehydroepiandrosterone sulfate (DHEA-S) levels: (a) Distribution of the DHEA-S levels per patient, (b) distribution of the mean DHEA-S level variation per patient compared with the DHEA-S levels per patient (c) distribution of the average DHEA-S level variation per patient compared with the global average DHEA-S level, unconditional linear mixed model. Id: Identification, DHEA-S: Dehydroepiandrosterone sulfate

Table 2: Intra-subject and inter-subject variability of the progesterone levels

Estimate of covariance parameters	Estimate±SE
Inter-subject	32,132,538±12,798,083
Intra-subject	30,931,756±6,904,590

SE=Standard error

Table 3: Intra-subject and inter-subject variability of the testosterone levels

Estimate of covariance parameters	Estimate±SE
Inter-subject	1.75±0.63
Intra-subject	1.44±0.31

SE=Standard error

Table 4: Intra-subject and inter-subject variability of the DHEA-S levels

Estimate of covariance parameters	Estimate±SE
Inter-subject	2485.17±728.07
Intra-subject	703.00±152.68

SE=Standard error, DHEA-S=Dehydroepiandrosterone sulfate

The results of several studies suggest that the local follicular environment may play a key role in the observed differences of the oocyte and embryo development capabilities.^[1-4]

To avoid the maximum possible interference in hormone levels and to generate a homogeneous sample, the present study was conducted under the most baseline conditions. Cases of infertility with an ovarian component, including

patients with a good quantitative ovarian reserve, were excluded. The patients within a particular age range were selected, and the same protocol of ovarian stimulation was applied in all cases. A long cycle with GnRH agonists and rFSH was used in mild patterns for ovarian stimulation, seeking the greatest resemblance to the physiological ovarian cycle.^[9]

The differences in the hormone levels might have been due to the different doses of stimulation employed. However, the criteria for follicular puncture were clearly established and were the same for all patients, and the individualization of dosage (based on each case) was necessary to optimize the results.

The first and second of each ovarian follicles were included as follicular fluid sample, selecting similar sized follicles with diameters >18 mm and <22 mm. The follicle size is related not only to oocyte maturation but also to the steroid hormone concentration;^[10,11] thus, when limiting the punctured follicles to a closed size, a comparison between different follicles becomes possible.

The follicular environment affects the development of oocytes and their reproductive outcomes. This fact has been demonstrated by various studies in which each individual oocyte was tracked after ICSI.^[2-4] However, by analyzing the

variation in the amount of steroid hormones from different follicles in 1 patient and between different patients, it has been observed that the percentage of the total variability due to inter-subject differences was not the same for all the studied hormone levels. For follicular progesterone and testosterone, the intra-patient and inter-patient variability were similar. By contrast, the DHEA-S variability was greater between subjects, whereas the estradiol variability was larger between different follicles of the same patient. When determining the DHEA-S level, its consideration as an indirect measurement of follicular DHEA should be addressed because cross-detection of DHEA and DHEA-S may appear in the results.

Thus, the amount of both follicular progesterone and testosterone should not be considered a good instrument for selecting between optimal follicles of a patient because the variability due to differences between patients and between follicles is similar. However, on one hand, estradiol levels may be considered an instrument for selecting follicles between different optimal follicles from 1 patient; on the other hand, DHEA-S may play a more relevant role in the selection between individuals.

Further studies with a larger number of patients and multiple follicles per patient would be required to define the specific role in each patient. According to previous results, the follicular progesterone and testosterone, indicators that are more involved in fertilization results,^[3] are those with similar intra-subject and inter-subject variabilities. Moreover, the estradiol levels, with more implications for pregnancy rates,^[3] are those with a larger variability between follicles of the same patient than between patients. Therefore, the amount of follicular estradiol may be considered an additional instrument for oocyte selection.

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