The relationship of premature progesterone rise with serum estradiol levels and number of follicles in GnRH antagonist/recombinant FSH-stimulated cycles

D. Kyrou [a,*], M. Al-Azemi [a], E.G. Papanikolaou [a], P. Donoso [a], K. Tziomalos [b], P. Devroey [a], H.M. Fatemi [a]

[a] Centre for Reproductive Medicine, Dutch-Speaking Free University Brussels, Laarbeeklaan 101, 1090 Brussels, Belgium
[b] First Propedeutic Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, AHEPA Hospital, Thessaloniki, Greece

A R T I C L E   I N F O

A B S T R A C T

Objective(s): To investigate the relationship between premature progesterone (P) rise and serum estradiol (E_2) levels and the number of follicles in GnRH antagonist/rec-FSH stimulated cycles.

Study design: Two hundred and seven patients treated by IVF/ICSI at the Centre for Reproductive Medicine of the Dutch-Speaking Brussels Free University were included in this observational study. They received 200 IU/day rec-FSH from day 2 of the cycle and daily GnRH antagonist starting on day 6 of stimulation. The criteria for hCG administration included the presence of ≥ 3 follicles of ≥ 17 mm diameter. Serum P, E_2 and LH were determined on the day of hCG administration. The outcome measure was to identify a threshold of E_2 and number of follicles on the day of hCG administration which would define a progesterone rise on the day of hCG administration (cut-off value of 1.5 ng/ml).

Result(s): Patients with a P >1.5 ng/ml had significantly higher concentrations of E_2 and increased number of follicles on the day of hCG administration compared to those with P ≤ 1.5 ng/ml. However, patients with a P >1.5 ng/ml the day of hCG showed lower pregnancy rates than those with P <1.5 ng/ml (17.8 vs. 32.7%, respectively; $p < 0.05$). A ROC curve was employed in order to estimate a cut-off for E_2 on day of hCG >1790.5 pg/ml and more than 9.5 follicles of ≥ 11 mm in diameter for progesterone rise over 1.5 ng/ml.

Conclusion(s): A significant impact is shown on progesterone rise by E_2 and number of follicles on the day of hCG administration in GnRH antagonist/rec-FSH-stimulated cycles. With this knowledge, an upcoming progesterone rise during follicular phase can be anticipated and prevented.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Serum progesterone (P) concentrations are low (<1.5 ng/ml) during the normal early follicular phase of ovulatory cycles, but tend to increase gradually 12–24 h before the onset of the LH surge [1]. The stimulation protocols in IVF treatment induce supraphysiological serum concentrations of estradiol (E_2) during the follicular phase. Despite the administration of GnRH analogues, however, moderate elevation in serum progesterone concentrations can be observed during the late follicular phase, which could influence endometrial development [2].

Many researchers have incorrectly described the progesterone rise during the late follicular phase as "premature luteinization" [3–6]. This process is defined as a rise in serum P levels above 1.5 ng/ml towards the end of the follicular phase [7]. No association has been reported between progesterone elevation

and fertilization rates or oocyte/embryo quality [8,9], which might indicate absence of a detrimental effect of elevated progesterone on the day of hCG administration on oocyte quality, as previously suggested [3–5,10–13]. However, a negative impact of elevated progesterone levels during the follicular phase on pregnancy rates has been described [3,14]. This may affect endometrial receptivity and embryo implantation.

Although progesterone rise has historically been associated with LH in the context of premature LH surges, more recent studies assign the elevated progesterone to FSH and estradiol exposure during the follicular phase [3,4,6]. Changes in paracrine regulation could explain an increase in progesterone production in the late follicular phase [15]. The pathologic mechanism is probably the high response of the ovary to the stimulation, which results in an excess number of follicles and proliferating granulosa cells. The high follicular P levels could lead to an advancement of the endometrium, disturbing the dialogue between embryo and endometrium and resulting in implantation failure [16,17].

Several studies [18,19] failed to demonstrate any negative impact of increased serum P levels during the follicular phase on pregnancy rates. Recent studies, however, did confirm significant

changes in endometrial gene expression and reduced ongoing pregnancy in patients with elevated serum P during the late follicular phase [7,14,16,20].

Changes in hormone concentrations carry significant biological messages. Understanding and proving this theory could give rise to better approaches to treatment and risk assessment. Therefore, the objective of this study was to investigate the relationship between premature P rise and serum E_2 levels and the number of follicles in GnRH antagonist/rec-FSH stimulated cycles.

2. Materials and methods

Two hundred and seven patients treated by IVF/ICSI at the Centre for Reproductive Medicine of the Dutch-Speaking Brussels Free University, were included in this observational study, between October 2007 and December 2008. Patients could enter the study only once. Inclusion criteria were age ≤ 39 years, body mass index (BMI) between 18 and 29 kg/m^2, presence of both ovaries and basal levels of estradiol (≤ 80 pg/ml) and progesterone (≤ 1.6 ng/ml) on day 1 of the cycle. Exclusion criteria were the presence of endometriosis stage ≥ 3 (American Fertility Society), polycystic ovarian syndrome (Rotterdam criteria), the need for preimplantation genetic diagnosis and azoospermia testicular sperm extraction.

Recombinant FSH (rec-FSH) (Puregon; Merck Sharpe Dome) was started on day 2 of the menstrual cycle at a dose of 200 IU/day in all patients. The dose of rec-FSH remained fixed during stimulation until day 10 of the cycle. If it was necessary to increase the dose of rec-FSH after 10 days of stimulation, or to decrease the dose of rec-FSH due to a risk of ovarian hyperstimulation syndrome (OHSS) patients were excluded from the study. GnRH antagonist (Orgalutran; Merck Sharpe Dome) was started on day 6 of rec-FSH stimulation.

Triggering of final oocyte maturation was performed by using 10,000 IU hCG (Pregnyl; Merck Sharpe Dome) as soon as at least three follicles ≥ 17 mm were present on ultrasound (US) scan. Oocyte retrieval was carried out 36 h after hCG administration by transvaginal ultrasound-guided puncture of follicles.

Hormonal assessment was performed at the initiation of stimulation, on day 6 of rec-FSH stimulation, on day 8 and on the day of hCG administration. Additional blood samples were taken as necessary between antagonist initiation and hCG administration. Serum LH, FSH, E_2, P and hCG were measured by means of the automated Elecsys immunoanalyzer (Roche Diagnostics, Mannheim, Germany). Intra-assay and interassay coefficients of variation (CVs) were <3 and $<4\%$ for LH, <3 and $<6\%$ for FSH, <5 and $<10\%$ for E_2, <3 and 5% for P and <5 and $<7\%$ for hCG, respectively.

US scan was performed on day 6 of stimulation and thereafter as necessary in order to ensure that hCG would be injected on the first day that the patient had ≥ 3 follicles of ≥ 17 mm.

The objective of the study was to identify a threshold of E_2 and number of follicles on the day of hCG administration which would define a progesterone rise on day hCG (cut-off value of 1.5 ng/ml; [14]).

The data are expressed as mean (SD). All continuous variables followed normal distribution as assessed with the Kolmogorov–Smirnov test. A logistic regression model was used to assess the relationship between E_2 and number of follicles with high progesterone on day hCG (>1.5 ng/ml). Maximum accuracy calculated via the ROC curve was then employed in order to estimate the best cut-off for E_2 and number of follicles on day hCG when progesterone is >1.5 ng/ml. JMP 8.0 (SAS Inst., Cary NC) was used for data analysis.

Table 1
Baseline characteristics and stimulation data of the study population.

Parameters	n = 207
Age (years)	32.9 ± 3.7
BMI (kg/m^2)	23.2 ± 3.3
No of trials	1.1 ± 0.5
Basal FSH (IU/l)	7.8 ± 2.6
Cause of infertility (%)	
Male	132 (63.8)
Ovulatory	4 (1.9)
Tubal	19 (9.2)
Idiopathic	48 (23.2)
Reproductive disorder	4 (1.9)
Treatment (%)	
ICSI	168 (81.2)
IVF	39 (18.8)
Stimulation	
Duration of stimulation (days)	9.3 ± 1.5
Total FSH administered (IU)	1859 ± 310.6
Progesterone on day of hCG (ng/ml)	1.2 ± 0.6

Values are means ± (standard deviation).

3. Results

Baseline characteristics and stimulation data of the participants are shown in Table 1. Forty-five women (21.7%) had serum progesterone levels >1.5 ng/ml on the day of hCG administration.

Patients with a P >1.5 ng/ml had significantly higher concentrations of E_2 and increased number of follicles on the day of hCG administration compared to those with P ≤ 1.5 ng/ml. However, patients with a P >1.5 ng/ml on the day of hCG showed lower pregnancy rates than those with P <1.5 ng/ml (17.8 vs. 32.7%, respectively; $p < 0.05$) (Table 2). In contrast, at P levels higher than 1.5 ng/ml on the day of hCG, there was no difference in pregnancy rates between women with P levels above any cut-off value and women with P levels below this cut-off value.

A logistic regression model was performed to assess the relationship between E_2 and number of follicles on hCG day with progesterone rise (over 1.5). The number of follicles on the day of hCG administration predicted serum progesterone levels >1.5 ng/ml on that day (odds ratio (OR) 1.090, 95% CI 1.026–1.159, $p = 0.005$) whereas serum E_2 levels on the day of hCG administration were not predictive (OR 1.000, 95% CI 0.999–1.000, $p = $ NS). The Nagelkerke R^2 of the model was 0.277.

The area under the ROC curve for prediction, from serum E_2 levels on the day of hCG administration, of a serum progesterone level >1.5 ng/ml on that day was 0.600 (95% confidence interval (CI) 0.505–0.695, $p < 0.05$), with a cut-off value of ≥ 1790.5 pg/ml for serum E_2 levels giving a sensitivity, specificity, positive and negative predictive value of 57.8, 61.1, 29.2 and 83.9%, respectively, for predicting serum progesterone levels >1.5 ng/ml. The area under the ROC curve for prediction, from number of follicles on the day of hCG administration, of a serum progesterone levels >1.5 ng/ml on that day was 0.590 (95% CI 0.501–0.679, $p < 0.05$), with a cut-off value of ≥ 9.5 follicles giving a sensitivity, specificity, positive and negative predictive value of 64.4, 51.2, 26.8 and 83.8%, respectively, for predicting serum progesterone levels >1.5 ng/ml (Fig. 1).

A serum progesterone concentration >1.5 ng/ml had sensitivity, specificity, positive and negative predictive value for predicting an unsuccessful attempt at pregnancy induction of 34.2, 80.3, 80.6 and 33.8%, respectively.

As expected, serum P levels on day 6 and 8 correlated with serum P levels on the day of hCG administration ($r = 0.321$ and $r = 0.486$, respectively; $p < 0.001$ for both correlations). In contrast, serum P levels on day 1 did not correlate with serum P levels on the day of hCG administration ($r = 0.112$, $p = 0.111$).

Table 2
Characteristics of women according to serum progesterone levels on the day of human chorionic gonadotropin (hCG) administration.

	Women with serum P >1.5 ng/ml on the day of hCG administration ($n=45$)	Women with serum P ≤ 1.5 ng/ml on the day of hCG administration ($n=162$)	p
Serum E_2 levels on the day of hCG administration (pg/ml)	2303.2 ± 1383.3	1881.1 ± 1175.8	<0.05
Number of follicles on the day of hCG administration	12.6 ± 5.5	11.1 ± 5.9	
Pregnancy rate (%)	17.8	32.7	

4. Comments

The results of the present study demonstrated that there is a relation between a premature progesterone rise during the late follicular phase and E_2 and the number of follicles on hCG day in GnRH antagonist/rec-FSH stimulated cycles.

Despite the use of GnRH analogues, subtle increases in serum progesterone levels beyond a defined threshold value have been observed at the end of the follicular phase during ovarian stimulation. Although the frequency of elevated serum progesterone levels varies, incidences as high as 35% of stimulated cycles in women treated with GnRH agonists [18,19] and 38% of cycles in women treated with GnRH antagonists [3,6] have been reported. In a large retrospective analysis of over 4000 cycles, however, the incidence of progesterone rise on the day of hCG administration above 1.5 ng/ml, was estimated to be 8.4% in agonist and antagonist cycles [14].

Recent publications confirmed that a premature progesterone rise during the follicular phase is associated with lower pregnancy and implantation rates [14,16,21].

Despite the overwhelming evidence for the crucial role of progesterone in ovulation, its origin and the regulation of its secretion throughout the follicular phase of the cycle remains poorly understood. Some publications [2,15] hypothesized that the premature progesterone rise is related to a lack of LH activity in the drugs used for ovarian stimulation. However, the source of progesterone present during the follicular phase is not only the ovary but also the adrenal gland.
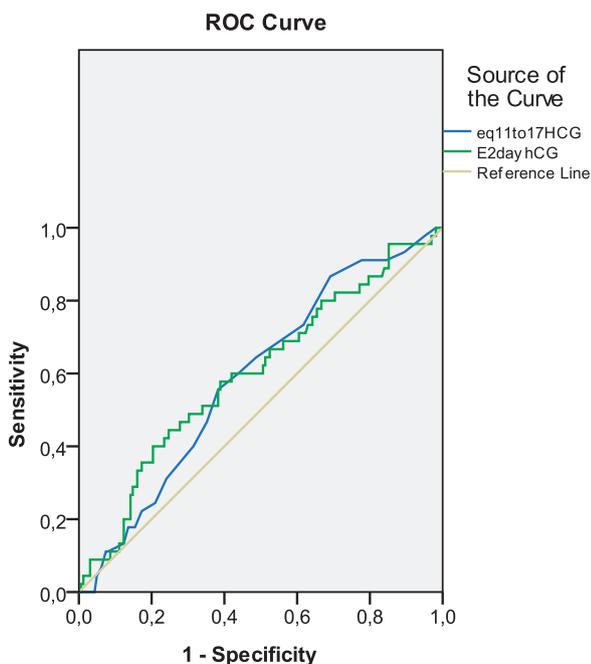

Fig. 1. Area under the receiver-operating characteristic (ROC) curve for serum E_2 and number of follicles measurement on the day of hCG administration.

In the ovary, FSH acts on granulosa cells promoting cell proliferation and steroid biosynthesis from cholesterol leading to progesterone biosynthesis. Granulosa cells are very active manufacturers of progesterone. Theca cells also make significant amounts of progesterone. According to the two-cell, two-gonadotropin theory of estrogen biosynthesis [22], progesterone is then metabolized to androgens (mainly androstenedione) by only theca cells under the influence of LH. Androgens are subsequently converted to estrogens through aromatization back in the granulosa cells. This step is limited by the amount of precursor available, which in turn depends on LH levels.

Recently, however, it was suggested that once the progesterone has been produced by either the theca or granulosa cells it cannot be converted into androgens and subsequent estradiol. If anything it might be converted into 17-hydroxyprogesterone, which also possesses progestogenic activity [23]. This conception is in disagreement with the theory that granulosa cell-derived progesterone can be converted into androgens in the theca cell compartment [2,14].

In summary, there are two sources of progesterone in the follicle, but only one step of further metabolism (conversion of progesterone to androgens), which is driven by LH activity in theca cells. Therefore, if progesterone production (by active granulosa cells) exceeds the limit of LH available, it is likely to leave more progesterone to find its way into the general circulation. Therefore, the origin of late follicular phase progesterone has been assumed to be a lack of LH in the late follicular phase of stimulated cycles [15]. On the other hand, the ovary is not the only source of progesterone production during the follicular phase [22]. The dominant source of progesterone in the follicular phase is the adrenal gland under the control of ACTH. Fanchin et al. [24] observed that during ovarian stimulation with HMG for IVF, peak concentrations of plasma progesterone, testosterone and androstenedione occurred during the early morning, coincidental with the circadian elevation in ACTH and adrenal function. Casson et al. [25] showed that adrenal DHEA-S secretion was augmented by ovarian hyperstimulation. This implies that ovarian hyperstimulation may induce adrenal steroid synthesis. In rats, estrogen administration causes an elevation in ACTH, which induces adrenal progesterone secretion by impairment of the glucocorticoid receptor-mediated negative feedback on CRF and ACTH secretion [26]. This subtle increase in serum progesterone stimulates secretion of LH. Whether similar or alternative interactions between the hypothalamic–pituitary–ovarian and the hypothalamic–pituitary–adrenal axes exist in human is as yet unknown. The increase in estradiol concentration in stimulated cycles does increase ACTH concentrations, which stimulate progesterone secretion from the adrenals [22].

Although premature progesterone rise is an important issue for implantation failure in IVF, this is the first study to propose a predictive model in order to anticipate this phenomenon. Since premature progesterone rise is related to estradiol levels and the number of follicles [15], with this study we have demonstrated that in rec-FSH/GnRH antagonist-stimulated cycles, a serum E_2 on day of hCG of ≥ 1790.5 pg/ml and more than 9.5 follicles of ≥ 11 mm in diameter have a significant effect on progesterone rise

over 1.5 ng/ml on the day of hCG administration for final oocyte maturation.

It becomes evident from the current analysis that even relatively low levels of serum E_2 (1.790.5 ng/l) and number of follicles (9.5), could lead to a premature progesterone rise.

Premature progesterone rise in stimulated IVF cycles is a frequent phenomenon, which is associated with lower pregnancy and implantation rates. With this knowledge, an upcoming progesterone rise during the follicular phase of GnRH antagonist/rec-FSH stimulated cycles can be anticipated and prevented.

Prevention could take two different approaches. A shift towards milder ovarian stimulation for IVF could be one of the possible methods. However, further sufficiently powered prospective studies applying mild treatment regimens for IVF are required to demonstrate their efficiency.

On the other hand, very limited data are available regarding the timing of the trigger for final oocyte maturation in rec-FSH/GnRH antagonist-stimulated IVF cycles. It has to be mentioned that the diameter of 17 mm in this study was arbitrary. Kolibianakis et al. [27] demonstrated clearly that prolongation of the follicular phase by delaying hCG administration decreases significantly the ongoing pregnancy rates, but the cutoff level for triggering final oocyte maturation was taken from a follicle size of 17 mm. Recently, Kyrou et al. [28] showed that earlier administration of hCG (as soon as there are three or more follicles of size $\geq$16 mm) which was expected to result in lower progesterone levels on the day of hCG administration, is not associated with the probability of pregnancy in cycles stimulated with rec-FSH and GnRH antagonists. Therefore future studies should focus on whether triggering at a smaller size of follicles (<17 mm) in high responder patients would jeopardize the outcome by having too many immature oocytes.

A further possible approach, however, once the progesterone level breached the level compatible with successful outcome, might be freezing of all embryos and embryo transfer in a natural cycle. This approach is supported by Melo et al. [8] who concluded that progesterone rise does not appear to have a negative impact on the ongoing pregnancy rate in an oocyte-donation programme.

In conclusion, these data demonstrate a significant effect on progesterone rise by E_2 and number of follicles in GnRH antagonist/rec-FSH stimulated cycles. Both of them have a predictive value on premature progesterone rise.

References

[1] Hoff JD, Quigley ME, Yen SS. Hormonal dynamics at midcycle: a reevaluation. J Clin Endocrinol Metab 1983;57:792–6.

[2] Fleming R, Jenkins J. The source and implications of progesterone rise during the follicular phase of assisted reproduction cycles. Reprod Biomed Online 2010;21:446–9.

[3] Bosch E, Valencia I, Escudero E, et al. Premature luteinization during gonadotropin-releasing hormone antagonist cycles and its relationship with in vitro fertilization outcome. Fertil Steril 2003;80:1444–9.

[4] Hofmann GE, Bentzien F, Bergh PA, et al. Premature luteinization in controlled ovarian hyperstimulation has no adverse effect on oocyte and embryo quality. Fertil Steril 1993;60:675–9.

[5] Legro RS, Ary BA, Paulson RJ, Stanczyk FZ, Sauer MV. Premature luteinization as detected by elevated serum progesterone is associated with a higher pregnancy rate in donor oocyte in vitro fertilization. Hum Reprod 1993;8:1506–11.

[6] Ubaldi F, Camus M, Smitz J, Bennink HC, Van Steirteghem A, Devroey P. Premature luteinization in in vitro fertilization cycles using gonadotropin-releasing hormone agonist (GnRH-a) and recombinant follicle-stimulating hormone (FSH) and GnRH-a and urinary FSH. Fertil Steril 1996;66:275–80.

[7] Van Vaerenbergh I, Fatemi HM, Blockeel C, et al. Progesterone rise on HCG day in GnRH antagonist/rFSH stimulated cycles affects endometrial gene expression. Reprod Biomed Online 2011;22:263–71.

[8] Melo MA, Meseguer M, Garrido N, Bosch E, Pellicer A, Remohi J. The significance of premature luteinization in an oocyte-donation programme. Hum Reprod 2006;21:1503–7.

[9] Polotsky AJ, Daif JL, Jindal S, Lieman HJ, Santoro N, Pal L. Serum progesterone on the day of human chorionic gonadotropin administration predicts clinical pregnancy of sibling frozen embryos. Fertil Steril 2009;92:1880–5.

[10] Fanchin R, Righini C, Olivennes F, de Ziegler D, Selva J, Frydman R. Premature progesterone elevation does not alter oocyte quality in in vitro fertilization. Fertil Steril 1996;65:1178–83.

[11] Martinez F, Coroleu B, Clua E, et al. Serum progesterone concentrations on the day of HCG administration cannot predict pregnancy in assisted reproduction cycles. Reprod Biomed Online 2004;8:183–90.

[12] Moffitt DV, Queenan Jr JT, Shaw R, Muasher SJ. Progesterone levels on the day of human chorionic gonadotropin do not predict pregnancy outcome from the transfer of fresh or cryopreserved embryos from the same cohort. Fertil Steril 1997;67:296–301.

[13] Shulman A, Ghetler Y, Beyth Y, Ben-Nun I. The significance of an early (premature) rise of plasma progesterone in in vitro fertilization cycles induced by a 'long protocol' of gonadotropin releasing hormone analogue and human menopausal gonadotropins. J Assist Reprod Genet 1996;13:207–11.

[14] Bosch E, Labarta E, Crespo J, et al. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization: analysis of over 4000 cycles. Hum Reprod 2010;25:2092–100.

[15] Smitz J, Andersen AN, Devroey P, Arce JC. Endocrine profile in serum and follicular fluid differs after ovarian stimulation with HP-hMG or recombinant FSH in IVF patients. Hum Reprod 2007;22:676–87.

[16] Kyrou D, Popovic-Todorovic B, Fatemi HM, et al. Does the estradiol level on the day of human chorionic gonadotropin administration have an impact on pregnancy rates in patients treated with rec-FSH/GnRH antagonist? Hum Reprod 2009;24:2902–9.

[17] Papanikolaou EG, Kolibianakis EM, Pozzobon C, et al. Progesterone rise on the day of human chorionic gonadotropin administration impairs pregnancy outcome in day 3 single-embryo transfer, while has no effect on day 5 single blastocyst transfer. Fertil Steril 2009;91:949–52.

[18] Edelstein MC, Seltman HJ, Cox BJ, Robinson SM, Shaw RA, Muasher SJ. Progesterone levels on the day of human chorionic gonadotropin administration in cycles with gonadotropin-releasing hormone agonist suppression are not predictive of pregnancy outcome. Fertil Steril 1990;54:853–7.

[19] Silverberg KM, Burns WN, Olive DL, Riehl RM, Schenken RS. Serum progesterone levels predict success of in vitro fertilization/embryo transfer in patients stimulated with leuprolide acetate and human menopausal gonadotropins. J Clin Endocrinol Metab 1991;73:797–803.

[20] Labarta E, Martínez-Conejero JA, Alamá P, et al. Endometrial receptivity is affected in women with high circulating progesterone levels at the end of the follicular phase: a functional genomics analysis. Hum Reprod 2011;26:1813–25.

[21] Kiliçdag EB, Haydardedeoglu B, Cok T, Hacivelioglu SO, Bagis T. Premature progesterone elevation impairs implantation and live birth rates in GnRH-agonist IVF/ICSI cycles. Arch Gynecol Obstet 2010;281:747–52.

[22] Eldar-Geva T, Margalioth EJ, Brooks B, Algur N, Zylber-Haran E, Diamant YZ. The origin of serum progesterone during the follicular phase of menotropin-stimulated cycles. Hum Reprod 1998;13:9–14.

[23] Yding Andersen C, Bungum L, Nyboe Andersen A, Humaidan P. Preovulatory progesterone concentration associates significantly to follicle number and LH concentration but not to pregnancy rate. Reprod Biomed Online 2011;23:187–95.

[24] Fanchin R, de Ziegler D, Castracane VD, Taieb J, Olivennes F, Frydman R. Physiopathology of premature progesterone elevation. Fertil Steril 1995;64:796–801.

[25] Casson PR, Kristiansen SB, Umstot E, Carson SA, Buster JE. Ovarian hyperstimulation augments adrenal dehydroepiandrosterone sulfate secretion. Fertil Steril 1996;65:950–3.

[26] Burgess LH, Handa RJ. Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. Endocrinology 1992;131:1261–9.

[27] Kolibianakis EM, Albano C, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Prolongation of the follicular phase in in vitro fertilization results in a lower ongoing pregnancy rate in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. Fertil Steril 2004;82:102–7.

[28] Kyrou D, Kolibianakis EM, Fatemi HM, Tarlatzis BC, Tournaye H, Devroey P. Is earlier administration of human chorionic gonadotropin (hCG) associated with the probability of pregnancy in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone (GnRH) antagonists? A prospective randomized trial. Fertil Steril 2011;96:1112–5.