

Gonadotrophin stimulation for *in vitro* fertilization significantly alters the hormone milieu in follicular fluid: a comparative study between natural cycle IVF and conventional IVF

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STUDY QUESTION: Is the steroid hormone profile in follicular fluid (FF) at the time of oocyte retrieval different in naturally matured follicles, as in natural cycle IVF (NC-IVF), compared with follicles stimulated with conventional gonadotrophin stimulated IVF (cIVF)?

SUMMARY ANSWER: Anti-Mullerian hormone (AMH), testosterone (T) and estradiol (E2) concentrations are ~3-fold higher, androstenedione (A2) is ~1.5-fold higher and luteinizing hormone (LH) is ~14-fold higher in NC-IVF than in cIVF follicles, suggesting an alteration of the follicular metabolism in conventional gonadotrophin stimulated IVF.

WHAT IS KNOWN ALREADY: In conventional IVF, the implantation rate of unselected embryos appears to be lower than in NC-IVF, which is possibly due to negative effects of the stimulation regimen on follicular metabolism. In NC-IVF, the intrafollicular concentration of AMH has been shown to be positively correlated with the oocyte fertilization and implantation rates. Furthermore, androgen treatment seems to improve the ovarian response in low responders.

STUDY DESIGN, SIZE, DURATION: This cross-sectional study involving 36 NC-IVF and 40 cIVF cycles was performed from 2011 to 2013. Within this population, 13 women each underwent 1 NC-IVF and 1 cIVF cycle. cIVF was performed by controlled ovarian stimulation with HMG and GnRH antagonists.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Follicular fluid was collected from the leading follicles. AMH, T, A2, dehydroepiandrosterone (DHEA), E2, FSH, LH and progesterone (P) were determined by immunoassays in 76 women. Aromatase activity in follicular fluid cells was analysed by a tritiated water release assay in 33 different women. For statistical analysis, the non-parametric Mann–Whitney *U* or Wilcoxon tests were used.

MAIN RESULTS AND ROLE OF CHANCE: In follicular fluid from NC-IVF and from cIVF, median levels were 32.8 and 10.7 pmol/l for AMH ($P < 0.0001$), 47.2 and 18.8 $\mu\text{mol/l}$ for T ($P < 0.0001$), 290 and 206 nmol/l for A2 ($P = 0.0035$), 6.7 and 5.6 pg/ml for DHEA (n.s.), 3292 and 1225 nmol/l for E2 ($P < 0.0001$), 4.9 and 7.2 mU/ml for FSH ($P < 0.05$), 14.4 and 0.9 mU/ml for LH ($P < 0.0001$) and 62 940 and 54 710 nmol/l for P (n.s.), respectively. Significant differences in follicular fluid concentrations for AMH, E2 and LH were also found in the 13 patients who underwent both NC-IVF and cIVF when they were analysed separately in pairs. Hormone analysis in serum excluded any relevant impact of AMH, T, A2, and E2 serum concentration on the follicular fluid hormone concentrations. Median serum concentrations were 29.4 and 0.9 mU/ml for LH ($P < 0.0001$) and 2.7 and 23.5 nmol/l for P ($P < 0.0001$) after NC-IVF and c-IVF, respectively. Positive correlations were seen for FF-AMH with FF-T ($r = 0.35$, $P = 0.0002$), FF-T with FF-LH ($r = 0.48$, $P < 0.0001$) and FF-E2 with FF-T ($r = 0.75$, $P < 0.0001$). The analysis of aromatase activity was not different in NC-IVF and cIVF follicular cells.

LIMITATION, REASONS FOR CAUTION: Any association between the hormone concentrations and the implantation potential of the oocytes could not be investigated as the oocytes in cIVF were not treated individually in the IVF laboratory. Since both c-IVF and NC-IVF follicles were stimulated by hCG before retrieval, the endocrine milieu in the natural cycle does not represent the pure physiological situation.

WIDER IMPLICATIONS OF THE FINDINGS: The endocrine follicular milieu and the concentration of putative markers of oocyte quality, such as AMH, are significantly different in gonadotrophin-stimulated conventional IVF compared with natural cycle IVF. This could be a cause for the suggested lower oocyte quality in cIVF compared with naturally matured oocytes. The reasons for the reduced AMH concentration might be low serum and follicular fluid LH concentrations due to LH suppression, leading initially to low follicular androgen concentrations and then to low follicular AMH production.

STUDY FUNDING/COMPETING INTERESTS: Funding for this study was obtained from public universities (for salaries) and private industry (for consumables). Additionally, the study was supported by an unrestricted grant from MSD Merck Sharp & Dohme GmbH and IBSA Institut Biochimique SA. The authors are clinically involved in low-dose monofollicular stimulation and IVF therapies, using gonadotrophins from all gonadotrophin distributors on the Swiss market, including Institut Biochimique SA and MSD Merck Sharp & Dohme GmbH. Otherwise, the authors have no competing interests.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: infertility / IVF outcome / follicular fluid / androgens / natural cycle

Introduction

Ever since gonadotrophins were introduced into IVF treatment, it has been discussed whether they might have an effect on follicular metabolism, and thereby affect the success rate of IVF therapies. This discussion has been further intensified by recent studies proposing co-treatment with androgens to modify the endocrine system in IVF therapies. These new co-treatments are based on studies providing some evidence that the IVF outcome is influenced by the kind of gonadotrophins being used.

In large clinical studies however, it has been shown that HMG, rFSH and rFSH+LH only show marginal differences in the pregnancy rate per collected oocyte (Andersen *et al.*, 2006; Durnerin *et al.*, 2008). Despite this, exogenous LH activity in a cycle stimulated with HMG or rFSH+LH seems to have a positive effect on the development of top-quality embryos (Lisi *et al.*, 2005; Andersen *et al.*, 2006).

These studies suggest that the gonadotrophins used might affect the outcome of IVF therapies as the gonadotrophins have a direct effect on the follicular metabolism. However, analysing the effect of different gonadotrophins is hardly possible as a large number of cases are necessary to detect a significant difference when applying the pregnancy rate as a primary end-point. Furthermore, a subtle dysregulation in folliculogenesis can hardly be discovered using the pregnancy rate as an end-point. Therefore, the analysis of the follicular endocrine milieu has been chosen in some studies to identify follicular markers for the effect of stimulation treatments and oocyte quality.

Intrafollicular AMH, measured at the time of follicular aspiration in conventional gonadotrophin-stimulated IVF therapies (cIVF), has been identified as a marker for oocyte implantation potential. In several studies, high AMH concentrations have correlated with the pregnancy rate (Fanchin *et al.*, 2007; Takahashi *et al.*, 2008; Pabuccu *et al.*, 2009). However, the investigation of the mechanisms which stimulate AMH production has only been rudimentally investigated to date. By comparing various cIVF stimulation regimens, Andersen and Lossl (2008) suggested that AMH secretion was stimulated by a HCG-induced, high follicular androgen concentration. These results again raise the question of whether the suppression of endogenous LH, which is achieved in cIVF

by the use of GnRH agonists and antagonists, reduces thecal cell androgen production and therefore the androgen concentration in the follicular fluid. The consequence could be a reduced AMH production, which directly or indirectly leads to reduced oocyte quality. Despite this, there is a lack of conclusive studies to demonstrate any effect of the type and dose of gonadotrophin stimulation on follicle metabolism and therefore on the oocyte quality in conventional IVF.

Thus the comparison of the endocrine milieu of naturally matured follicles with follicles after high-dose gonadotrophin stimulation allows new insights into the effect of gonadotrophins on follicular physiology. The follicular fluid from natural cycle IVF (NC-IVF) follicles can be considered to be a model for the ideal follicle as evolution has probably perfected folliculogenesis and every endocrine manipulation is likely to demonstrate an adverse disruption of the endocrine milieu. This concept is also supported by the discovery that when the follicle matures naturally, as in natural cycle IVF, higher implantation rates are achieved than with conventional IVF (Aanesen *et al.*, 2010).

Therefore, in this study, we compared the endocrine milieu in NC-IVF with that of cIVF and investigated the concentrations of AMH, androgens and gonadotrophins in serum and follicular fluid at the time of follicular aspiration. The endocrine milieu of naturally matured, and therefore supposedly ideal, follicles is thereby characterized and the effects of high-dose gonadotrophin stimulation on the endocrine milieu are described.

Materials and Methods

In this study, 76 patients underwent 1 NC-IVF ($n = 36$) and/or 1 cIVF ($n = 40$) cycle and 13 among them were treated with both therapies. Aromatase assays were performed with another set of 33 patients (NC-IVF $n = 15$; cIVF $n = 18$). The study was approved by the local ethical committee and each patient's approval was given by written consent.

cIVF patients were stimulated with HMG (150–300 IU per day of human menopausal gonadotrophin, Menogon HP[®], Ferring AG, Baar, Switzerland) which was initiated between Day 3–5 of the menstrual cycle. GnRH antagonists (Orgalutran[®], MSD Merck Sharp & Dohme GmbH, Lucerne, Switzerland) were first administered between Day 6 and 7 of the menstrual cycle and continued until ovulation induction. Once an adequate ovarian response

had been confirmed, 10 000 IU of urinary human chorionic gonadotrophin (hCG) (Predalon[®], MSD Merck Sharp & Dohme GmbH, Lucerne, Switzerland) was administered to induce ovulation. Transvaginal oocyte retrieval was scheduled 36 h after hCG administration and performed under general anaesthesia. To minimize contamination with blood and to reduce cross contamination from other follicles, follicular fluid was collected only from the first follicle aspirated. The follicle chosen was at least 18 mm in diameter.

NC-IVF patients were monitored by ultrasound and analysis of luteinizing hormone (LH) and 17 β -estradiol (E2) concentrations. When the follicle diameter reached at least 18 mm and E2 concentration was >800 pmol/l, 5000 IU of hCG was administered and oocyte retrieval was performed without anaesthesia 36 h later.

For both types of cycle, the cumulus oophorus complex was isolated and the follicular fluid was clarified by centrifugation, firstly at 600 \times g for 10 min and then followed by a second 10 min centrifugation at 1300 \times g. The supernatant fluids were stored at -30°C until further analysis. Venous blood was collected at the time of follicle aspiration and the obtained serum was stored at -30°C .

We intended to analyse a broad spectrum of those hormones which stimulate the follicles, such as FSH and LH, and those which are produced in response by thecal and granulosa cells or which are metabolized in the process of follicular hormone production. Testosterone (T), follicle stimulating hormone (FSH), progesterone (P) in serum and follicular fluid, and serum E2 concentrations were analysed by electrochemiluminescent immunoassay (ECLIA) on a COBAS 6000 (e601 Modul) (Roche Diagnostics GmbH, Mannheim, Germany). The inter-assay coefficients of variation (CV) of these assays were <4%. Dehydroepiandrosterone (DHEA) was measured with an ELISA from IBL (IBL-International, Hamburg, Germany). The inter-assay CV of this test was <12%. Androstenedione (A2) was analysed by radio-immunoassay (RIA, Coat-a-count[®], Siemens Healthcare Diagnostics, Inc., Los Angeles, CA, USA). This method was also used for the determination of E2 in the follicular fluid as it required a 1:500 dilution. The CV of these RIAs were <11%. AMH and LH were determined manually with commercially available microplate enzyme immunometric methods (ELISA). The assay for AMH was obtained from Immunotech (Marseille, France). Both sera and follicular fluids were introduced without dilution and the protocol of the manufacturer was followed. The inter-assay CV was <14.2%. The method used for the determination of LH was from Cayman, Ann Arbor, MI, USA. Serum was used directly in the assay, as recommended by the manufacturer. Follicular fluid was similarly used without dilution since the expected concentrations were

lower (but still above the sensitivity limit of 0.5 mIU/ml). The inter-assay CV was <7.9%.

For aromatase activity measurements, follicular aspirates were each centrifuged at 1000 \times g for 5 min and then stored at -40°C . After thawing, the material was again centrifuged, the supernatant discarded and the cell pellet was redissolved in KiP buffer before it was vortexed vigorously. Aromatase activity measurements were then performed by the tritiated water release assay as described elsewhere (Lephart and Simpson, 1991; Pandey *et al.*, 2007) on this material. In brief, reactions were performed at 37°C in 15-ml Falcon tubes each with a final volume of 200 μl consisting of 176 μl cell lysate and 10 nM androstenedione labelled with [1β - ^3H (N)]-androstene-3,17-dione (30 000 cpm/reaction) in 50 mM K-phosphate buffer (pH 7.4). The reactions were initiated by adding 1 mM NADPH and stopped by adding 1 ml of chloroform. After vortexing for 30 s, 1 ml of water was added, and the mix was centrifuged at 1000 rpm for 5 min. Aliquots (0.5 ml) of the water phase were extracted with an equal volume of 5% charcoal/0.5% dextran for 40 s, then centrifuged at 12 000 rpm for 15 min, and 0.5-ml aliquots of supernatants were collected for counting of ^3H radioactivity. The conversion rate of androstenedione to estrone was assessed for both groups. For the internal control purposes, total protein contents of the follicular cell material (as used for the aromatase assay) was measured in each sample by a modified Lowry assay according to the manufacturer's protocol (DC Protein Assay, Bio-Rad Laboratories AG, Cressier, Switzerland).

Statistical analyses were performed by the non-parametrical Mann-Whitney *U*-test for the comparison of hormone levels between the two patient groups. Regression analyses were done using the Spearman rank correlation method. *P*-values <0.05 were considered as statistically significant.

Results

The mean patient ages were 34.9 years (SD 4.9, range 21–42) for the NC-IVF group and 33.8 years (SD 3.7, range 25–41) for the cIVF group. The mean follicle sizes at the time of aspiration in the NC-IVF and c-IVF groups were 19.6 mm (SD 1.2) and 19.5 mm (SD 1.2), respectively (Table I). Both parameters, as well as the AMH serum concentrations, were not significantly different between the two groups. The mean ages of the additional set of patients for the aromatase assay were 36.1 years (SD 4.4, range 27–42) for the NC-IVF group and 35.5 years (SD 3.8, range 27–42) for the cIVF group (Table IV).

Table I Characteristics of trial participants for follicular fluid hormone assays.

	NC-IVF ^a (n = 36)	cIVF ^b (n = 40)	NC-IVF + cIVF ^c (n = 13)
Mean age (years)	34.9 (\pm 4.9, 21–42)	33.8 (\pm 3.7, 25–41)	37.2 (\pm 4.3, 31–42)
Aetiology of infertility (n/total)			
Male factor	28/38	23/40	7/13
Tubal factor	5/38	4/40	2/13
Endometriosis	0/38	0/40	0/13
Idiopathic	2/38	1/40	1/13
Others	3/38	12/40	3/13
Anti-Mullerian hormone (pmol/l)	17.7 (\pm 14.6)	22.9 (\pm 15.3)	17.3 (\pm 8.9)
Mean follicle size (mm)	19.6 (\pm 1.2)	19.5 (\pm 1.2)	

^aNatural cycle IVF.

^bConventional gonadotrophin-stimulated IVF.

^cSubgroup of patients, undergoing both therapies.

In addition to follicular fluid, serum was analysed to exclude any relevant impact of serum concentrations on the difference of follicular fluid protein concentrations in follicles from NC-IVF and cIVF. The relevant impact from the hormones present in the circulation could be excluded for AMH, T, A2 and E2 as the follicular fluid concentrations were higher than that found in serum (Table II).

For DHEA, FSH and LH concentrations were similar or even higher in serum than in follicular fluid, suggesting that follicular fluid concentrations of these hormones are strongly influenced by serum.

In the follicular fluid, AMH, T, A2, E2 and LH concentrations were significantly different in NC-IVF compared with that in c-IVF follicles (Table II). The results for these six parameters are given in Fig. 1 as box and whisker plots, illustrating the variations of the individual concentrations. AMH, T and E2 concentrations were ~3-fold higher, A2 was ~1.5-fold higher and LH was ~14-fold higher in NC-IVF than in cIVF follicles. A subgroup analysis of those patients, receiving both therapies ($n = 13$), confirmed the significant differences in the concentrations of AMH, E2 and LH between NC-IVF and cIVF follicles (Table III).

Progesterone concentrations were similar in NC-IVF and cIVF follicles. However, the serum concentrations were markedly different. In NC-IVF, serum concentrations were 2.7 nmol/l and in cIVF, serum concentrations were 23.5 nmol/l at the time of follicle aspiration (Fig. 2).

The markedly increased concentration of the putative implantation marker AMH in NC-IVF follicles raised the question whether the concentration of AMH would correlate with other follicular fluid and serum parameters and thus suggest a metabolic link. Therefore a regression analysis of AMH and testosterone was performed. As shown in Fig. 3B testosterone concentrations were positively correlated ($r = 0.35$, $P = 0.0002$) with AMH concentrations.

The significantly increased testosterone concentration in NC-IVF follicles might be either due to increased testosterone production due to increased LH activity in NC-IVF or due to an inhibition of the follicular aromatase, inhibiting the conversion of T into E2. An inhibition of the aromatase activity, i.e. a reduced conversion of T into E2 seems to be unlikely as such an effect would result in an accumulation of testosterone with a non-linear correlation of T and E2 and not a linear correlation as shown in Fig. 3. Furthermore, aromatase activities were similar in NC-IVF and cIVF follicular cells as analysed by tritiated water release assays. NC-IVF follicular cells showed a conversion rate of 1360 ± 277 arbitrary units

(au) and cIVF follicular cells showed a conversion rate of 1144 ± 182 au ($P = 0.23$) (Table IV).

The alternative concept, a link between testosterone production and LH activity in NC-IVF follicles, was supported by a positive correlation of testosterone and LH concentrations ($r = 0.48$, $P < 0.0001$).

Discussion

Our study describes the endocrine profile of follicles which were, apart from the ovulation induction with hCG, naturally matured. We assume that these entities represent an evolutionary model of the ideal follicle. Naturally matured follicles may accordingly generate the highest implantation potential for the embryo derived from the oocyte they are harbouring.

Our study compared the endocrine profile of naturally matured (NC-IVF) follicles with that of follicles generated after conventional high-dose gonadotrophin stimulation using LH-suppressing GnRH antagonists (cIVF). This comparison referred to a study group of 76 different subjects in both treatment groups and a sub-analysis with 13 subjects who underwent both therapies to minimize intra-individual variation. The results in the small subgroup were largely identical to those in the complete, cross-sectional treatment groups.

For the analysis, we chose hormones which reflect the steroid metabolism in the follicle. In theca cells, LH induces the conversion of pregnenolone into progesterone or DHEA which is then converted into A2 and T. A2 and T are transported to the granulosa cells where they are converted under the influence of FSH by the enzyme aromatase into E2. AMH is also produced by granulosa cells and large amounts of progesterone are synthesized by luteinized granulosa and thecal cells.

The main differences in the comparison of NC-IVF and cIVF were the significantly lower intrafollicular AMH concentrations, androgen concentrations and LH concentrations in the cIVF follicles. In addition, serum progesterone concentrations were much lower in NC-IVF cycles.

AMH has already been identified as a marker for a high oocyte implantation potential in several studies (Fanchin et al., 2007; Takahashi et al., 2008; Pabuccu et al., 2009). Therefore, this means that the oocyte quality in naturally matured follicles might be higher when compared with cIVF as higher AMH concentrations were measured for NC-IVF. Various studies support this theory. Implantation rates of ~25% have

Table II Concentrations of various hormones in follicular fluid and serum on the day of oocyte retrieval in all natural cycle (NC-IVF) and conventional gonadotrophin-stimulated (cIVF) IVF cycles (cross-sectional analysis).

Hormone	Follicular fluid concentration			Serum concentration		
	NC-IVF ($n = 36$)	cIVF ($n = 40$)	P-value	NC-IVF ($n = 30$)	cIVF ($n = 33$)	P-value
AMH (pmol/l)	32.8 (0.5–281)	10.7 (1.0–238)	<0.0001	17.2 (3.7–66.4)	12.7 (4.5–68.0)	0.0287
Testosterone (μ mol/l)	47.2 (1.5–>52)	18.8 (2.8–<52)	<0.0001	1.0 (0.1–3.2)	2.0 (0.6–4.1)	<0.0001
A2 (nmol/l)	290 (8.0–>350)	206 (29–>350)	0.0035	9.8 (4.2–29.4)	14.5 (5.8–47.0)	0.0003
DHEA (pg/ml)	6.7 (2.3–16.7)	5.6 (1.4–13.0)	0.0744	6.3 (2.8–12.6)	5.2 (2.3–12.0)	0.2305
E2 (nmol/l)	3292 (369–7153)	1225 (109–5020)	<0.0001	0.6 (0.2–5.3)	4.0 (0.4–10.7)	<0.0001
FSH (mIU/ml)	4.9 (0.2–15.6)	7.2 (1.3–17.3)	0.0414	11.8 (5.2–39.7)	11.1 (2.7–18.1)	0.3604
LH (mIU/ml)	14.4 (0.3–60.0)	0.9 (0.2–12.2)	<0.0001	29.4 (10.5–81.9)	0.9 (<0.2–9.8)	<0.0001

The results are given as median and range. P-values were obtained by non-parametrical Mann–Whitney U-test.

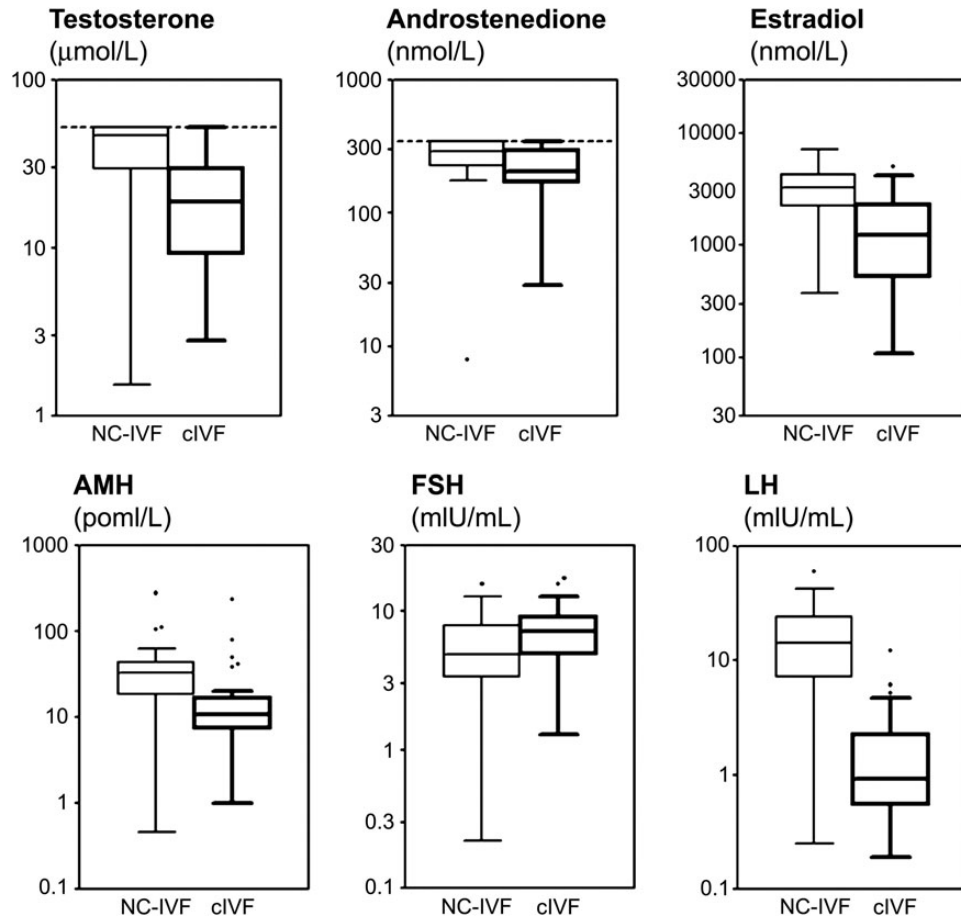


Figure 1 Tukey Box and Whisker plot of those hormones in the follicular fluid in natural cycles (NC-IVF, $n = 36$, fine lines) and conventional gonadotrophin stimulated IVF cycles (cIVF, $n = 40$, bold lines) which showed significantly different concentrations (Table II). Boxes represent the 25th and 75th centiles, and the bottom and top whiskers are defined by the 25th centile minus 1.5 times the interquartile range (IQR) and the 75th centile plus 1.5 times the IQR, respectively. Data points outside this range are plotted as individual points. Please note the logarithmic scale.

Table III Subanalysis of follicular fluid and serum hormone concentration of those 13 patients undergoing both a natural cycle (NC-IVF) and a conventional gonadotrophin-stimulated (cIVF) IVF cycle (paired analysis).

Hormone	Follicular fluid concentration ($n = 13$)			Serum concentration ($n = 7$)		
	NC-IVF	cIVF	P-value	NC-IVF	cIVF	P-value
AMH (pmol/l)	35.0 (0.5–281)	10.4 (6.8–16.8)	0.0134	18.7 (9.5–42.8)	7.5 (5.4–17.3)	0.0156
Testosterone ($\mu\text{mol/l}$)	47.2 (1.5–>52)	25.4 (5.4–>52)	0.1016	0.8 (0.1–1.2)	1.5 (0.6–2.6)	0.0313
A2 (nmol/l)	228 (8.0–>350)	246 (36–>350)	0.5186	8.1 (4.2–12.4)	11.8 (5.8–16.3)	0.1094
DHEA (pg/ml)	5.6 (2.3–13.9)	6.8 (3.0–13.0)	1.0	6.5 (3.0–12.0)	6.3 (3.6–8.1)	1.0
E2 (nmol/l)	2948 (369–7153)	1768 (320–5020)	0.0327	0.6 (0.2–1.3)	3.7 (1.1–8.3)	0.0156
FSH (mIU/ml)	5.0 (0.2–12.8)	8.4 (1.9–15.8)	0.2163	13.3 (10.4–39.7)	13.0 (8.8–15.1)	0.1875
LH (mIU/ml)	16.3 (0.3–60.0)	0.6 (0.2–6.2)	0.0012	40.2 (22.1–77.6)	0.9 (0.5–2.0)	0.0156

been described for NC-IVF (Aanesen *et al.*, 2010), whereas for cIVF without embryo selection they are only ~15% (DIR, FIVNAT). However, it must be stressed that a comparison of studies with data from large registers is only possible to a limited extent and a correct comparison as part of a randomized study had not been carried out to date. Nevertheless, the increased AMH concentrations in naturally matured follicles were confirmed and cannot be due to the different stages of follicle maturation as previously suggested

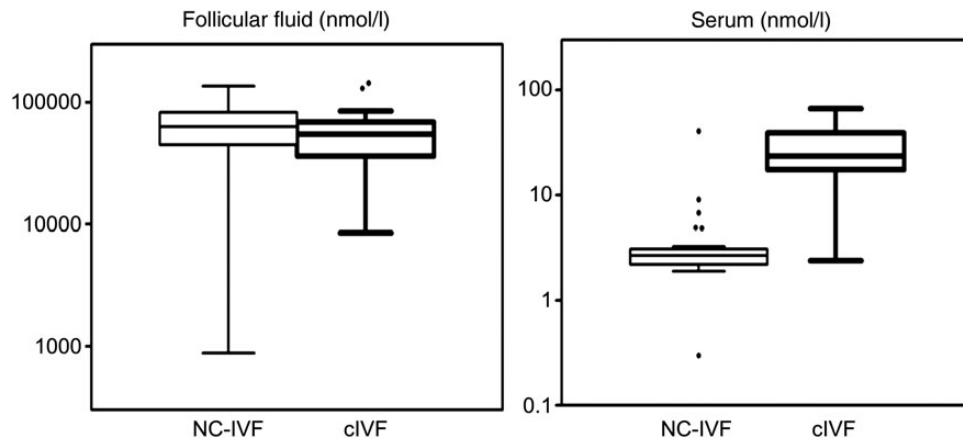


Figure 2 Progesterone concentration in the follicular fluid in natural cycles (NC-IVF, $n = 36$, fine lines) and conventional gonadotrophin stimulated cycles (cIVF, $n = 40$, bold lines). The data are presented according to Fig. 1.

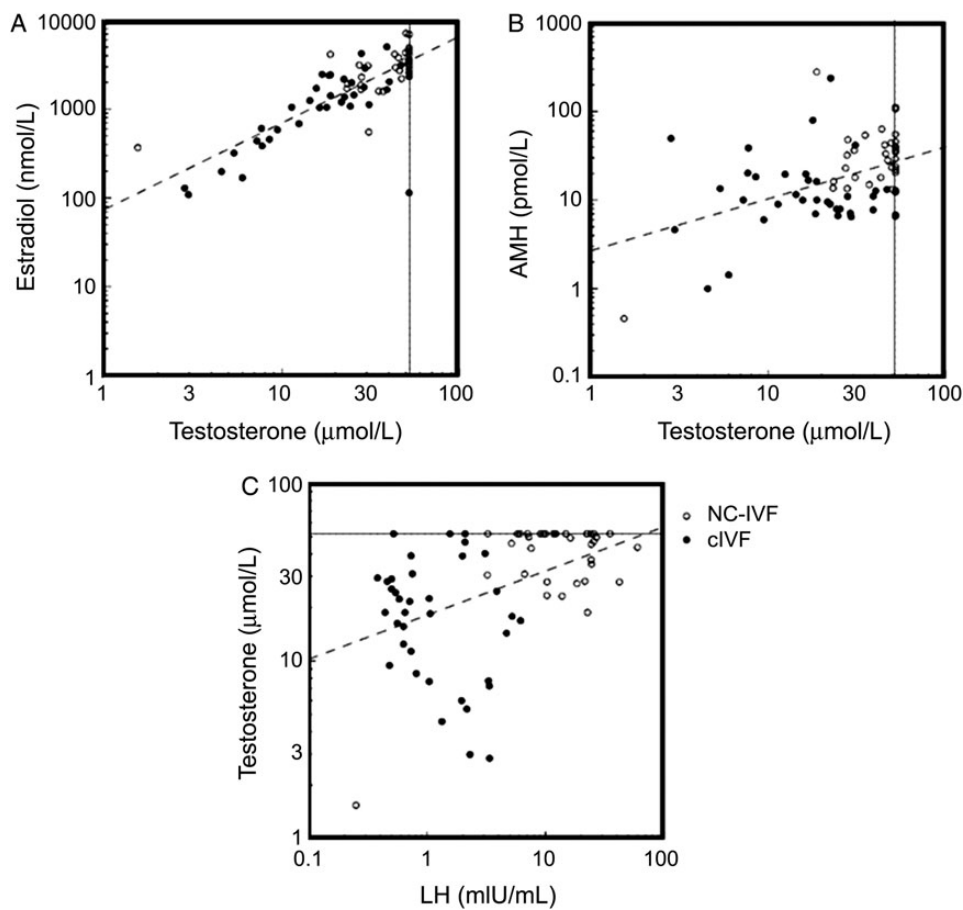


Figure 3 Correlations between different hormones in follicular fluid of natural cycle (NC-IVF, open circles) and conventional gonadotrophin stimulated (cIVF, closed circles) IVF cycles. **(A)** estradiol as a function of testosterone; **(B)** AMH as a function of testosterone; **(C)** testosterone as a function of LH. The dotted vertical or horizontal line in each graph represents the maximum testosterone concentration which could be quantified (52.2 $\mu\text{mol/l}$). Please note the logarithmic scale. The dashed regression line shown pertains to the total patient population (NC-IVF plus cIVF).

Table IV Characteristics of trial participants and aromatase activity in follicular fluid cells from NC-IVF and cIVF follicles. There were no significant differences between the two groups.

	NC-IVF ^a (n = 15)	cIVF ^b (n = 18)
Mean age (years)	36.1 ± 4.4 (27–42)	35.5 ± 3.8 (27–42)
Anti-Müllerian hormone (pmol/l)	10.7 (± 8.5)	18.5 (± 7.6)
Mean follicle size (mm)	18.0 (± 1.2)	19.3 (± 1.3)
Activity (au)	1360 ± 277	1144 ± 182

^aNatural cycle IVF.

^bConventional gonadotrophin-stimulated IVF.

(Fanchin *et al.*, 2005) because the mean size of the follicles in the groups compared was identical.

While higher AMH concentrations in NC-IVF follicles could be the reason for the higher oocyte quality, leading to higher implantation rates in NC-IVF cycles, it cannot be ruled out that other factors might be involved. One of these factors could be the serum progesterone concentration, which we found to be nine times higher in cIVF compared with NC-IVF at the time of oocyte aspiration, 1.5 days after hCG application. A meta-analysis (Kolibianakis *et al.*, 2012) has shown that high serum progesterone concentrations at the time of hCG application correlate with lower pregnancy rates per cycles. However, these findings have recently been questioned by a pooled analysis (Griesinger *et al.*, 2013) which differentiated between low, normal and high responders and thereby did not find an impact of high progesterone concentrations on pregnancy rates. Furthermore, these studies were focused on progesterone concentrations at the time of hCG application. Data about the outcome in relation to serum progesterone concentrations at a later stage are very sparse and not conclusive (Check *et al.*, 2008). Therefore, it seems plausible that rather than serum progesterone concentrations, the follicular endocrine milieu itself is responsible for the higher implantation rate in the naturally matured follicle.

Three essential questions can be derived from these results related to the follicular endocrine milieu. (i) What is the significance of the increased AMH concentrations? (ii) Which regulatory mechanisms lead to the increased AMH concentrations? And (iii) Is it possible to draw conclusions for conventional IVF stimulation?

The first question, the significance of follicular AMH on oocyte quality, is difficult to answer as the significance of AMH is still unclear. It has only been shown that AMH is produced by granulosa cells (Vigier *et al.*, 1984) and that atretic granulosa cells do not produce AMH (De Vet *et al.*, 2002). The degree of apoptosis in granulosa cells correlates with the developmental competence of the oocyte (Nakahara *et al.*, 1997). These relationships lead to the hypothesis that a high AMH concentration may have no direct effect on the oocyte, but is a marker for the granulosa cell function, and as such is of relevance for the function of the oocyte. In contrast, it has been suggested that there is a direct link in the opposite direction between oocyte function and intrafollicular AMH production of the granulosa cells. The oocyte seems to activate various physiological processes in the surrounding granulosa cells. In mice, it has been shown that the oocyte influences the AMH expression via this mechanism (Salmon *et al.*, 2004).

The second question, which regulatory mechanisms leads to the AMH production, is similarly difficult to address. It has been shown that during IVF treatment, induction with hCG before gonadotrophin stimulation leads to higher intrafollicular androgen concentrations as well as increased follicular AMH concentrations (Andersen and Lossl, 2008). It was suggested that intrafollicular testosterone possibly stimulates the AMH production. Based on our study results, this means that the increased androgen concentrations in the naturally matured follicles would be the reason for the increased AMH concentrations. The precise mechanisms for the stimulation of AMH production are, however, still unknown. Androgens may induce FSH receptor expression in the granulosa cells (Weil *et al.*, 1999). A direct stimulatory effect of LH is also possible as a stimulatory effect of hCG/LH on the AMH production in granulosa cells of PCO patients, but not in the granulosa cells of healthy women, has been found (Phy *et al.*, 2004). In our study, we detected a correlation between the follicular testosterone concentration and the AMH concentration, which supports but does not prove the supposition of a dependency of the AMH concentration on the testosterone concentration.

This in turn raises the question of which mechanisms would lead to an increased follicular testosterone concentration in naturally matured follicles. Elevated androgen levels can be the consequence of two different mechanisms. Either AMH inhibits the aromatase, as a result of which androgens accumulate, or androgenesis is stimulated by increased LH-mediated stimulation of theca cells.

One study in which an *in vitro* inhibition of aromatase by AMH was detected, speaks in favour of the aromatase inhibition hypothesis (Grossman *et al.*, 2008). However the results of our study argue against this hypothesis. Inhibition of aromatase would rather lead to an increase in the testosterone/estradiol ratio. But this ratio was not increased in the follicles with an increased AMH concentration (Fig. 3). Furthermore, the analysis of aromatase activity revealed similar activity in NC-IVF and cIVF follicular cells.

The second hypothesis implies that androgen synthesis is increased in naturally matured follicles due to an increased stimulation of theca cells by LH. Indeed, we found higher LH concentrations in serum as well as in follicular fluid in naturally matured follicles, because LH suppression using GnRH agonists or GnRH antagonists, as in cIVF, is not performed in NC-IVF.

From this comes the third question of whether conclusions for conventional IVF stimulation could be drawn from the study results. Assuming that high intrafollicular AMH concentrations are a marker for high oocyte quality, stimulation processes which lead to high intrafollicular androgen concentrations, and consequently to high AMH concentrations, would be beneficial. Accordingly this could be achieved with a sufficient level of theca cell stimulation by LH, either by reduced suppression of the physiologically endogenous pituitary LH secretion or by exogenous LH supplementation with HMG or LH.

In line with this hypothesis, a comparison of IVF stimulation with HMG versus rFSH has shown that HMG stimulation significantly increased concentrations of intrafollicular testosterone and estradiol (Smits *et al.*, 2007). The ongoing implantation rate was 24% after HMG stimulation compared with 20% after rFSH stimulation ($P = 0.25$) (Andersen *et al.*, 2006). In a comparison of stimulation with rFSH versus rFSH plus rLH, a meta-analysis of five studies showed a significantly higher number of mature oocytes with LH-supplementation ($P = 0.0098$) but no significant differences in the implantation rate (Baruffi *et al.*, 2007).

In women aged ≥ 35 years, the implantation rate under the addition of rLH was shown to be significantly increased in a meta-analysis with an OR

of 1.36 (95% CI 1.05–1.78) (Hill *et al.*, 2012). In five studies comparing the long GnRH agonist with the GnRH antagonist protocol, the latter produced a higher implantation rate with an OR of 1.56 (95% CI 1.04–2.33) (Hill *et al.*, 2012). These results are in line with our hypothesis as LH concentrations are less suppressed in antagonist protocols than in long protocols.

Lower apoptosis rates in human cumulus cells in rLH-substituted IVF cycles also support a positive effect of LH (Ruvolo *et al.*, 2007). Whether this effect directly or indirectly leads to better granulosa cell function via an increased androgen production (the consequence of which is possibly increased AMH production) has not been demonstrated to date; however, it seems likely to us to play a role.

What implications can be made from our study in the context with the other clinical studies listed? The present study supports previous results, this time from an as yet unreported perspective, that high intrafollicular AMH and androgen concentrations may be physiological and therefore can probably be markers for normal follicular function. Our study also supports the results of the meta-analyses showing that rLH supplementation may be advantageous for follicular function. According to our investigations, LH probably stimulated the androgen-AMH-axis.

For future studies, these results indicate that in defined subgroups, such as older women for example, exogenous LH/hCG supplementation or decreased suppression of endogenous LH production could be considered. Using the physiological endocrine profile in naturally matured follicles as a reference value could also be an advantage.

In summary, the comparison of the endocrine profile of naturally matured follicles (NC-IVF) with the profile after conventional gonadotrophin stimulation (cIVF) shows a significant suppression of LH, androgen and AMH concentrations after cIVF. A comparison of our results with those of other clinical studies suggests a negative effect of suppression of the hypophyseal LH production from cIVF on the follicular endocrine system.

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Authors' roles

M.v.W. was responsible for the study conception and design, acquisition, analysis and interpretation of data, drafting of the article, and final approval. Z.K. was responsible for the study conception, acquisition and analysis of data, revising the article, and final approval. C.E.F. was responsible for the conception and design of the aromatase assays, analysis and interpretation of data, drafting of the article, and final approval. P.S. was responsible for analysis and interpretation of data, revising the article, and final approval. B.W. was responsible for acquisition of data, revising the article, and final approval. U.M. was responsible for analysis and interpretation of data, revising the article, and final approval. N.A.B. was responsible for the protein hormone assays, data analysis, tables and figures, revising the article and final approval.

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Conflict of interest

The authors are clinically involved in low-dose monofollicular stimulation and IVF therapies, using gonadotrophins from all gonadotrophin distributors on the Swiss market, including Institut Biochimique SA and MSD Merck Sharp & Dohme GmbH.

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