

# Steroids and protein markers in the follicular fluid as indicators of oocyte quality in patients with and without endometriosis

Dorothea M. Wunder,<sup>1,2</sup> Michael D. Mueller,<sup>1</sup> Martin H. Birkhäuser,<sup>1</sup> and Nick A. Bersinger<sup>1</sup>

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**Purpose:** To investigate the concentrations of steroid hormones (estradiol, progesterone), pregnancy-associated protein-A, IGF-binding protein-4 and leptin in the follicular fluid of infertile patients with and without endometriosis.

**Methods:** Follicular fluid of IVF patients with and without endometriosis was aspirated, centrifuged and stored to analyze the above mentioned hormones and to compare their concentrations between women with and without endometriosis.

**Results:** Follicular fluid estradiol levels were significantly higher in controls than in affected women. The concentrations of the other markers did not differ between the two groups.

**Conclusions:** Since not only the follicular fluid concentration of estradiol, but also the oocyte quality is decreased in women with endometriosis, we suggest that estradiol can be considered as a marker not only of oocyte maturity but also of oocyte quality.

**KEY WORDS:** Endometriosis; follicular fluid; leptin; PAPP-A; steroid hormones.

## INTRODUCTION

Endometriosis, which is characterized by the presence of endometrial tissue outside the uterus, is an enigmatic, chronic, progressive and inflammatory disease, associated with local and systemic abnormalities in the immune response and leading to infertility and pain. Leptin, the hormone encoded by the obesity (*ob*) gene, was firstly thought to have only anti-obesity properties, but many studies (1,2) have shown that leptin also had proinflammatory, immunoregulatory and angiogenic effects, all of which play a role in the pathogenesis of endometriosis. Leptin influences reproduction and fertility (3) and may be involved in the pathogenesis of endometriosis (4). Pregnancy-associated protein-A (PAPP-A)

is a high molecular weight, highly glycosylated 2 × 2-heterotetrameric polypeptide linked to the pro-form of the eosinophilic major basic protein (5). Its biological role has been unclear, but it has been recently found (6) to have a protease activity towards insulin-like growth factor-binding protein 4 (IGF-bp4) and has been suggested to play a role in follicle selection and granulosa luteinization (7). Recently, increased concentrations of PAPP-A in the peritoneal fluid of women with endometriosis were reported (8). IGFs are stimulating steroidogenesis and IGF-bps are effective antigonadotropins; they both play a key role in ovarian follicular development (9). Insulin-like growth factor binding protein-4 (IGF-bp4) is an inhibitor of IGF action and with that a potent inhibitor of FSH stimulated granulosa steroidogenesis (10). By regulating FSH action, both IGF and IGF-bps play a key role in ovarian follicular development (9). By cleaving IGF-bp4, PAPP-A is providing higher IGF-II activity in the follicles, which is important for follicle growth and survival.

<sup>1</sup>Department of Obstetrics and Gynaecology, University of Berne, Switzerland.

<sup>2</sup>To whom correspondence should be addressed at Universitäts-Frauenklinik Inselspital Effingerstrasse 102 3010 Berne, Switzerland; e-mail: dorothea.wunder@insel.ch.

The outcome of in vitro fertilization (IVF) treatment is poorer for patients with endometriosis than for patients with other causes of infertility (11,12,13,14,15), but whether the reduced pregnancy rate in endometriosis is due to embryo quality, the endometrial environment, or both is controversial. Consequently, it is most critical to elucidate the pathophysiological mechanism of endometriosis and to find out why this disease has a negative influence on fertility. It was shown that oocytes of women with endometriosis are of inferior quality and have lower implantation rates in IVF than oocytes from women with other origins of infertility (12). There is increasing evidence that not only the gonadotropins, but also cytokines, growth factors as well as the locally produced steroid hormones themselves are modulating the folliculogenesis. It is to be expected that the follicular fluid in patients with endometriosis is different from the one from other women (fertile or infertile) and this may be responsible for the inferior quality and implantation potential of these oocytes.

Controlled ovarian stimulation in assisted reproduction gives us the unique opportunity to study the dynamic changes occurring in follicular fluid (FF) composition under the influence of gonadotropins. FF contains a variety of growth factors, cytokines, prostaglandins and steroid hormones secreted by the cells of the ovarian follicle. The objective of the present study was to investigate the mechanisms associated with infertility in patients with endometriosis through the analysis of the steroid hormones estradiol ( $E_2$ ) and progesterone (Prog), the hormone leptin and the protein markers pregnancy-associated plasma protein-A (PAPP-A) and insulin-like growth factor-binding protein-4 (IGF-bp4) in the follicular fluid of infertile patients with and without the disease.

## MATERIALS AND METHODS

### Patient Characteristics

The present study contained 326 analyses of follicular fluid of patients undergoing assisted reproduction between September 1999 and December 2003. The fluids were collected at oocyte pick-up from patients undergoing IVF and embryo transfer. For being included in the study, the patient in reproductive age (maximum 42 years) had to provide written consent for the treatment by intracytoplasmic sperm injection (ICSI). Only ICSI-cycles were included to

have a more homogenous population. Hormonal values, gynecological ultrasound results and cervical smears had to be normal, and no infectious diseases (Rubella, HIV, Hepatitis B, Hepatitis C, Syphilis) were allowed to be present. Criteria for exclusion from an IVF/ICSI-treatment were acute or chronic infectious diseases, severe psychiatric problems, or being a carrier of severe genetic diseases. Diagnosis of endometriosis was based on histological analyses of laparoscopically taken biopsies. In our study group there were 47 patients with diagnosed endometriosis, of which 33 were mild (rAFS stages I or II) and 14 severe (rAFS stages III or IV) cases and 279 controls.

### Protocol for Controlled Ovarian Stimulation

Ovarian stimulation and oocyte retrieval were usually performed using a "long" protocol, with downregulation starting in the luteal phase of the previous cycle with the agonist Triptorelin, 0.1 mg per day s.c. (Decapeptyl<sup>®</sup>, Ferring pharmaceuticals, Wallisellen, Switzerland) until complete pituitary desensitization was documented. Stimulation was initiated, once sonographic evidence indicated no ovarian follicular activity and serum levels of human chorionic gonadotropin (hCG) and estradiol were below 2 mIU/mL and 130 pmol/L, respectively, in most cases with recombinant FSH (Gonal-F<sup>®</sup>, Serono Pharma, Geneva, Switzerland or Puregon<sup>®</sup>, Organon Pharmaceuticals, Pfäffikon, Switzerland) or with menopausal gonadotropin (hMG, Pergonal<sup>®</sup>, Serono or Humegon<sup>®</sup>, Organon). Follicular maturation was assessed by ultrasound and serum estradiol measurements. hCG (5,000–10,000 IU, Profasi<sup>®</sup>, Serono or Pregnyl<sup>®</sup>, Organon) was administered when at least three follicles exceeded 17 mm in diameter and the estradiol concentration per mature follicle exceeded 1000 pmol/L.

For low responders the short protocol was used. Downregulation was performed on the first day of the cycle with Triptorelin, 0.05 mg per day. Gonadotrophin stimulation (see above) was initiated on the second day of the cycle after sonographic evidence indicated no ovarian follicular activity and serum hCG was below 2 mIU/mL. hCG (10,000 IU) was given as above to induce ovulation.

Short and long protocols were equally distributed in both groups, the short protocol accounting for less than 5% in both groups. Similar distribution was

also the case with the different gonadotrophins used (rFSH or HMG).

In the case of hyper-responders with  $E_2$  levels greater than 10,000 pmol/L and consequently an increased risk of developing a hyperstimulation syndrome, only 5,000 IU hCG were administered. In the endometriosis group, there were nine hyperresponders (of totally 47 patients), in the control group there were 65 (of 279),  $p = 0.7066$ , (n.s. by Fishers Exact test). Concerning the low response, there were nine (in a total of 47 patients) in the endometriosis group, and 38 (of 279) in the control group,  $p = 0.1544$  (n.s. by Fishers Exact test).

Thirty-five to 36 h after the administration of hCG, the fluid containing the oocytes was retrieved from all follicles by needle aspiration, with transvaginal ultrasound guidance and under routine intravenous sedation.

#### **Assessment for Oocyte Maturity, ICSI and Embryo Culture**

Hyaluronidase denuded oocytes were assessed for maturity. Only metaphase-II oocytes, identified by the presence of the first polar body, were chosen for fertilization. ICSI was performed 3–6 h after oocyte recovery by using previously described (16) techniques and instrumentation.

#### **Embryo Grading, Selection and Transfer**

Fertilization was assessed 17 h after IVF or ICSI. Only normally fertilized oocytes (two pronuclei (PN) and two polar bodies) were considered for embryo transfer, and cryopreservation was performed at this stage when more than two normally fertilized oocytes were available. The cells with the highest PN score and with the best morphological grade were selected for transfer and cultured for another 20–30 h at 37°C in fresh CO<sub>2</sub> equilibrated IVF medium (Vitrolife®, Göteborg, Sweden). The remaining, normally fertilized oocytes were cryopreserved in the PN stage according to the Swiss law. Embryo development was evaluated 2 to 3 days after ICSI or insemination by determining the number of blastomeres and the relative proportion of anucleate cell fragments. Embryos with less than 10% fragments, with 10–20% fragments, and with 20–30% fragments were referred to as grade 1, 2 and 3, respectively.

#### **Measurement of Hormones and Cytokines in Follicular Fluid**

FF samples from individual follicles were pooled and centrifuged for 10 min at 500 × *g* and the supernatants were stored at –30°C until analyzed further. Fractions of FF with massive blood contamination were excluded.

**Steroid Hormones.** Estradiol-17 $\beta$  and progesterone were determined by competitive radioimmunoassay using coated tube methodology. The kits ("Coat-a-Count") were manufactured by DPC, Los Angeles, USA and obtained from Buhmann Laboratories, Basle, Switzerland. Samples were diluted 1:250 (estradiol) and 1:500 (progesterone) in the diluent provided. The 3-h incubation protocol was used. Intra- and interassay coefficients of variance at halfmaximal binding were 4.3 and 6.8% for estradiol, and 3.9 and 5.5% for progesterone, respectively.

**Pregnancy-Associated Plasma Protein A (PAPP-A).** This protein was determined with a double antibody microplate enzyme immunometric assay (ELISA), marketed by Buhmann Laboratories, Switzerland and involving a capture antibody purified by negative affinity chromatography (17). A dilution of 1:11 was used; the method and assay parameters are described elsewhere (17).

**IGF-Binding Protein 4 (IGF-bp4).** This analyte was quantified similarly by microplate ELISA. The monoclonal capture antibody, the biotinylated polyclonal (goat) detection antibody and the recombinant human IGF-bp4 (Asp22-Glu258) standard were obtained from R&D systems. Briefly, microplate wells (Maxisorp®, Nunc, Denmark) were coated with 100  $\mu$ L of the capture antibody (2  $\mu$ g/mL) in phosphate buffered saline (PBS) overnight at 4°C, and excess sites were then blocked, without previous washing, with bovine serum albumin (BSA, 0.5 mg/mL, Fluka, Buchs, Switzerland) in PBS for 1 h at 30°C. Then the plates were washed in two aspiration cycles using PBS with Tween-20 (PBST, 0.1% v/v; Sigma, Fluka, Buchs, Switzerland) and follicular fluid samples (1:50) and standard (16 to 0.25 ng/mL in serial 1:2 steps) were diluted in PBS containing 5% (v/v) Tween-20 without carrier protein, and incubated for 2 h at 30°C under slow shaking (300 rpm) in a ThermoStar® dry incubator/shaker. After three washing steps with PBST, the detection antibody (400 ng/mL in PBS containing 5% (v/v) Tween-20 and 2% (v/v) heat-inactivated normal goat serum obtained from the Scottish Antibody

Production Unit, Carlisle, Scotland) was added and the plates incubated as above. After three more washing cycles, streptavidin-conjugated horseradish peroxidase compound (Southern Biotech, USA; Bioreba, Reinach, Switzerland), diluted 1:4000 in Blotto® (Pierce, USA; Socochim, Lausanne, Switzerland), was added and the plates incubated for 30 min under the same conditions. After extensive washing (five aspirations) the colour was developed using the same protocol as for leptin (18).

**Leptin.** This hormone was quantified with an ELISA developed in our laboratory using monoclonal “matched-pair” (capture and detection) antibodies obtained from R&D Systems, Abingdon, England. The recently described (18) method was followed with the exception that the samples (1:50 dilution) and standards (1000–15.6 pg/mL in serial 1:2 steps) were incubated in RD5P medium (R&D Systems, England). Subsequent incubations were in Blotto® (Pierce, USA). The chromogenic detection method and the coefficients of variance have been reported (18).

### Statistics

Except for the normally distributed clinical variables (Table I) non-parametric, two-tailed Mann–Whitney *U* test and  $2 \times 2$  contingency tables were used as indicated. A *p* value of 0.05 or less was accepted as significant.

## RESULTS

### Clinical Data and IVF Treatment Outcome

In our total sample of 326 women undergoing IVF treatment and fulfilling the inclusion criteria, 47 were diagnosed with endometriosis. Table I, top section, shows the pretreatment and clinical data. There were no statistically significant differences, including ovarian reserve, between the groups. The results for the outcome parameters of the IVF treatment are given in the second part of Table I.

### Steroid Hormones, PAPP-A, IGFBP-4 and Leptin Concentrations in Follicular Fluids

Follicular fluid concentrations for estradiol were significantly reduced in endometriosis patients in comparison to the non-endometriosis controls. On the other hand, no significantly different follicular fluid levels between the two groups were observed

**Table I.** Pre-IVF Treatment and Clinical Parameters and IVF Outcome Data for the Study Patients of the Two Groups, Controls and Cases of Endometriosis

Clinical parameter	Control group ( <i>n</i> = 279)	Endometriosis group ( <i>n</i> = 47)	<i>p</i> value
Maternal age (years) (Mean ± SD)	33.2 ± 4.1	33.0 ± 3.7	0.7254 <sup>a</sup>
Body mass index (kg/m <sup>2</sup> ) (Mean ± SD)	23.9 ± 3.8	22.8 ± 3.7	0.0575 <sup>a</sup>
Basal FSH (mIU/mL) (Median, Range)	7.7 (2.4–24.8)	7.6 (2.8–19.8)	0.7044 <sup>b</sup>
Oocytes retrieved (N)	8.6 ± 4.5	7.8 ± 4.4	0.3767 <sup>a</sup>
Embryos transferred/ET	1.72 ± 0.67	1.51 ± 0.69	0.0498 <sup>a</sup>
Cancelled transfers (N) <sup>c</sup>	24 of 279	5 of 47	0.5869 <sup>d</sup>
No Pregnancy (all cycles)	202 of 279	37 of 47	0.4760 <sup>d</sup>
Biochemical pregnancies	15 of 279	3 of 47	0.7276 <sup>d</sup>
Miscarriages	7 of 279	1 of 47	1.00
Ongoing pregnancies	55 of 279	6 of 47	0.3157 <sup>d</sup>
Pregnancy rate per transfer (%)	30.2	23.8	

<sup>a</sup>By Student's *t*-test.

<sup>b</sup>By Mann–Whitney *U*-test.

<sup>c</sup>Fertilization and cleavage failures or threatening hyperstimulation syndrome.

<sup>d</sup>By Fisher's Exact test ( $2 \times 2$  contingency table).

for progesterone, leptin, PAPP-A and IGF-bp4. The results are presented in Table II.

Leptin concentrations in the follicular fluid correlated strongly and significantly ( $p < 0.0001$ ) with the body mass index, irrespective of the absence or presence of endometriosis but in an even more pronounced way in the latter group. The results are presented in Fig. 1. PAPP-A levels were strongly ( $p < 0.0001$ ) and positively correlated to progesterone in both groups, and negatively to estradiol in the controls ( $p = 0.0201$ ). Moreover, PAPP-A was positively correlated to IGF-bp4 in the control group ( $p = 0.0293$ , Fig. 2A), with leptin presenting the same pattern to an even stronger extent ( $p = 0.0005$ , Fig. 2B). No associations, however, were found between steroid hormones and leptin.

## DISCUSSION

We have found a significantly decreased follicular fluid concentration of estradiol in endometriosis patients compared to controls, but no difference in progesterone levels which is in contrast to Pellicer *et al.* (19) who found that these levels were increasing

**Table II.** Ovarian Steroid Hormones, Leptin, PAPP-A and IGF-Binding Protein-4 (Median, Range) in the Pooled Follicular Fluid of Women with and Without Endometriosis

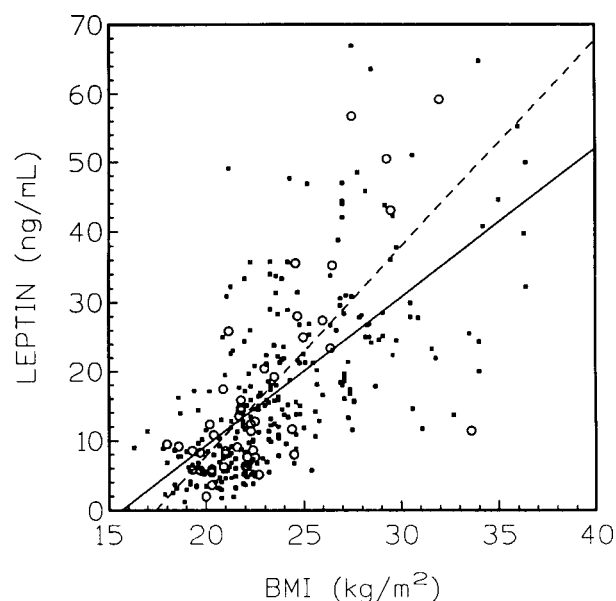
Hormone or growth factor	Control group (n = 279)	Endometriosis group (n = 47)	p value
Estradiol (nmol/L)	823 (79–5832)	641 (134–3545)	0.0279
Progesterone ( $\mu$ mol/L)	28.92 (1.59–119.2)	28.31 (7.29–45.09)	0.9328
Leptin (ng/mL)	14.15 (1.23–66.95)	11.38 (2.01–56.73)	0.1655
PAPP-A (mIU/L)	1.37 (0.02–2.89)	1.31 (0.66–2.58)	0.6143
IGF-bp4 (ng/mL)	123 (64–184)	122 (84–188)	0.7722

with the severity of the disease. This discrepancy is difficult to explain, but it may be related to lower numbers in the mentioned study (particularly the controls) and to different distribution of the patients between the various types of infertility (tubal vs. male) in the control group. Other studies of natural and stimulated cycles did not indicate any differences of steroid levels in the serum and FF in endometriosis patients compared with controls. We have, however, also observed slightly lower estradiol levels in the serum (on the day of oocyte retrieval) in our endometriosis patients (median, 2.88 nmol/L) than in the controls (median, 4.34 nmol/L), and serum and follicular fluid estradiol levels correlated

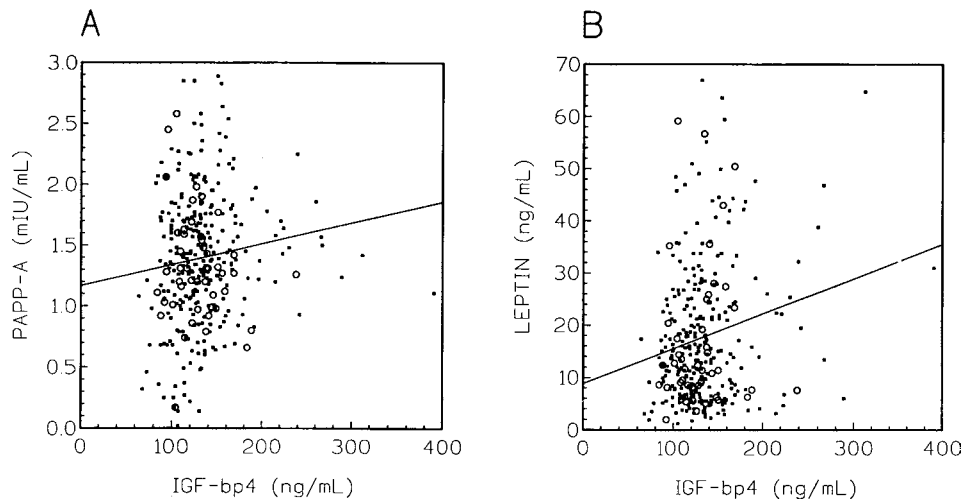
strongly with each other in both groups ( $p < 0.01$  by Spearman Rank correlation analysis). In our IVF centre, more than 90% of the couples were referred for reasons of male infertility; our control population could thus be closer to the normal, fertile one which is reacting better to the gonadotrophin stimulation, resulting in higher FF estradiol levels. Our significantly increased estradiol levels together with the better pregnancy rates of the control group in our study correspond to the finding that fertilized oocytes came from follicles whose FF contained higher estradiol levels, and the best cleavage rate is obtained from follicles containing more estradiol (20).

Data on leptin in the context of fertility are controversial. An earlier report suggested low serum levels to be related to infertility (3), a finding which has not been confirmed. One study reported an increased leptin concentration in peritoneal fluid in women with peritoneal endometriosis (21). Information on leptin levels in the follicular fluid of endometriosis patients is scarce, but we are confirming the parallelism between serum and follicular fluid via correlation of the latter with the BMI. The effect of leptin on ovarian steroidogenesis is altogether controversial, and only limited data exist regarding the ovarian function and leptin. A positive relationship between the increase in estrogen and leptin was reported (22), and another study suggested a direct stimulation by leptin of estradiol, but not of progesterone production in cultured granulosa cells (23). Another study (24), however, indicated no effect of leptin on estradiol or progesterone production in granulosa cell culture and Ghizzoni *et al.* (25) even suggested an inhibition of steroid biosynthesis by leptin in such cultures. In this study we did not observe any associations amongst follicular fluid leptin and steroid hormone levels.

The results of studies trying to establish a correlation between leptin levels and the outcome of IVF treatment are controversial, too. Better pregnancy rates in the case of lower serum leptin levels at



**Fig. 1.** Follicular fluid leptin concentrations as a function of the body mass index. The individual values and the regression lines are represented as dots and a solid line for the controls and as open circles and a dashed line for the cases of endometriosis. Regression parameters for slope,  $r$  and  $p$  are 2.136, 0.4192, and  $< 0.0001$  for controls and 3.002, 0.5334, and  $< 0.0001$  for endometriosis, respectively.



**Fig. 2.** Follicular fluid PAPP-A (A, left panel) and leptin (B, right panel) concentrations as a function of the concentration of IGF-bp4. The individual values and the regression lines are represented as dots and a solid line for the controls and as open circles for the cases of endometriosis. Regression parameters for slope,  $r$  and  $p$  values in the control group are  $1.72 \times 10^{-3}$ , 0.125, and 0.0365 for PAPP-A, and 0.067, 0.206, and 0.0005 for leptin, respectively. No significant association was observed for these parameters in the endometriosis group.

baseline or at the day of the administration of hCG were reported (26), and the same was suggested to be the case for follicular fluid in a conference abstract at a recent Annual Meeting of ESHRE (2003). These observations would be in theoretical agreement with the reported decreased pregnancy rates and concomitantly elevated levels of leptin in endometriosis patients (11). Moreover, the reduced estradiol levels in the follicular fluid observed here may strengthen the hypothesis of the inhibitory role of leptin at the level of the granulosa cell, although the causal relationship of this interaction can only be elucidated by in vitro studies at the cellular level. Another report (27), however, did not find any association of FF leptin levels and outcome, which is in agreement with our own observation from this study ( $p = 0.5386$ ).

Data on the body mass index (BMI) in women with endometriosis are scarce and controversial. One study (28) reported endometriosis patients to be underweight more frequently, which is in contrast to McCann *et al.* (29) who did not observe a significant difference of body fat distribution or BMI between women with endometriosis and controls. In the population of our own study presented here, the BMI in the endometriosis group was indeed lower than in the controls (Table I), but statistical significance was not reached.

The knowledge on the possible function and clinical usefulness of PAPP-A outside pregnancy is still

very thin. Increased PAPP-A levels have been found in the peritoneal fluid of women with endometriosis in one study (8), but results on PAPP-A in the FF of such patients are not available. Concerning the outcome, a positive relationship between elevated follicular fluid PAPP-A levels and the chance of achieving a pregnancy in that cycle is supported by an observation (30) that PAPP-A gene expression in human ovaries is restricted to healthy follicles. We have observed a significant, positive correlation between PAPP-A and IGF-bp4 levels in the FF of our control group ( $p = 0.0365$ ) which is difficult to explain: The reported role of PAPP-A as a specific protease to IGF-bp4 would rather suggest a negative association. The situation in the ovary, however, has not been specifically explored and may differ from the one in fibroblasts, trophoblast, and endometrium. An autoregulatory “short” loop may thus be functioning for PAPP-A at the level of the follicle. On the other hand, we have observed a pronounced positive correlation between follicular fluid levels of PAPP-A and progesterone, while the association with estradiol is negative. The further investigation of this extremely complicated pattern for the role of ovarian PAPP-A will be very challenging; one of the major problems is certainly the difficulty to select a proper control population with absolutely fertile women undergoing ICSI treatment only because of pure male factor. It will thus always remain difficult to

compare the observations from different projects and to reconcile results which seem inconsistent or even contradictory.

A recent report has demonstrated that pre-IVF surgery did not improve the subsequent embryo and outcome parameters (31). The gained knowledge on the basics of folliculogenesis, however, will improve long-term treatment of infertility.

## CONCLUSIONS

In conclusion, while the follicular fluid levels of progesterone, PAPP-A, IGF-bp4 and leptin showed no significant differences between IVF patients with and without endometriosis, the results of our study indicate that FF estradiol concentrations contrast significantly between the two groups. As the oocyte quality is also decreased in women with endometriosis, estradiol can be considered as a marker not only of oocyte maturity but also of oocyte quality, but this without the reduced estradiol necessarily being the consequence of poor quality, i.e. the causal relationship cannot be determined in this type of study. In vitro investigations would be required to address this point, but they are illegal in Switzerland. Moreover, the assessment of the role of the other markers and their clinical potential in fertility related investigations requires a larger number of endometriosis cases as well as in vitro studies.

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