

Concentrations of leptin and C-reactive protein in serum and follicular fluid during assisted reproductive cycles

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BACKGROUND: There are only a few studies that have investigated inflammatory processes during ovarian hyperstimulation, with contradictory results especially concerning outcome. The aim of the study was to investigate the inflammatory markers C-reactive protein and leptin in serum and follicular fluid and to correlate these with the outcome. **METHODS:** One hundred and sixty-two gonadotrophin stimulated cycles were evaluated. Serum concentrations of leptin and C-reactive protein were measured at the initiation of stimulation, on the day of hCG administration or the day before, and on the day of oocyte retrieval. They were also determined in the follicular fluid. **RESULTS:** Serum leptin and C-reactive protein levels increased significantly during stimulation until the day of oocyte pick up, but following different patterns. After stimulation, they correlated with each other in serum and follicular fluid, but not with estradiol or progesterone concentration, embryo quality, or the pregnancy rate. **CONCLUSIONS:** Leptin and C-reactive protein levels change significantly during assisted reproductive treatment. In contrast to estradiol they are, however, not a marker of success.

Key words: C-reactive protein/follicular fluid/IVF/leptin/serum

Introduction

Leptin, the hormone encoded by the obesity (*ob*) gene and initially found to have anti-obesity properties, has since been shown to be proinflammatory (Janik *et al.*, 1997), immunoregulatory (Lord *et al.*, 1998) and angiogenic (Lord *et al.*, 1998; Sierra-Honigmann *et al.*, 1998). It influences reproduction (Masuzaki *et al.*, 1997) and may be involved in the pathogenesis of endometriosis (Matarese *et al.*, 2000) and infertility (Barash *et al.*, 1996; Brzechffa *et al.*, 1996). Leptin was found in oocytes and follicular fluid (FF) (Karlsson *et al.*, 1997), but its production by granulosa cells is a conflicting issue. Cioffi *et al.* found leptin expression at the mRNA and protein levels by granulosa and cumulus cells and the presence of leptin in mature human oocytes (Cioffi *et al.*, 1997); so did Loeffler *et al.* (2001). Karlsson *et al.*, however, reported the absence of *ob* gene expression in human granulosa cells (Karlsson *et al.*, 1997) and Karamouti *et al.* confirmed their findings, demonstrating that leptin is not secreted by human luteinized granulosa cells in cultures (Karamouti *et al.*, 2003). Lower mean follicular fluid and serum leptin concentrations were suggested to be associated with an increased pregnancy rate in some studies (Mantzoros *et al.*, 2000; Tsai *et al.*, 2002). Welt *et al.* (2003) have not seen any association of follicular fluid leptin concentrations with oocyte quality, fertilization or embryo grade in contrast to Barroso *et al.* (1999), where leptin follicular fluid levels correlated negatively with embryo quality. Concentrations of

circulating leptin are increased by inflammatory mediators (Saraff *et al.*, 1997).

C-reactive protein (CRP) is an acute phase protein and is produced by the liver. Several studies have found that body mass index (BMI) is strongly associated not only with leptin but also with CRP levels (Mendall *et al.*, 1996; Ford, 1999; Visser *et al.*, 1999; Yudkin *et al.*, 1999). CRP was found to be increased after estrogen administration (Kluft *et al.*, 2002). An association between plasma CRP and leptin has been shown in humans (Shamsuzzaman *et al.*, 2004) and Bullo *et al.* even demonstrated that leptin could induce the production of CRP (Bullo *et al.*, 2003). Maruna *et al.* identified leptin as an acute phase reactant with potential haematopoietic, immunomodulatory and hepatocyte stimulating activity and also found a significant correlation between CRP and leptin (Maruna *et al.*, 2001). The available results on the usefulness of CRP concentrations as a marker of success in assisted reproductive technologies (ART) are controversial. In the study of Sacks *et al.*, CRP levels have been measured before IVF stimulation and 14 days after egg collection. Women pregnant after IVF, especially pregnant women after ovarian hyperstimulation had significantly higher CRP levels compared to non-pregnant women 14 days after egg collection (Sacks *et al.*, 2004). Almagor *et al.* found serum CRP levels, measured on the day of embryo transfer to be correlated with the outcome of *in vitro* fertilization (IVF)-treatment (Almagor *et al.*, 2004), while Orvieto *et al.* (2004a) did not.

Ovulation is an inflammatory process, and the interactions and correlations of leptin and CRP are thus of great interest in this context. To our knowledge only a few studies on CRP in assisted reproduction, and no data comparing CRP with leptin and steroids in this field, are available in the literature.

The aim of the present study was to examine whether leptin and CRP, which are both involved in inflammatory processes, are correlated with each other and whether they could be an outcome marker in IVF treatment.

Materials and methods

Patients and IVF procedure

The present study contains serum and follicular fluid from 162 women undergoing IVF with intracytoplasmic sperm injection (ICSI). Inclusion criteria were a patient age of 42 years maximum, normal pre-treatment hormonal values, gynecological ultrasound results and cervical smears. 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. Written consent for the treatment by IVF/ICSI was given by all patients, and the investigation was approved by the ethical committee of the University of Berne.

For ovarian stimulation, the 'long' protocol was used. With the agonist Triptorelin, 0.1 mg per day s.c. (Decapeptyl[®], Ferring pharmaceuticals, Wallisellen, Switzerland), downregulation was performed either in the luteal phase of the previous or on the first day of the treatment cycle until complete pituitary desensitization was documented. After sonographic evidence indicated no ovarian follicular activity and serum levels of human chorionic gonadotrophin (hCG) and estradiol were below 2 mIU/ml and 130 pmol/l, respectively, stimulation was initiated in most cases with recombinant FSH (Gonal-F[®], Serono Pharma, Geneva, Switzerland or Puregon[®], Organon Pharmaceuticals, Pfäffikon, Switzerland) or with menopausal gonadotrophin (hMG, Pergonal[®], Serono or Humegon[®], Organon). The starting dose was adapted according to age and ovarian response; usually it was 150–225 IU daily and for patients with an already known low response it was maximally 300–450 IU daily. Ultrasound and serum estradiol measurements were performed to assess follicular maturation; hCG (5000 to 10 000 IU, Profasi[®], Serono or Pregnyl[®], Organon) was administered when at least three follicles exceeded 17 mm in diameter and the estradiol concentration per mature follicle exceeded 1000 pmol/l. 35–36 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.

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 techniques and instrumentation (Tesarik and Sousa, 1995). Fertilization was assessed 17 h after ICSI. One to two normally fertilized oocytes (two pronuclei and two polar bodies) with the highest PN score and with the best morphological grade were considered for embryo transfer. Cryopreservation of the remaining PN was performed at this stage according to the Swiss law. The fertilized oocytes selected for transfer were cultured for another 20–30 h at 37 °C in fresh CO₂ equilibrated IVF medium (Vitrolife[®], Göteborg, Sweden). Embryo development was evaluated 2 and 3 days after ICSI by determining the number of blastomeres and the relative proportion of anucleate cell fragments. Embryo scores were calculated by multiplying the

fragmentation grade (<10% fragments, grade 4; 10–20%, grade 3; 20–30%, grade 2; >30% fragments, grade 1) with the number of blastomeres. In most cases two embryos (range 1–3) were transferred 2 or 3 days after oocyte retrieval. The Cumulated embryo score (CES) was calculated by adding up the individual scores of the transferred embryos for each patient.

Collection of serum and follicular fluid

Blood samples were drawn at three points: on the day of initiation of ovarian stimulation, on the day of or one day before hCG administration, and on the day of the oocyte pick-up, withdrawn immediately before the procedure. Only cycles with an available serum from all three time points were included in the study. Sera were prepared in the central routine laboratory and stored, after the immediate determination of estradiol, at –30 °C until assayed for the other analytes (see below) in batches.

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

Measurement of steroid hormones, leptin and CRP in serum and follicular fluid

Serum 17 β -estradiol (E2) was assayed on the day of collection in the routine laboratory using an automated method ('Elecsys', Roche, Basle, Switzerland). Progesterone was determined by competitive radio-immunoassay (RIA), using the coated tube ('Coat-a-Count') kit manufactured by DPC, Los Angeles, CA, and obtained from Buhlmann Laboratories, Basle, Switzerland. Sera, obtained at the day of OPU, were used without dilution while the follicular fluids were diluted 1:500 in the diluent provided, and the 3 h incubation protocol was followed. Estradiol in the follicular fluids was similarly determined with the same RIA methodology, in an 1:250 dilution and following the protocol supplied by DPC.

Leptin 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 method (Malek *et al.*, 2001) was followed with the exception that the samples (1:50 dilution for both sera and FF) and standards (1000–15.6 pg/ml in serial 1:2 steps) were incubated in RD5P medium (R&D Systems Europe, Abingdon, England). Subsequent incubations were in PBS containing 0.5% non-fat milk proteins (Blotto[®], Pierce USA, obtained from Socochim, Lausanne, Switzerland) as described. The intra- and inter-assay coefficients of variance at midstandard level (125 pg/ml) were 2.7 and 8.9%, respectively.

CRP was determined by microplate ELISA in our laboratory as follows: rabbit polyclonal anti-human CRP (Sigma C3527) was added at 2 μ g/ml in PBS (incubation volume = 100 μ l/well) for overnight 'coating' at 4 °C, then the contents were aspirated and the excess sites blocked with 250 μ l BSA (0.5% w/v in PBS) during 1 h at 37 °C. After a washing step (two aspiration cycles using PBS containing Tween-20, Sigma, 0.1% v/v), standards (CRP Sigma C4064, 100–0.39 ng/ml) or serum/FF samples (diluted in Blotto[®]) were added (100 μ l/well) and the plates incubated for 2 h at 30 °C with a shaking speed of 500 r.p.m. Initial sample dilution was 1:250, but it was necessary to repeat some measurements at higher or lower dilution. After washing the wells three times with PBST, horseradish peroxidase conjugated rabbit anti-human CRP (Dako P227, Glostrup, Denmark, diluted 1:10 000 in Blotto) was added and the incubation continued for 1 h under the same conditions. Then the wells were washed four times with PBST, and ready-to-use TMB substrate solution (Zymed, obtained from Staehelin, Basle,

Switzerland; 100 µl/well) was added in a timed sequence. After 10–20 min of incubation at room temperature in the dark, the reaction was stopped by the addition of 100 µl 2M hydrochloric acid in the same sequence. The optical density was determined with a dual channel microplate reader (Bio-Rad, Hercules, USA, model 550) at 450 nm against a reference at 590 nm. The sensitivity of the assay was 0.30 ng/ml, but the lowest CRP concentration measured in this study was 18 ng/ml. The intra- and inter-assay coefficients of variance in the geometric mean of the standard curve were 5.54 and 10.65%, respectively (Malek *et al.*, submitted).

Statistical methods

Concentrations of leptin, CRP and steroids between the subsequently pregnant ($n = 47$) and non-pregnant ($n = 102$) groups were compared non-parametrically with the Mann–Whitney U test. The same was done, over the whole study population ($n = 162$, including the 13 cycles not leading to embryo transfer for reasons of fertilization or cleavage failure, i.e. CES = 0), when the concentrations of these markers were compared between the different time points. Correlations between the different markers were assessed by linear regression after logarithmic transformation and non-

parametrically by Spearman rank correlation analysis. A P -value of 0.05 or less was considered to identify a significant difference.

Results

Clinical data

Table I shows the pre-IVF treatment data and clinical parameters for the two groups, those women that subsequently became pregnant (pregnant group) and those women who did not become pregnant (non pregnant women). The maternal age, but not the pre-treatment FSH (assessment of ovarian reserve) was lower in the pregnant group. The number of retrieved oocytes at pickup and the cumulated embryo score (CES) were significantly higher in the pregnancy group.

Steroid hormone, leptin and CRP concentrations in serum and follicular fluids

Comparison between pregnant and non-pregnant groups

These results are given in Table II. Before the initiation of stimulation with gonadotrophins, none of the tested markers

Table I. Pre-IVF treatment data and clinical parameters for the two groups, pregnant and non pregnant women

Clinical parameter	Pregnant women ^a	Non-pregnant women ^b	P -value
Maternal age (years)	31.2 ± 4.3	33.7 ± 3.1	<0.001 ^d
Body mass index (kg/m ²)	23.8 ± 3.1	23.6 ± 3.7	0.774
Basal FSH (mIU/ml)	8.34 ± 3.60	8.28 ± 2.96	0.916
Oocytes retrieved	10.7 ± 4.7	9.1 ± 4.5	0.045
Embryos transferred	1.98 ± 0.39	1.91 ± 0.38	0.319
CES ^c	46.2 ± 11.7	39.1 ± 15.8	0.003 ^d

Values given are means ± SD (where applicable). P -values were obtained by Student's t -test.

^a $n = 47$, including 10 biochemical pregnancies and six first or second trimester abortions; the other pregnancies were delivered and included three twin and one triplet gestation.

^b $n = 102$, excluding 13 cycles not leading to embryo transfer.

^cCumulated embryo score.

^dAfter Welch's correction.

Table II. Steroid hormones, leptin (with and without correction for BMI) and CRP concentrations in serum and follicular fluid (FF) of pregnant and non-pregnant women (for numbers and definitions see footnote to Table I)

Time point	Marker	Pregnant women	Non-pregnant women	P -value
Day 1	Leptin ¹	10.72 (1.48–39.39)	11.19 (1.00–45.96)	0.544
	Leptin/BMI	0.39 (0.07–1.35)	0.51 (0.05–1.64)	0.445
	CRP ²	1.26 (0.13–35.46)	1.76 (0.12–36.99)	0.196
	Estradiol ³	0.071 (0.021–0.195)	0.060 (0.020–0.199)	0.162
hCG/hCG-1	Leptin ¹	14.08 (2.65–55.61)	16.26 (3.46–58.42)	0.493
	Leptin/BMI	0.60 (0.148–2.18)	0.70 (0.166–2.58)	0.379
	CRP ²	1.25 (0.018–34.25)	1.40 (0.195–87.25)	0.203
	Estradiol ³	10.39 (2.14–24.11)	7.71 (0.97–27.87)	0.006
OPU (serum)	Leptin ¹	15.70 (1.52–53.10)	16.86 (2.34–64.53)	0.496
	Leptin/BMI	0.69 (0.080–1.87)	0.75 (0.112–2.67)	0.368
	CRP ²	2.87 (0.07–33.05)	3.48 (0.21–167.5)	0.481
	Estradiol ³	5.07 (0.37–12.39)	3.55 (0.25–81.56)	0.012
OPU (FF)	Prog. ³	38.1 (7.5–102)	36.8 (8.3–1400)	0.877
	Leptin ¹	14.84 (1.23–51.00)	14.47 (1.79–55.16)	0.741
	Leptin/BMI	0.65 (0.065–1.96)	0.63 (0.086–2.32)	0.630
	CRP ²	1.48 (0.05–51.11)	1.32 (0.11–119.3)	0.655
	Estradiol ³	869 (225–2335)	694 (79–3291)	0.035
	Prog. ⁴	25.2 (4.5–50.7)	29.3 (3.8–69.8)	0.128

Values given are medians and range; P -values were obtained by non-parametric Mann–Whitney U test (significant differences marked in bold). Units are stated in the footnote (please note differences between serum and FF).

¹ng/ml.

²µg/ml.

³nmol/l.

⁴µmol/l.

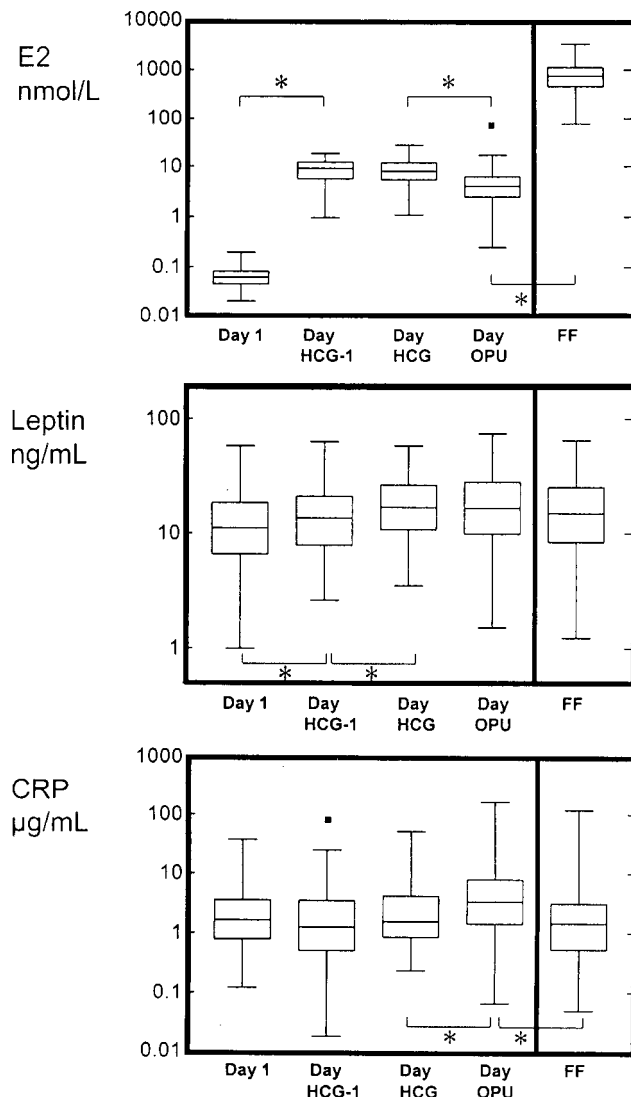


Figure 1. Box and whisker plot of estradiol, leptin and CRP levels in serum as a function of the stimulation phase, and in follicular fluid. Boxes represent the median (central line) and quartiles; whiskers show the minima and maxima. Two outliers are shown as single closed squares. Note the logarithmic scale. Statistically significant differences ($P < 0.05$ by Mann–Whitney analysis) between neighbouring groups are illustrated with a bracket and asterisk. Day ‘hCG-1’ = 3 days before OPU; day ‘hCG’ = 2 days before OPU.

was able to distinguish between the two groups. On the day hCG/hCG-1 (serum) and OPU (serum and FF), estradiol was clearly higher in the group which achieved a pregnancy compared to the one who did not.

Time course of leptin and CRP concentrations during ovarian stimulation

The serum concentrations of all markers were significantly higher on the day of OPU than at initiation of the stimulation phase (day 1), but the time course of this increase was different between leptin and CRP. Levels of the former significantly increased between day 1 of stimulation and day hCG-1/hCG, but did not anymore between then and the day of OPU. This was comparable to estradiol whose levels behaved similarly, but with a decrease between the day of hCG-1/hCG and OPU. On the other hand, CRP did not show an increase between day 1 and day hCG-1/hCG, but its serum levels doubled in the 72/48 h time interval between then and OPU. When the hCG-1 and hCG time points were compared and analysed separately ($n = 70$ and 92 , respectively), leptin was found to significantly increase during this 24 h interval while estradiol had already ceased doing so and CRP was beginning to rise but not with statistical significance yet. The results of these longitudinal investigations are illustrated in Figure 1. In contrast to estradiol and progesterone with their respectively 200- and 600-fold increase over the serum level on the day of OPU, the follicular fluid concentrations of leptin and CRP were lower than in the serum. This reduction in FF level was significant for CRP but not for leptin (Figure 1).

Correlations between leptin and CRP levels, and with gonadal steroids and clinical parameters

On the day of OPU, an extremely high significance level ($P < 0.0001$) was found for the correlation with the body mass index not only for leptin, but also for CRP in the serum (Figure 2A) and in the follicular fluid (Figure 2B). Similarly to leptin, the CRP concentrations were in strong correlation between the two compartments ($r = 0.897$, Figure 2C). A strong positive association was also observed between CRP and leptin in the serum as well as in the follicular fluid ($P < 0.0001$, both compartments, Spearman Rank correlation).

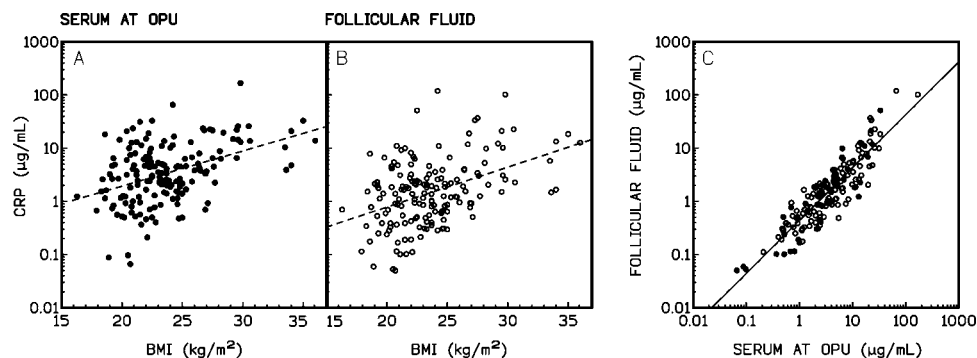


Figure 2. CRP levels on the day of OPU. Correlation between CRP in the serum (Figure 2A, closed circles) or in the follicular fluid (Figure 2B, open circles) and the body mass index (BMI). These associations are highly significant ($P < 0.0001$). Figure 2C compares the CRP levels between serum and follicular fluid. Closed circles, pregnant ($n = 47$); open circles, not pregnant ($n = 115$).

On the other hand, no correlations were observed between the gonadal steroids (estradiol or progesterone) and leptin or CRP in the serum or in the follicular fluid (all $P > 0.31$). This was also the case when leptin and CRP levels were used as a ratio between the concentration of the protein and the body mass index of the patient (all $P > 0.12$).

Discussion

There is not yet much information on CRP in IVF treatment and it is interesting that, to date, there does not seem to be a consensus between the few different studies. In this respect, the considerable differences in the number of patients included have to be noted, ranging from 16 in the paper from Orvieto *et al.* (2004a) to ten times this number in our study.

In agreement to several other studies (Mendall *et al.*, 1996; Ford, 1999; Visser *et al.*, 1999; Yudkin *et al.*, 1999), we have found a positive correlation between CRP and BMI. In the other study on CRP relating to IVF (Almagor *et al.*, 2004), data on BMI were not given. In contrast to Orvieto *et al.* (2004a), we also found a significant ($P < 0.001$) difference in CRP levels between follicular fluid and serum taken on the day of OPU, with the FF concentration being lower. We believe that, for both observations comparing our study with the one cited (Orvieto *et al.*, 2004a), statistical significance was reached in ours because of the higher number of cases included, since the trend was the same and the range of measured values was wide.

Our results concur with the other studies (Almagor *et al.*, 2004; Orvieto *et al.*, 2004a) showing no difference in serum CRP levels on the day of ovarian pick-up (OPU) between women subsequently becoming pregnant and those who did not. Leptin was also found not to be a prognostic marker in this context, which is however in contrast to others who did observe lower leptin levels to be associated with treatment success (Mantzoros *et al.*, 2000; Tsai *et al.*, 2002). In our study the estradiol concentration was the only prognostic parameter that was increased in the serum (as well as in the follicular fluid.) without a lot of women who became pregnant over those who did not on the day of hCG administration or hCG-1 and at OPU.

We could confirm the association of CRP and leptin levels found by others (Maruna *et al.*, 2001; Shamsuzzaman *et al.*, 2004), and we concur with the findings of Orvieto *et al.* (2004a) who did not find any correlations between steroid hormones and CRP or between steroid hormones and leptin. Our findings are also in full agreement with the recently published study by Chen *et al.* (2004), with an absence of correlation between serum or follicular fluid leptin levels and gonadal steroid levels or IVF outcome. Regarding CRP, our results as well as the findings by Orvieto *et al.* (2004a) showed a significant increase in concentration on day of OPU when compared to the day hCG-1 in spite of the decrease in E2 levels at this stage.

To our knowledge no data comparing CRP with leptin and steroids in this field are available in the literature. Our study is therefore unique since we have demonstrated that leptin and CRP, which are both involved in inflammatory processes,

showed a significant difference in their serum concentration time course during ovarian stimulation. In contrast to leptin levels, which continually rose during the ovarian stimulation phase until the day of hCG/hCG-1, the concentrations of CRP did not change between the first day of stimulation and the day of hCG/hCG-1. This latter finding is in contrast to the one by Orvieto *et al.* (2004a) who reported a significant increase in serum CRP levels during stimulation and concluded that the controlled ovarian hyperstimulation potentiated a state of systemic inflammation. But our observations are in line with the recent report by Orvieto (2004b) where he described a significant increase in CRP level on the day of ovum pick-up compared to the day of hCG administration despite the decrease in E2 level, and where he speculated that the inflammatory response was significantly stimulated by the exogenous hCG. Orvieto *et al.* also reported on another inflammatory marker, leukocyte selectin (L-selectin), which was even found to be decreased during controlled ovarian hyperstimulation until peak estradiol was reached, and then significantly increased after hCG administration (Orvieto *et al.*, 2001). It seems that the situation is quite complex. An explanation for the discrepancies in the time course of the two inflammatory mediators leptin and CRP could be that they might be unrelated to each other or that they follow different pathways in the inflammatory process.

It has to be noted that blood sampling for measuring CRP and leptin was obtained before oocyte collection was started, and thus no ovulation or stress induction had taken place. Moreover, the lower CRP concentration in the FF than in the serum is indicating an absent or very low production of this protein in the ovary, and there are no data on *in vitro* CRP production by granulosa cells in the literature.

Conclusions

In conclusion, we could show that ovulation induction is an inflammatory process leading to increased levels of CRP as well as of leptin, but with different patterns. However, according to our study results, they cannot be used as markers of successful outcome of the IVF treatment.

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