Expression and Function of 3 β Hydroxysteroid Dehydrogenase (3 β HSD) Type II and Corticosteroid Binding Globulin (CBG) in Granulosa Cells from Ovaries of Women with and Without Endometriosis¹

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Purpose: To investigate the secretion of progesterone (P4) and corticosteroid binding globulin (CBG) by granulosa luteal cells (GC) as well as the mRNA levels of CBG and 3 β hydroxysteroid dehydrogenase (3 β HSD), in women with and without endometriosis in vivo and in vitro.

Methods: Prospective study in a private, university-affiliated assisted reproduction unit, including women with severe endometriosis ($n = 14$) or without the disease ($n = 20$) undergoing in vitro fertilization/intracytoplasmic sperm injection and embryo transfer. GC were obtained from each follicle aspirated, pooled for each patient, and follicular and blood contaminating leukocytes depleted through immunomagnetic purification. Secreted P4 and CBG, and mRNA for both 3 β HSD and CBG were determined in vivo and in vitro using RIA and reverse transcription followed by competitive polymerase chain reaction (cRT-PCR).

Results: The pattern of expression of 3 β HSD and CBG mRNAs in vivo and in vitro was similar in both groups. Also, GC from patients with endometriosis produced equal amounts of P4 and CBG than controls without the disease, either in freshly isolated cells or in 24-h cultures.

Conclusions: The GC function in terms of 3 β HSD and CBG mRNA expression and P4/CBG secretion does not seem to be altered in patients with endometriosis in comparison with those without this condition.

KEY WORDS: CBG; endometriosis; follicular environment; granulosa cells; 3 β HSD.

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INTRODUCTION

Endometriosis is a mysterious disease that consists in the presence of endometrial tissue implanted out of the uterine cavity. Innumerable studies have attempted to explain the causes of the subfertile status that endometriosis-affected women suffer, but without a distortion of the anatomical tract, the reasons are unclear, and many hypotheses have been raised (1).

The application of in vitro fertilization (IVF) permitted a closer analysis of phenomena that otherwise

were occult such as fertilization, folliculogenesis, early embryo development, or implantation. Based on previous data from our Institution (2,3), we hypothesized that there is decreased pregnancy and implantation rates in women with endometriosis compared to controls, and that the presence of endometriomas in women receiving oocytes from young healthy donors was not different in terms of pregnancy rates, compared with those recipients without the disease (4). This is a clear sign of low quality oocytes, may be caused by a detrimental background in the cases of endometriosis that could potentially affect the quality of the resulting embryo; it has also been demonstrated to be worse in these women (5,6).

Our clinical findings were further studied addressing the characteristics of the endocrine intrafollicular milieu in these patients analyzing granulosa cell function in short-term cultures and follicular fluid (FF) (7) for steroids. Promising results were obtained, showing an increase in the secretion of progesterone (P4) by granulosa cell cultures in women with endometriosis, whereas no differences were found in estradiol, testosterone, and androstenedione.

Thus, the endocrine microenvironment within the follicles needed further investigation, since the causes of these P4 concentrations within the follicle are unclear. Many factors can affect intrafollicular P4 concentration, and among them, those related with its production or transport are serious candidates to be implicated.

Pregnenolone is converted in P4 in the human ovaries by the granulosa luteal cells (GC). The enzyme responsible for its biosynthesis in the human gonads is 3β hydroxysteroid dehydrogenase (3β HSD) Type II, in a two-step reaction, transforming first an hydroxyl group into ketone, and second, transferring the double bond to the position 4–5, from the position 5–6 in the molecule (8).

Otherwise, one of the molecules implicated in P4 bioavailability is the corticosteroid binding globulin (CBG), which is also expressed in the GC and present in FF. Moreover, a high concentration of this molecule within the follicles has been correlated with success in the IVF cycles (9). An increase in the expression of these molecules can be directly related with an increase in the intrafollicular progesterone, thus suggesting endocrinological defects in these patients.

Our aim in this study was to ascertain these molecules in women with endometriosis, compared with those without the disease, *in vivo* (analyzing the granulosa cells that have been conditioned by other follicular cells immediately after purification, and the

follicular fluids), and *in vitro* (with 24-h cell cultures after purification, to analyze granulosa cell function itself).

MATERIALS AND METHODS

Luteinized GC and FF were obtained from a total of 34 women undergoing oocyte retrieval for IVF at the Instituto Valenciano de Infertilidad from December 1999 to December 2000. All patients have undergone laparoscopy for a period not greater than 1 year before the IVF cycle, and were grouped according to the findings (10) as healthy ($n = 14$) or having severe endometriosis ($n = 20$) because of the presence of ovarian endometriomas that were still present after laparoscopy (all of them stage III–IV). The Institutional Review Board of our clinic approved the project, and written consent was obtained from each patient.

The protocol for ovarian stimulation was started by pituitary desensitization with daily subcutaneous administration of 1 mg leuprolide acetate (Procrin, Abbott S.A., Madrid, Spain) and began in the luteal phase of the menstrual cycle. Serum E_2 levels <60 pg/mL (220 pmol/L) and negative vaginal ultrasonographic scans were used to define ovarian quiescence. Days 1 and 2 of ovarian stimulation, two ampoules/day hMG (hMG Lepori, Farma-Lepori, Madrid, Spain) were administered together with two ampoules of high purity FSH (Neo-Fertinorm, Serono Laboratories, Madrid, Spain). Days 3, 4, and 5 of ovarian stimulation, one ampoule/day hMG plus one ampoule/day FSH were administered to each patient. Beginning Day 6, hMG and FSH were administered on an individual basis according to serum E_2 and transvaginal ovarian ultrasound scans. The criteria for hCG administration (10,000 IU, Profasi, Serono Laboratories, Madrid, Spain) were the presence of two or more follicles >1.9 cm in greatest diameter, and serum E_2 levels >800 pg/mL (2940 pmol/L). Leuprolide acetate, FSH, and hMG injections were discontinued the day of hCG administration. Oocyte retrieval was scheduled 36 h after hCG injection. The luteal phase was supported with vaginal administration of micronized P (400 mg/day, Progeffik, Laboratorios Effik, Madrid, Spain).

Granulosa Cells and Follicular Fluid Preparation

FFs without obvious blood contamination from individual patients were centrifuged, supernatants pooled, and stored at -20°C until the radioimmunoassay (RIA) was performed.

Follicular cells from all follicles greater than 16 mm in individual patients were pooled (total number of follicles included in this study was 154), and contaminating blood cells were eliminated using both salt treatment method (11), and immunobead treatment, using mouse monoclonal antihuman CD14 and mouse monoclonal antihuman CD45 (Sigma, St. Louis, MO), and then with specific secondary sheep antimouse IgG antibodies, coupled with magnetic beads (Dynal, Oslo, Norway). After incubation, bead-bound cells were removed. Isolated GC were >99% pure as assessed by flow cytometry analysis (data not shown).

GC Cultures

GC were plated triplicate at a density of 2.5×10^5 viable cells per well (assessed by Trypan Blue staining, Sigma, St. Louis, MO), in 36-mm diameter culture dishes (Falcon, Beckton-Dickinson, NJ) with 1 mL M-199 medium (Sigma, St. Louis, MO), supplemented with 1% fetal bovine serum (FBS, Gibco, Life Technologies, Glasgow, Scotland), gentamicin 50 $\mu\text{g}/\text{mL}$ of medium (Gibco, Life Technologies, Glasgow, Scotland), and fungizone, 250 ng/mL of medium (Gibco, Life Technologies, Glasgow, Scotland), for 24 h at 37°C, 5% CO₂.

Once cells adhered, media were discarded to remove nonadherent cells, and wells were washed twice with M-199, and cultured for 24 h in the media above described. Subsequently, the conditioned culture media were stored at -20°C until RIA assays and cells were detached from plastic with the use of RNastat 60 reagent (Tel-Test, Friendswood, TX) for RNA purification.

RNA Extraction

The RNA extraction was performed from both freshly isolated and cultured GC, according to the Chomczynski and Sacchi (12) method, and quantified by spectrophotometry on a Shimadzu 1201 (Izasa, Madrid, Spain) spectrophotometer.

Reverse Transcription (RT)

RT was carried out using Advantage RT-for-PCR KIT (Clontech, Palo Alto, CA). Mastermix per sample was prepared as follows: 4 μL of 5X reaction buffer, 1 μL of dNTP mix (10 mM each), 0.5 μL of Recombinant RNase Inhibitor, 1 μL of MMLV (Moloney-Murine Leukemia Virus) reverse transcriptase. One μg of each sample was diluted to a final

volume of 12.5 μL in diethylpyrocarbonate (DEPC) treated water; and then 1 μL of oligo (dT) 18 was added, heated at 70°C, 2 min, and kept on ice until mastermix (6.5 μL) was added. Once all components were mixed, the reaction was incubated at 42°C for 1 h, and heated at 94°C for 5 min to stop cDNA synthesis and destroy DNase activity. The product was diluted to a final volume of 100 μL with DEPC treated water and stored at -20°C until the PCR.

Primers for PCR

Sequences for the mRNAs to be detected in human GC were obtained from the GenBank Database of the National Center for Biotechnology Information (NCBI) of the National Institute of Health (NIH, Internet Address: <http://www2.ncbi.nlm.nih.gov/cgi-bin/genbank>). Each set of primer sequences was designed by using the program OLIGO 5.0 Primer Analysis Software (National Bioscience, Plymouth, MN) and synthesized by GIBCO-BRL Custom Primers (Gibco, Life Technologies, Glasgow, Scotland). Design was performed to cross intron/exon boundaries to ensure that amplified products were from cDNAs and not from contaminating genomic DNA. Primer locations on cDNA, sizes of amplified fragments, and sequences are listed in Table I.

Construction of Competitive Fragments

Floating primers for 3 β HSD, CBG, and β -actin were designed from the sequence of cDNA between the 3' and 5' binding sites followed by the reverse complementary 3'-binding site. RT products from isolated GC were amplified in 35 cycles of PCR with the use of an Eppendorf Mastercycler personal (Eppendorf, Hamburg, Germany); the products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide (EtBr), and extracted from the gel with the Sephaglass™ Band Prep Kit (Pharmacia Biotech, Cambridge, UK). The concentration was quantified by spectrophotometry. Resulting products had a deletion between 3' and 5' primer binding sites, compared with the target cDNA to be detected. PCR products were confirmed by independent sequence analysis. Sequences of all primers, and data on fragment sizes are described in Table I.

cPCR

Volumes of RT products (Table II) were mixed with PCR mastermix containing 5 μL 10X buffer, 2.5 μL 50 mM MgCl₂, 0.5 μL Taq Polymerase (all

Table I. Primers Used for cPCR

mRNA	GeneBank accession number	Size of amplified fragment	Position of primers on cDNA	Primer	Sequence of oligonucleotide
β -actin	X00351, J00074, M10278	838 bp (target)	294–325	–5'	5' $\rightarrow$ ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG $\rightarrow$ 3'
			1131–1100	–3'	5' $\rightarrow$ CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC $\rightarrow$ 3'
		434 bp (comp.)	1131–1100 + 837–809 + 676–656	–3'-float	5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GCA GGA AGG CTG GAA GAG TGC ATG TCA CGC ACG ATT TCC CGC -3'
3β -HSD	M-77144	446 bp (target)	177–195	–5'	5'-GGA GGG CTT CTG GGT CAG-3'
			602–623	–3'	5'-TTT TTG CTG TAC GGG TAT GGA-3'
		211 bp (comp.)	602–623 + 348–367	–3'-float	5'-TTT TTG CTG TAC GGG TAT GGA CCT GCG AGG CTC TTT TCA G-3'
CBG	M-31662	528 bp (target)	467–487	–5'	5'-ACG AGA CAT CAA CGACTA CT-3'
			625–634	–3'	5'-CCC ATT TCC TCC ACG ACA TC-3'
		188 bp (comp.)	625–634 + 975–994	–3'-float	5'-CCC ATT TCC TCC ACG ACA TCT AGT TGA CCA GGA CGA GGA T -3'

Bioline, London, UK), 1 μ L dNTPs mix (Sigma, St. Louis, MO), 1 μ L primer mix (5 mM each, Gibco, Life Technologies, Glasgow, Scotland) for either CBG, 3β HSD, or β -actin. Volumes of competitor (Table II), and sterile distilled water were added to a final volume of 50 μ L.

These volumes of competitor cDNAs to be added in the cPCR experiments were decided after previous cPCRs fixing the amount of sample target cDNAs and adding serial dilutions of competitor, and were chosen with the aim of keeping the ratios of competitor versus target cDNAs within 0.5 and 1.5 in as much samples as possible. The cPCR reactions were carried out using an Eppendorf Personal Mastercycler (Eppendorf, Hamburg, Germany), with the following program using a heated lid (100°C): first heated to 94°C, 5 min, following *n* cycles of 94°C, 45 s; T°C (Table II), 45 s; 72°C, 45 s, with a final extension of 72°C, 5 min, and cooled down to 4°C. Products were then electrophoresed in a 1% agarose gel in the presence of EtBr, and bands analyzed in an image analysis system (Gelprinter Plus, TDI, Madrid, Spain), using the 1D Software (TDI, Madrid, Spain). Each cPCR experiment was performed a minimum of 2–3 times with very similar results. In the negative controls,

an equal volume of sterile distilled water substituted competitor and target.

All the results of the expression analysis were normalized with previous β -actin cPCR results.

Radioimmunoassay (RIA)

P4 and CBG concentrations in FF and conditioned GC media were determined in duplicate by RIA with the use of a commercially available kit (ICN Biomedicals, Costa Mesa, CA) and following manufacturer’s instructions.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD). Student’s *t* test was carried out between groups, since data were normally distributed. Significance was defined as *p* < 0.05. The statistical analysis was performed using the Statistical Package for Social Sciences 9.0 (SPSS, Chicago, IL).

RESULTS

The presence of specific mRNAs for 3β HSD and CBG molecules was detected in all samples together with control β -actin.

The analysis of the expression of mRNA for 3β HSD in GC did not reveal any difference when patients with and without endometriosis were compared in the cPCR experiments (Fig. 1). In vivo 3β HSD expression in endometriosis patients was 1.2 ± 0.5 while in women without the disease it was 1.0 ± 0.1 , both expressed as ratios between amplification of target

Table II. cPCR Data

mRNA	Volume of sample (μ L)	Annealing temperature (°C)	Amount of competitive DNA	Number of PCR samples
β -actin	2.5	56	24.7 ng	30
3β -HSD	10	57	3 fg	35
CBG	10	51	6.8 fg	35

Fig. 1. cRT-PCR results for 3β HSD expression in GC from women with endometriosis and controls without the disease.

versus competitor molecules. In the in vitro experiments, the expression of 3β HSD was 0.6 ± 0.3 in the endometriosis group and 0.7 ± 0.1 in the control group, indicating that GC express this mRNA in the same manner.

The cPCR results on mRNA expression for CBG (Fig. 2) did not reveal any difference when patients with endometriosis were compared with healthy patients. The ratios of the optical densities of target versus competitor in vivo were 1.3 ± 0.6 in those patients with the disease and 1.2 ± 0.3 in healthy women. In the cultured GC from patients with endometriosis, the expression of CBG was 0.7 ± 0.1 , while in women without endometriosis the ratio was 0.8 ± 0.3 .

Both secreted CBG and P4 were detected in all GC culture supernatants and in every FF analyzed. Secreted CBG levels in vivo and in vitro, for endometriosis and control group, were comparable (Table III), as measured by RIA, hence reinforcing

again the results of mRNA expression and intracellular protein. RIA measurements demonstrated an equal pattern of secretion of P4 in both groups in vivo and in vitro, adding strength to the previous mRNA findings regarding this molecule (Table III).

DISCUSSION

In this study we wanted to find an explanation to preceding studies with controversial results regarding the intrafollicular presence of P4 in women with endometriosis as compared with healthy controls and their implications in the pathophysiology of endometriosis.

The past results yielded by our research line on this topic demonstrated that there was an increase in the GC secretion of P4 in short-term cultures, and also in its presence in the FF of women with endometriosis compared with nonendometriosis patients, that could

Fig. 2. cRT-PCR results for CBG expression in GC from women with endometriosis and controls without the disease.

Table III. Secreted P4 and CBG Concentrations (Expressed as mean $\pm$ SEM) In Vivo and In Vitro in Women with Endometriosis Versus Those Without the Disease

Molecule	In vivo (FFs)		In vitro (Culture supernatants)	
	Control	Endo	Control	Endo
CBG (ng/ mL)	110.3 $\pm$ 12.5	124.0 $\pm$ 17.4	12.9 $\pm$ 0.6	14.1 $\pm$ 0.6
P4 (ng/mL)	31571 $\pm$ 3703	40670 $\pm$ 2111	1097 $\pm$ 391	1664 $\pm$ 764

be involved in the clinical results of our IVF and ovum donation program (7).

Moreover, in another related study by Harlow *et al.* (13), trying to give rationale to the reduced fertilization that they found in women with endometriosis (14) as the cause of endometriosis-associated infertility, they found, together with an aromatase dysfunction, that the ability to secrete progesterone in the GC from these women was diminished as compared to controls, again in vitro in cell cultures of 3 h. Nevertheless, their population included tubal damage or unexplained infertility as controls (with absence of endometriosis confirmed by laparoscopy in all of them), and infertile couples with treated or untreated endometriosis. Measurements were done by fluoroimmunoassay, but no determination of leukocytes in the cultures or in vivo experiments was attempted.

Other studies by Skrzypczak (15) also found an impairment in the progesterone production of GC from women with severe ovarian endometriosis, as in the actual work we did, compared with healthy women, but low number of patients (only 11) were included in the study.

These initial data together suggested an impairment of the follicular function that could convey to subtle alteration of the oocytes, mediated by the altered presence of P4 inside the follicle, although they were contradictory.

This work aimed to explore the secretion of two molecules, CBG and P4, as well as the mRNA expression of the CBG and of the enzyme that catalyses the ovarian formation of P4 (3β HSD Type II), in the GC of women with endometriosis in comparison to patients without the disease in experiments in vivo and in vitro. Thus, the present results are in discordance with them, since no differences in P4 were found in a very similar design, and the molecules related to P4 biology studied were synthesized in a similar way.

Herein we took special care in avoiding the presence of leukocytes (both from inside the follicle and

contaminating blood) by purification and subsequent analysis of GCs for their presence within the FF. These cells are able to modulate steroidogenesis (16,17), and they have been demonstrated to be approximately 15% of the cells present in the FF without peripheral blood contamination (18), and near to 60% in contaminated FF (author's own unpublished data). In addition, we only pooled for the in vivo determinations in both groups, those FF coming from the early follicles, before blood contamination was evident, because of inherent follicular puncture limitations. P4 amounts in peripheral blood could mask the real situation in the follicle, thus interfering with the results, if this step is not carefully performed. We firmly believe that GC model of study, applied to any research field, is a useful tool but also requires these previous steps to be completely sure of its reliability and reproducibility.

Moreover, the statistical power of the study because of the sample size, and the fact that we only included patients with a laparoscopy performed less than 1 year before the ovum pickup, indicating the absence or presence of endometriosis (in the latter, with ovarian endometriomas), adds strength to our results.

It is difficult to give rationale to the different results that counteract what has been formerly reported in the literature on the measurement of P4. In this study, the use of adequate controls was included, the results in vivo and in vitro were correlated, and two molecules implicated in the concentrations of P4 inside the follicles had been determined with the analysis of gene expression and of the secreted molecule, ensuring a more complete approximation to the situation.

Nevertheless, we must consider that active implants behave differently than inactive disease, and we did not discriminate among them. Perhaps the selection of patients should include a parallel evaluation of the degree of activation of the disease to compare results, since the disease could display different degrees of activation (19,20).

Other problems within the GC including defects in the apoptosis pathway (21,22), in diverse paracrine factors (23), and even intrafollicular leukocytes (24) have been described and could explain a different status within the follicle, but recent studies compared to the present one again gave conflicting results (25). Anyhow, the available literature is insufficient, and efforts are needed to ascertain which factors or processes are really implicated in the infertility-related aspects of the disease in the molecular dimension. But, before this is done, a standardization of the design of

any basic study on this complicated topic is needed; this is necessary to make results comparable and so that a consensus may be reached, paving the way for determinant advances in this field.

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REFERENCES

- Garrido N, Navarro J, Remohí J, Simón C, Pellicer A: Follicular hormonal environment and embryo quality in women with endometriosis. *Hum Reprod Update* 2000;6(1):67–74
- Simón C, Gutiérrez A, Vidal A, de los Santos MJ, Tarín, JJ, Remohí J, Pellicer A: Outcome of patients with endometriosis in assisted reproduction: Results from in vitro fertilization and oocyte donation. *Hum Reprod* 1994;9:725–729
- Pellicer A, Oliveira N, Gutierrez A, Remohí J, Cano A, Ruiz A, Simón C: Implantation in endometriosis: Lessons learned from IVF and oocyte donation. *In Progress in Endometriosis*, Spinola P and Coutinho EM (eds), Casterton-Hill, Parthenon, 1994, pp 177–183
- Díaz I, Navarro J, Blasco L, Simon C, Pellicer A, Remohí J: Impact of stages III-IV of endometriosis on recipients of sibling oocytes. Matched case-control study. *Fertil Steril* 2000;74(1):31–34
- Brizek CL, Schlaff S, Pellegrini VA, Frank JB, Worriow KC: Increased incidence of aberrant morphological phenotypes in human embryogenesis—an association with endometriosis. *J Assist Reprod Genet* 1996;12:106–112
- Pellicer A, Oliveira N, Ruiz A, Remohí J, Simón C: Exploring the mechanisms of endometriosis-associated infertility: An analysis of embryo development and implantation in assisted reproduction. *Hum Reprod* 1994;9:725–729
- Pellicer A, Valbuena D, Bauset C, Albert C, Bonilla-Musoles F, Remohí J, Simón C: The follicular endocrine environment in stimulated cycles of women with endometriosis: Steroid levels and embryo quality. *Fertil Steril* 1998;69:1135–1141
- Speroff L, Glass RH, Kase NG: *Clinical Gynecologic Endocrinology and Infertility*. Baltimore, Williams and Wilkins, 1973
- Andersen CY: Levels of steroid-binding proteins and steroids in human preovulatory follicle fluid and serum as predictors of success in *in vitro* fertilization-embryo transfer treatment. *J Clin Endocrinol Metab* 1990;71(5):1375–1381
- American Fertility Society: Revised American Fertility Society Classification of endometriosis. *Fertil Steril* 1985;43:351–352
- Beckmann MW, Polacek D, Seung L, Schreiber JR: Human ovarian granulosa cell culture: Determination of blood cell contamination and evaluation of possible culture purification steps. *Fertil Steril* 1991;56(5):881–887
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;16:156–159
- Harlow ChR, Cahill DJ, Maile LA, Talbot WM, Mears J, Wardle PG *et al.*: Reduced pre-ovulatory granulosa cell steroidogenesis in women with endometriosis. *J Clin Endocrinol Metab* 1996; 81:426–429
- Cahill DJ, Wardle PG, Maile LA, Harlow ChR, Hull MGR: Pituitary-ovarian dysfunction as a cause for endometriosis-associated and unexplained infertility. *Hum Reprod* 1995; 10:3142–3146
- Norman RJ, Bonello N, Jasper MJ, Van der Hoek KH: Leukocytes: Essential cells in ovarian function and ovulation. *Reprod Med Rev* 1997;6:97–111
- Skrzypack J: Morphology and steroidogenesis of cultured granulosa cells from endometrioidally changed ovaries. *Exp Clin Endocrinol Diabetes* 1996;103(4):228–232
- Adashi EY: The potential relevance of cytokines to ovarian physiology: The emerging role of resident ovarian cells of white blood cell series. *Endocr Rev* 1990;11:454–456
- Garrido N, Albert C, Mercader A, Remohí J, Simón C, Pellicer A: Leukocyte subpopulations in ovulatory follicles in patients with endometriosis (abstract No 172). *In 15th Annual Meeting of the European Society of Human Reproduction and Embryology*, Tours, France. *Hum Reprod* 1999;14:227 (Abstract Book 1)
- Nisolle M, Donnez J: Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. *Fertil Steril* 1997;68(4):585–596
- Brosens I: Diagnosis of endometriosis. *Semin Reprod Endocrinol* 1997;15(3):229–233
- Toya M, Saito H, Ohta N, Saito T, Kaneko T, Hiroi M: Moderate and severe endometriosis is associated with alterations in the cell cycle of granulosa cells in patients undergoing *in vitro* fertilization and embryo transfer. *Fertil Steril* 2000;73(2):344–350
- Nakahara K, Saito H, Saito T, Ito M, Ohta N, Takahashi T, Hiroi M: Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. *Fertil Steril* 1998;69(5):931–935
- Carlberg M, Nejaty J, Froya B, Guan Y, Soder O, Berqvist A: Elevated expression of tumor necrosis factor alpha in cultured granulosa cells from women with endometriosis. *Hum Reprod* 2000;15(6):1250–1255
- Lachapelle MH, Hemmings R, Roy DC, Falcone T, Miron P: Flow cytometric evaluation of leukocyte subpopulations in the follicular fluids of infertile patients. *Fertil Steril* 1996;65(6): 1135–1140
- Garrido N, Albert C, Krüssel JS, O'Connor J, Remohí J, Simón C, Pellicer A: No differences in expression, production and secretion of vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) by granulosa cells in women with endometriosis compared to those without the disease. *Fertil Steril* 2001;76(3):568–575