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Endometrial receptivity is affected in women with high circulating progesterone levels at the end of the follicular phase: a functional genomics analysis

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BACKGROUND: Elevated serum progesterone levels at the end of the follicular phase in controlled ovarian stimulation (COS) leads to a poorer ongoing pregnancy rate in IVF cycles due to reduced endometrial receptivity. The objective of this study was to use microarray technology to compare endometrial gene expression profiles at the window of implantation according to the levels of circulating progesterone.

METHODS: For this prospective cohort study, microarray data were obtained from endometrial biopsies from 12 young healthy oocyte donors undergoing COS with pituitary suppression by either gonadotrophin-releasing hormone (GnRH) agonists or antagonists, and recombinant FSH. On the day of recombinant chorionic gonadotrophin (rCG) administration, six women had serum progesterone levels (P) >1.5 ng/ml (study group) and six had serum P levels <1.5 ng/ml (control group). Endometrial samples were collected using a Pipelle catheter 7 days after the rCG injection.

RESULTS: Using the parametric test, we identified 140 genes significantly dysregulated (64 up- and 76 down-regulated) in the study group endometria compared with the control endometria, regardless of the GnRH analogue employed. These genes are related to cell adhesion, developmental processes, the immune system and others, which are all required for normal endometrial function development. Of the 25 gene targets previously proposed as markers for endometrial receptivity, 13 appeared over-regulated in the study group.

CONCLUSIONS: Our results reveal that elevated progesterone levels on the day of rCG administration can induce significant alterations in the gene expression profile of the endometrium.

Key words: endometrial receptivity / progesterone / gene expression / ovarian stimulation

Introduction

Embryo implantation continues to be the most uncertain event of the whole reproductive process. Among the diverse factors affecting its prognosis, elevation of circulating progesterone levels at the end of the follicular phase in controlled ovarian stimulation (COS) for IVF-embryo transfer seems to have a negative impact on embryo implantation and therefore on cycle outcome (Schoolcraft *et al.*, 1991; Silverberg *et al.*, 1991; Mio *et al.*, 1992; Check *et al.*, 1993; Fanchin *et al.*, 1993, Harada *et al.*, 1995, Randall *et al.*, 1996; Shulman *et al.*, 1996; Fanchin *et al.*, 1997; Bosch *et al.*, 2003; Kiliçdag *et al*; 2010). In fact, it has been recently reported that serum progesterone levels >1.5 ng/ml on the last day of COS are related to a significant decrease in the ongoing pregnancy rate following IVF cycles, irrespective of the gonadotrophin-releasing hormone (GnRH) analogue used for pituitary down-regulation (Bosch *et al.*, 2010).

Although the mechanism by which increased serum progesterone concentrations affect cycle outcome is still unclear, some data suggest that it impair endometrial receptivity rather than oocyte quality (Fanchin *et al.*, 1996; Fanchin *et al.*, 1997; Fanchin *et al.*,

1999; Smitz et al., 2007). Both the oocyte donation and embryo cryopreservation models are the best clinical tools to distinguish the effects on oocytes from those on the endometrium. Elevated progesterone in donors has been found to have no adverse effect on oocyte quality and the implantation rate in recipients (Hofmann et al., 1993; Legro et al., 1993; Check et al., 1994; Shulman et al., 1996; Melo et al., 2006; Check et al., 2010), thus corroborating that the putative adverse impact of progesterone in IVF is likely to be on the endometrium (Chetkowski et al., 1997; Fanchin et al., 1997; Shapiro et al., 2010).

Some authors have suggested that the mechanism underlying the deleterious effect of an elevated progesterone level is an abnormally accelerated endometrial maturation leading to impaired endometrial receptivity (Forman et al., 1989; Sharma et al., 1990; Silverberg et al., 1991; Melo et al., 2006). This secretory endometrial transformation has been observed on the day of oocyte retrieval in both the GnRH agonist (Chetkowski et al., 1997; Ubaldi et al., 1997) and antagonist cycles (Kolibianakis et al., 2002; Van Vaerenbergh et al., 2009).

Furthermore, immunohistochemistry assays have shown that estrogen receptor and progesterone receptor expression in the endometrium on the day of human chorionic gonadotrophin (hCG) administration is similar to that described on the first days of the luteal phase in natural cycles. This indicates an accentuated maturation of the endometrium exposed to supraphysiological concentrations of progesterone in the late follicular phase of IVF cycles (Papanikolaou et al., 2005). This endometrial advancement anticipates the window of implantation, which is a self-limited period, in which the endometrial epithelium acquires a functional ability to support blastocyst adhesion (Horcajadas et al., 2006).

There are several studies that have focused on the gene profile of the endometrium during the window of implantation. By comparing gene expression in three different situations: (i) the natural cycle (Riesewijk et al., 2003), (ii) the IVF-stimulated cycle (Horcajadas et al., 2005) and (iii) non-receptive conditions, such as insertion of an intrauterine device (Horcajadas et al., 2006), we were able to find 25 genes that seem relevant during the window of implantation (Horcajadas et al., 2007). In this work, we investigate endometrial gene expression during the peri-implantation period in young women under COS in accordance with the presence or absence of high circulating progesterone levels at the end of the follicular phase.

Materials and Methods

Study design

This was a single-centre, prospective cohort study carried out in a university-affiliated private infertility clinic between April 2007 and July 2009. Twelve women were included in the study, which was designed to compare the gene expression profile in the endometrium between two groups: (i) study group (six women) with a progesterone serum level of >1.5 ng/ml and (ii) control group (six women) with a progesterone serum level of <1.5 ng/ml on the day of recombinant chorionic gonadotrophin (rCG) administration. Each group included three cases under a GnRH agonist long protocol and three others under a GnRH antagonist multiple-dose protocol for pituitary down-regulation. The cut-off level of 1.5 ng/ml was chosen in accordance with our most recent published results which confirm that a progesterone serum level surpassing this threshold is detrimental for cycle outcome (Bosch et al., 2010).

The primary end-point was to analyse the impact of high progesterone levels on the last day of COS on the gene expression profile of the endometrium during the window of implantation.

The Institutional Review Board's and the Institution's Ethics Committee approvals were obtained. The clinicaltrials.gov registration number is NCT00447850.

Study population and protocol

A total of 12 young and healthy women (oocyte donors) were enrolled in the study. Inclusion criteria were: (i) age between 18 and 35 years; (ii) regular menstrual cycle (25–35 days); (iii) normal cycle day 3 hormones (FSH <10 IU/L; LH <10 IU/L and E_2 <60 pg/ml; (iv) body mass index (BMI) between 18 and 25 kg/m^2 and (v) normal karyotype. Exclusion criteria were: (i) endometriosis and (ii) polycystic ovary syndrome.

Donors underwent COS using either a GnRH agonist long protocol (Procrin®, Abbot, Madrid, Spain) or a GnRH antagonist multiple-dose protocol (Cetrotide®, Merck-Serono, Geneva, Switzerland) for pituitary suppression. Ovarian stimulation was carried out with a fixed starting dose of 225 IU/day s.c. of recombinant follicle-stimulating hormone (rFSH) (Gonal-F®, Merck-Serono, Geneva, Switzerland) for the first 3 days of stimulation, when doses were adjusted according to the ovarian response.

Ovulation induction was performed with rCG, 250 μg, s.c. (Ovitrelle®, Merck-Serono, Geneva, Switzerland) when at least three follicles reached 18 mm in diameter, and oocyte retrieval was carried out 36 h later by a transvaginal ultrasound-guided puncture of follicles. Serum oestradiol (E_2) and progesterone levels were determined on the day of rCG administration. The luteal phase of the oocyte donor was supplemented with a vaginal administration of 400 mg/day of natural micronized progesterone (Progeffik®, Effik, Madrid, Spain), starting 1 day after oocyte retrieval in order to simulate a patient's cycle in which an embryo transfer is performed after COS. All the donors gave their written informed consent.

Progesterone measurement

Serum progesterone levels were measured on the day of rCG administration. Samples were tested with a microparticle enzyme immunoassay Axsym System (Abbott Cientifica S.A., Madrid, Spain), which had a sensitivity of 0.2 ng/ml. Intraobserver and interobserver variation coefficients were 9.6 and 3.9%, respectively. As previously described, besides the internal quality control checks performed daily in the institution's laboratory, assays were calibrated whenever a new reactive batch was used or whenever an outcome outside the normal range was observed. Furthermore, external quality control assessment of every hormone assay was performed monthly at the Spanish Society of Clinical Biochemistry and Molecular Pathology, as previously reported (Bosch et al., 2010).

Tissue collection

A total of 12 endometrial samples were collected using a Pipelle catheter (Pipelle de Cornier®, Prodimed, Neuilly-en-Thelle, France) under sterile conditions from the uterine fundus. The biopsy was performed 7 days after the rCG injection (rCG + 7) as it is considered the window of implantation. Endometrial dating was performed using Noyes' criteria (Noyes et al., 1950) by a pathologist who was blind to the day on which the specimen was obtained.

RNA isolation

A portion (one half) of each sample was snap frozen in liquid nitrogen and stored at −70°C until further processing. Total RNA was extracted using the 'Trizol method' according to the protocol recommended by the manufacturer (Life Technologies, Inc., Gaithersburg, MD, USA). In short,

homogenized biopsies (1 ml TRIzol reagent/75 mg tissue) were incubated at room temperature for 5 min, chloroform (0.2 volumes of TRIzol) was then added and samples were incubated for 2.5 min at room temperature. Thereafter, the aforementioned samples were centrifuged for 15 min at 12 000 g (4°C). The aqueous phase was precipitated with an equal volume of 2-propanol, stored in ice for 5 min and centrifuged for 30 min at 12 000 g (4°C). The pellet was washed with 75% ethanol and dissolved in DEPC-treated water. The integrity of the RNA samples (RNA quality control procedure) was assessed with the 2100 Bioanalyzer (Agilent Technologies, Madrid, Spain) by running an aliquot of the RNA samples in the RNA 6000 Nano LabChip (Agilent Technologies, Madrid, Spain).

Microarray hybridization

All samples were hybridized in the Whole Human Genome Oligo Microarray (Agilent Technologies, Madrid, Spain) that encompasses more than 44 000 human DNA probes. The sample preparation and hybridization protocols to be followed with the endometrial samples were adapted from the Agilent Technical Manual. In short, first-strand cDNA was transcribed from 1 μg of total RNA using the T7-Oligo(dT) Promotor Primer. Samples were *in vitro* transcribed and Cy-3 labelled [all with the QuickAMP labelling kit (Agilent Technologies, Madrid, Spain)]. The cRNA synthesis typically yielded between 10 and 15 μg. Following a further clean-up round (QIAGEN, Barcelona, Spain), cRNA was fragmented into pieces ranging from 35 to 200 bases that were confirmed using the Agilent 2100 Bioanalyzer technology. Fragmented cRNA samples (1.65 μg) were hybridized onto chips by means of a 17-h of incubation at 65°C with constant rotation, then microarrays were washed in 2 × 1-min steps in two washing buffers (Agilent Technologies, Madrid, Spain). Hybridized microarrays were scanned in an Axon 4100A scanner (Molecular Devices, Sunnyvale, CA, USA), and data were extracted with the GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA).

Data processing and data analysis

The GenePix Pro 6.0 software was used for array image analysis and the calculation of spot intensity measurements, which are considered raw data.

Spot intensities (medians) without background subtraction were transformed to the log 2 scale. Before quantile normalization, the data were represented on a box plot to know data distribution and to subtract any abnormal microarray data. The replicates by gene symbol were merged and the data were filtered in order to delete the unknown sequences or probes without a gene description.

The R-statistical software system was used as a tool for these purposes and for the downstream analysis (R Development Core Team, 2004).

The gene expression profile was determined by comparing the experimental groups with the control group (2-by-2 comparisons) with parametric tests (Significance Analysis of Microarrays (SAM)) and non-parametric tests (Rank Product). Two criteria were used to define the genes that had altered mRNA abundance among the different sample sets: an absolute fold change of 2.0 or more and a corresponding corrected *P*-value <0.05.

Functional analysis of the results

To detect activations or inactivations in biological functions or pathways, we used the database for annotation, visualization and integrated discovery (DAVID) (Dennis *et al.*, 2003), a gene-set based algorithm that detects the significant representation of functionally related genes in lists of genes ordered by differential expression. DAVID can search blocks of functionally related genes by different criteria such as Gene Ontology (GO) terms, KEGG pathways and others.

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (von Mering *et al.*, 2007) then represents the connections among the differentially expressed genes.

Clustering and principal component analysis

The expression data were normalized by Z-score. Hierarchical clustering was performed with the MeV 4.2.02 software (http://www.tm4.org) (Saeed *et al.*, 2003) with a complete-linkage hierarchical clustering algorithm to be then visualized by the same software. Euclidean distance was chosen as the similarity measure.

A principal component analysis (PCA) was performed using the MeV 4.2.02 software. The data table of rows (genes) and columns (endometrial samples) was transposed and a PCA was run to reduce the number of variables to two or three principal components, which represent the majority of data set variability. A two- or three-dimensional scatterplot was produced to visualize the differences in sample sets based on each sample's gene expression profile.

Results

Table I summarizes the baseline characteristics and ovarian stimulation parameters of all 12 women. The mean age of patients who participated in the study was 24.7 ± 4.3 years. There was a significant difference in the mean age of women from the study group when compared with the control group (27.5 ± 4.2 versus 22 ± 2.2 years, respectively, $P = 0.017$). The progesterone serum levels on the last day of COS were significantly higher in the study group (2.39 ± 0.8 versus 0.54 ± 0.2 ng/ml, respectively).

Regarding embryo quality, the mean number of blastomeres of the whole embryo cohort on cleavage Day 3 was 7.43 ± 0.73 in the control group and 7.38 ± 0.65 for the study group ($P = 0.87$), while fragmentation rates were 5.89 ± 3.32 and 11.22 ± 5.56, respectively ($P = 0.007$). The clinical pregnancy rate was 57.1% in patients who received oocytes from donors with P <1.5 ng/ml on the day

Table I Oocyte donor parameters according to P levels on the day of rCG administration.

	P level <1.5 ng/ml (*n* = 6)	P level > 1.5 ng/ml (*n* = 6)	*P*-value
Age (years)	27.5 ± 4.2	22 ± 2.2	0.017
Previous pregnancies	3/6 (50%)	1/6 (16.7%)	0.545
BMI (kg/m^2)	22.8 ± 2.7	22.4 ± 2.3	0.807
Total dose of rFSH (IU)	1550 ± 512.8	1970.8 ± 152.0	0.083
Number of oocytes retrieved	20.5 ± 6.1	23.5 ± 1.5	0.287
Days of stimulation	9.5 ± 1.9	9.6 ± 0.8	0.845
E2 (pg/ml) on day of rCG administration	2236.8 ± 808.7	2649.3 ± 801.9	0.396
P (ng/ml) on day of rCG administration	0.54 ± 0.2	2.39 ± 0.8	<0.001

Data are expressed as mean (±SD) or percentage; P, progesterone; E2, estradiol; rCG, recombinant chorionic gonadotrophin.
P-value represents the significance value of the studied comparison; $P < 0.05$ = significant.

of rCG administration, and 46.7% in those whose donors showing P > 1.5 ng/ml ($P = 0.58$; OR = 0.66 (CI: 0.15–2.84).

Histological endometrial dating

According to Noyes' criteria, the histological dating of the endometrium on day rCG + 7 in the study group showed no significant ($P = 0.628$) advancement in dating (10.0 ± 1.7, mean of days after ovulation ± SD) compared with the control group (9.3 ± 2.8).

Differential gene expression

After RNA isolation, all the samples were of good enough quality to be included in the microarray experiments. In all, 12 endometrial samples were analysed, as was the normalized data used for further bioinformatics analyses. To find differentially expressed genes, two different methods were used: parametric (SAM) and non-parametric (Rank product). Using SAM, 140 genes were found to be differentially expressed between the two groups (64 up- and 76 down-regulated genes) using a fold change >2 and a corrected *P*-value <0.05. Using Rank product, the number of dysregulated genes were 161 up- and 209 down-regulated in the higher progesterone level group. The differentially expressed genes found with SAM are listed in Table II. Gene symbol, gene description, fold change and percentage of false discovered rate (FDR) are indicated for the parametric dysregulated genes.

Finally, we compared the list of dysregulated genes in women with high circulating progesterone levels with the 25 window of implantation genes strongly related to receptiveness and the implantation process (Horcajadas *et al.*, 2007). These genes are regulated in the natural cycle in one sense, showing a dysregulation under subfertile conditions, and stimulated cycles (Horcajadas *et al.*, 2005; Simón *et al.*, 2005; Horcajadas *et al.*, 2008a) or refractory situations in the presence of an intrauterine device (Horcajadas *et al.*, 2006). Of these 25 genes, 13 showed a dysregulation in women with high progesterone levels: 7 were over up-regulated and 6 were over-down-regulated. All these genes showed higher changes than those observed in the normal natural cycle. Those genes, which appeared up-regulated in the natural cycle, were more markedly up-regulated in women who had high progesterone levels. On the other hand, those genes which were down-regulated in the normal natural cycles were observed to be more down-regulated in women with high progesterone levels. Furthermore, 8 of these 13 genes were seen to have putative progesterone response elements (PRE) (DNA-binding sites, AACAGT) in their regulatory sequences. This search was performed on the PROMO Home Page, a virtual laboratory for the identification of putative transcription factor-binding sites in DNA sequences (http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The list of these 25 genes analysed in this part is shown in Table III. Their regulation in natural cycle and their dysregulation under suboptimal receptive conditions, their over-expression with high level of progesterone and the number of PREs are also indicated. Over-expressed genes are highlighted in bold.

Sample clustering

Using the expression of all the genes and PCA, those samples belonging to the same group tended to be in the same cluster (data not shown). Using the 140 differentially expressed genes and PCA, we distinguished two clear groups (data not shown). We also performed hierarchical clustering by using Pearson' correlation, and samples were classified into two main groups: one containing all the control samples and the other containing the high progesterone level samples (Figure 1).

GO of the differentially expressed genes

Using the GO implemented in DAVID, we investigated the biological sense of the 140 dysregulated genes. We analysed the biological processes, cellular component, molecular functions and KEGG pathways. We discovered several biological over-represented processes with statistical significance (FDR <0.05). These over-represented biological processes were mainly related to cell adhesion, developmental process, immune system process and others (see Table IV). The cellular components and molecular functions pathways were statistically and significantly over-represented, and they are also summarized in Table IV. Finally, only one KEGG pathway was over-represented with statistical significance for antigen processing and presentation.

Discussion

Despite the wide use of GnRH analogues for pituitary down-regulation in COS cycles for IVF, subtle increases in serum progesterone levels are still observed at the end of the follicular phase in many cases (Edelstein *et al.*, 1990; Silverberg *et al.*, 1991; Ubaldi *et al.*, 1996; Bosch *et al.*, 2003; Papanikolaou *et al.*, 2009; Bosch *et al.*, 2010).

The clinical impact of this has been highly controversial for a good many years, with some studies that could not find any association between progesterone levels and pregnancy rates (Givens *et al.*, 1994; Bustillo *et al.*, 1995; Levy *et al.*, 1995; Ubaldi *et al.*, 1995; Abuzeid and Sasy, 1996; Doldi *et al.*, 1999; Venetis *et al.*, 2007), whereas others have reported a negative impact on cycle outcome when serum progesterone levels are increased on the day of hCG administration (Check *et al.*, 1993, Fanchin *et al.*, 1993, 1997; Harada *et al.*, 1995; Shulman *et al.*, 1996; Bosch *et al.*, 2003). Nevertheless, we recently showed with a sample of more than 4000 IVF cycles that a serum progesterone level above the threshold of 1.5 ng/ml on the last day of COS is related to a significant decrease in the ongoing pregnancy rate in all types of patients and in any circumstances related to ovarian response (Bosch *et al.*, 2010). It must be taken into consideration that the majority of studies that failed to demonstrate an association between serum progesterone levels and pregnancy rate used a threshold value of 0.9 ng/ml, which was mostly chosen arbitrarily without performing a trend analysis to identify an association between progesterone levels and pregnancy (Bosch, 2008).

To ascertain whether these negative results are a consequence of impairment in endometrial receptiveness, we conducted the present study in which the gene expression of endometria of young healthy women (oocyte donors), under COS with rFSH and pituitary suppression, has been analysed according to the serum progesterone levels (< or >1.5 ng/ml) on the day of rCG administration. To the best of our knowledge, this is the first study to analyse the impact of progesterone levels on the gene expression profile of the endometrium in the window of implantation period because to date, the impact of supraphysiological levels of progesterone on the endometrium has

Table II List of the differentially expressed genes with a fold change (FC) higher than 2 or lower than −2, calculated by a parametric test (SAM).

Gene name	FC	*P*-value	FDR (%)	ACCN	Description
AOX1	6.94	0.00	0.01	NM_001159	Aldehyde oxidase 1
FGB	6.22	0.52	3.66	NM_005141	Fibrinogen beta chain
DPP4	3.92	0.52	1.56	NM_001935	Dipeptidyl-peptidase 4
SLC1A1	3.87	0.52	2.23	NM_004170	Solute carrier family 1 member 1
ENST00000381298	3.77	0.00	0.00	ENST00000381298	Interleukin-6 receptor subunit beta precursor (IL-6R-beta) (interleukin-6 signal transducer) (membrane glycoprotein 130) (gp130) (oncostatin-M receptor alpha subunit) (CD130 antigen) (CDw130).
HAPLN1	3.71	0.00	0.00	NM_001884	Hyaluronan and proteoglycan link protein 1
PTPRR	3.61	0.00	0.00	NM_002849	Protein tyrosine phosphatase, receptor type, R transcript variant 1
GLT1D1	3.42	0.52	1.21	NM_144669	Glycosyltransferase 1 domain containing 1
RARRES1	3.20	0.00	0.15	NM_002888	Retinoic acid receptor responder transcript variant 2
HABP2	3.02	0.52	2.58	NM_004132	Hyaluronan-binding protein 2
IMPA2	2.86	0.00	0.00	NM_014214	Inositol(myo)-1(or 4)-monophosphatase 2
MFAP4	2.86	0.52	3.09	NM_002404	Microfibrillar-associated protein 4
SNX10	2.83	0.52	0.87	NM_013322	Sorting nexin 10
FCGR3A	2.83	0.00	0.06	NM_000569	Fc fragment of IgG, low affinity IIIa, receptor
TLE2	2.73	0.00	0.00	NM_003260	Transducin-like enhancer of split 2
PRL	2.68	0.00	0.26	NM_000948	Prolactin
LCP1	2.64	0.00	0.90	NM_002298	Lymphocyte cytosolic protein 1
TGM2	2.53	0.00	0.27	NM_198951	Transglutaminase 2 transcript variant 2
RND3	2.53	0.00	0.00	NM_005168	Rho family GTPase 3
LOC387763	2.53	0.52	3.55	ENST00000339446	Hypothetical LOC387763, partial cds.
LAMA4	2.52	0.00	0.00	NM_002290	Laminin, alpha 4
ENST00000383706	2.51	0.52	1.31	ENST00000383706	ADAMTS-9 precursor (EC 3.4.24.-) (A disintegrin and metalloproteinase with thrombospondin motifs 9) (ADAM-TS 9) (ADAM-TS9).
MLPH	2.51	0.00	0.00	NM_024101	Melanophilin transcript variant 1
ALOX15B	2.48	0.00	0.00	NM_001141	Arachidonate 15-lipoxygenase, type B transcript variant d
LBP	2.47	0.52	3.13	NM_004139	Lipopolysaccharide binding protein
LUM	2.46	0.52	2.28	NM_002345	Lumican
DHRS3	2.44	0.52	4.57	NM_004753	Dehydrogenase/reductase
GATA6	2.44	0.52	1.93	NM_005257	GATA-binding protein 6
CHST11	2.44	0.00	0.86	NM_018413	Carbohydrate
HTR2A	2.43	0.52	3.13	NM_000621	5-hydroxytryptamine
MLLT11	2.40	0.52	4.01	NM_006818	Myeloid/lymphoid or mixed-lineage leukemia
RP1-93H18.5	2.36	0.00	0.00	NM_001010919	Hypothetical protein LOC441168
XCL1	2.35	0.00	0.00	NM_002995	Chemokine
GLIPR1	2.33	0.52	0.52	NM_006851	GLI pathogenesis-related 1
ENPEP	2.31	0.52	4.17	NM_001977	Glutamyl aminopeptidase
FILIP1L	2.30	0.00	0.00	NM_182909	Filamin A interacting protein 1-like transcript variant 1
SERPING1	2.30	0.00	0.37	NM_000062	Serpin peptidase inhibitor, clade G member 1, transcript variant 1
DEPDC1B	2.26	0.52	1.68	NM_018369	DEP-domain containing 1B
MMRN1	2.26	0.81	4.95	NM_007351	Multimerin 1
KLRC3	2.24	0.52	3.36	NM_007333	Killer cell lectin-like receptor subfamily C, member 3 transcript variant 2
ENST00000370395	2.24	0.52	1.87	ENST00000370395	CSL-type zinc finger-containing protein 1
HCLS1	2.23	0.52	0.97	NM_005335	Hematopoietic cell-specific Lyn substrate 1
ITGB2	2.22	0.00	0.40	NM_000211	Integrin, beta 2

Continued

Table II *Continued*

Gene name	FC	*P*-value	FDR (%)	ACCN	Description
IL1B	2.16	0.00	0.00	NM_000576	Interleukin 1, beta
LEPREL1	2.15	0.00	0.89	NM_018192	Leprecan-like 1
PROK1	2.12	0.52	2.05	NM_032414	Prokineticin 1
LOC388610	2.12	0.00	0.00	NM_001013642	Hypothetical LOC388610
RASGRP2	2.12	0.52	1.08	NM_005825	RAS guanyl-releasing protein 2 transcript variant 1
ADAMTS9	2.12	0.52	1.32	NM_182920	ADAM metallopeptidase with thrombospondin type 1 motif, 9
CADPS2	2.12	0.81	4.89	NM_017954	Ca^{2+}-dependent activator protein for secretion 2 transcript variant 1
TOP2A	2.12	0.52	0.61	NM_001067	Topoisomerase
EDNRB	2.11	0.81	4.86	NM_003991	Endothelin receptor type B transcript variant 2
MGC33846	2.10	0.81	4.90	NM_175885	Hypothetical protein MGC33846
SLC22A5	2.08	0.52	2.61	NM_003060	Solute carrier family 22 member 5
GGTLA1	2.07	0.52	2.06	NM_004121	Gamma-glutamyltransferase-like activity 1
SPBC25	2.06	0.00	0.16	NM_020675	Spindle pole body component 25 homolog
TMEM45A	2.06	0.00	0.00	NM_018004	Transmembrane protein 45A
KRT86	2.05	0.52	3.65	NM_002284	Keratin 86
LHFP	2.04	0.52	0.93	NM_005780	Lipoma HMGIC fusion partner
MFNG	2.03	0.00	0.00	NM_002405	MFNG *O*-fucosylpeptide 3-beta-*N*-acetylglucosaminyltransferase
RGS1	2.02	0.52	3.78	NM_002922	Regulator of G-protein signalling 1
RPL22L1	2.02	0.00	0.00	BC049823	Ribosomal protein L22-like 1, (cDNA clone IMAGE:4865966)
ACTA2	2.02	0.52	1.13	NM_001613	Actin, alpha 2, smooth muscle, aorta
GAS1	2.01	0.00	0.00	NM_002048	Growth arrest-specific 1
SLC25A29	−2.04	0.00	0.46	NM_001039355	Solute carrier family 25, member 29 transcript variant 1
CNDP2	−2.04	0.76	2.88	NM_018235	CNDP dipeptidase 2
TLR5	−2.04	0.00	0.71	NM_003268	Toll-like receptor 5
CBLC	−2.05	0.76	3.89	NM_012116	Cas-Br-M
COL1A2	−2.06	0.00	0.00	NM_000089	Collagen, type I, alpha 2
SH3RF2	−2.08	0.76	2.90	NM_152550	SH3-domain containing ring finger 2
IFNGR1	−2.13	0.00	0.00	NM_000416	Interferon gamma receptor 1
MAN2B2	−2.13	0.00	0.00	NM_015274	Mannosidase, alpha, class 2B, member 2
TPD52L1	−2.14	0.00	0.00	NM_001003395	Tumor protein D52-like 1 transcript variant 2
ANXA3	−2.14	0.00	1.23	NM_005139	Annexin A3
TMEM139	−2.15	0.76	2.89	NM_153345	Transmembrane protein 139
ABCG1	−2.16	0.00	0.00	NM_207630	ATP-binding cassette, sub-family G member 1 transcript variant 1
RAB15	−2.16	0.00	0.82	NM_198686	RAB15, member RAS onocogene family
SMAD9	−2.17	0.00	0.00	NM_005905	SMAD family member 9
SORBS2	−2.17	0.00	2.45	AF090937	Clone HQ0618 PRO0618, complete cds
KRTCAP3	−2.18	0.00	0.01	AY358993	Clone DNA129535 MRV222 complete cds
ENST00000368025	−2.22	0.00	0.00	ENST00000368025	Junctional adhesion molecule A precursor (JAM-A) (junctional adhesion molecule 1) (JAM-1) (Platelet adhesion molecule 1) (PAM-1) (platelet F11 receptor) (CD321 antigen)
TMEM142B	−2.22	0.00	0.00	NM_032831	Transmembrane protein 142B
UCA1	−2.23	0.00	1.93	DQ249310	Clone DU2 UCA1 protein complete cds
PPP1R1A	−2.23	0.00	2.41	NM_006741	Protein phosphatase 1, regulatory
ATP1B1	−2.23	0.00	0.00	NM_001677	ATPase, Na+/K+ transporting, beta 1 polypeptide transcript variant 1
EVA1	−2.25	0.00	0.46	NM_144765	Epithelial V-like antigen 1 transcript variant 2
ABCB9	−2.26	0.00	0.00	NM_019625	ATP-binding cassette, sub-family B member 9 transcript variant 1
SCNN1A	−2.27	0.00	0.00	NM_001038	Sodium channel, non-voltage-gated 1 alpha

Continued

Table II *Continued*

Gene name	FC	*P*-value	FDR (%)	ACCN	Description
CRIP1	−2.27	0.00	1.17	NM_001311	Cysteine-rich protein 1
ASRGL1	−2.28	0.00	0.00	NM_025080	Asparaginase like 1
ANK3	−2.28	0.00	0.33	NM_020987	Ankyrin 3, node of Ranvier transcript variant 1
KAZALD1	−2.28	0.76	4.51	NM_030929	Kazal-type serine peptidase inhibitor domain 1
ECHDC2	−2.29	0.00	0.30	NM_018281	Enoyl Co-enzyme A hydratase domain containing 2
AIM1L	−2.29	0.00	1.50	NM_017977	Absent in melanoma 1-like
USP53	−2.29	0.76	3.51	BC017382	Ubiquitin-specific peptidase 53, complete cds
PCCA	−2.29	0.00	0.17	NM_000282	Propionyl Co-enzyme A carboxylase, alpha polypeptide
FAM110C	−2.30	0.00	0.41	NM_001077710	Family with sequence similarity 110 member C
ENST00000370892	−2.30	0.00	0.10	ENST00000370892	Leucine-rich repeat-containing protein 1 (LAP and no PDZ protein) (LANO adapter protein)
CLGN	−2.32	0.76	4.58	NM_004362	Calmegin
TRPM4	−2.33	0.00	0.00	NM_017636	Transient receptor potential cation channel, subfamily M, member 4
CHST4	−2.37	0.76	3.90	NM_005769	Carbohydrate
PDE9A	−2.39	0.00	0.00	NM_002606	Phosphodiesterase 9A transcript variant 1
FLJ37464	−2.39	0.00	0.00	NM_173815	Hypothetical protein FLJ37464
RNASET2	−2.40	0.00	1.45	NM_003730	Ribonuclease T2
NRXN3	−2.40	0.00	0.27	NM_004796	Neurexin 3 transcript variant alpha
ZNF589	−2.51	0.00	2.05	NM_016089	Zinc finger protein 589
SLAIN1	−2.52	0.00	1.09	NM_001040153	SLAIN motif family, member 1 transcript variant 1
ALPL	−2.56	0.00	0.00	NM_000478	Alkaline phosphatase, liver/bone/kidney
ANKRD35	−2.58	0.00	1.23	NM_144698	Ankyrin repeat domain 35
SLC15A2	−2.58	0.00	0.00	NM_021082	Solute carrier family 15 member 2
LOC388135	−2.61	0.76	3.07	NM_001039614	Similar to RIKEN cDNA 6030419C18 gene
OCIAD2	−2.64	0.00	0.46	NM_001014446	OCIA-domain containing 2 transcript variant 1
DGKD	−2.64	0.00	0.88	NM_152879	Diacylglycerol kinase, delta 130kDa transcript variant 2
FLJ21511	−2.64	0.00	0.00	NM_025087	Hypothetical protein FLJ21511
FLJ20366	−2.66	0.00	0.00	NM_017786	Hypothetical protein FLJ20366
LRRC1	−2.66	0.00	1.22	NM_018214	Leucine-rich repeat containing 1
GYLTL1B	−2.66	0.00	3.08	NM_152312	Glycosyltransferase-like 1B
DDX43	−2.67	0.00	1.27	NM_018665	DEAD
NDRG2	−2.67	0.76	2.96	NM_201535	NDRG family member 2 transcript variant 1
KRT23	−2.80	0.00	0.00	NM_015515	Keratin 23
LOC284422	−2.85	0.76	3.83	ENST00000211092	HSPC323, partial cds
C10orf116	−2.87	0.00	0.31	NM_006829	Chromosome 10 open reading frame 116
GMPR	−2.95	0.00	1.06	NM_006877	Guanosine monophosphate reductase
HES5	−3.04	0.00	1.09	NM_001010926	Hairy and enhancer of split 5
KRT8	−3.07	0.00	0.14	NM_002273	Keratin 8
CAMK2B	−3.12	0.76	4.35	NM_172082	Calcium/calmodulin-dependent protein kinase transcript variant 6
HGD	−3.17	0.00	0.92	NM_000187	Homogentisate 1,2-dioxygenase
ZBED2	−3.21	0.00	0.50	NM_024508	Zinc finger, BED-type containing 2
HSD11B2	−3.35	0.76	2.91	NM_000196	Hydroxysteroid
ZDHHC11	−3.46	0.00	2.26	NM_024786	zinc finger, DHHC-type containing 11
SFTPG	−3.66	0.00	0.00	NM_205854	Surfactant-associated protein G
DUOX1	−3.69	0.00	0.67	AL137592	mRNA; cDNA DKFZp434L0610 (from clone DKFZp434L0610); partial cds
FXYD4	−3.72	0.00	1.56	NM_173160	FXYD-domain containing ion transport regulator 4

Continued

Table II *Continued*

Gene name	FC	*P*-value	FDR (%)	ACCN	Description
WFDC2	−3.76	0.76	2.67	NM_080734	WAP four-disulfide core domain 2 transcript variant 4
SLC37A2	−3.83	0.00	0.00	NM_198277	Solute carrier family 37 member 2
TFCP2L1	−4.05	0.00	0.22	NM_014553	Transcription factor CP2-like 1
VTCN1	−4.24	0.76	3.64	NM_024626	V-set domain containing T cell activation inhibitor 1
MOSC1	−4.79	0.00	0.00	NM_022746	MOCO sulphurase C-terminal domain containing 1
SLC7A4	−4.95	0.00	0.00	NM_004173	Solute carrier family 7 member 4
TNNC1	−7.57	0.00	0.21	NM_003280	Troponin C type 1

FDR, false discovery rate; ACCN, accession number.

been studied by means of histological and immunohistochemical changes, but not by gene expression profiling.

The present study shows, by using parametric and non-parametric methods, that a total of 140 and 370 genes, respectively, are dysregulated by more than 2-fold in women with high serum progesterone levels. The biological processes over-represented in a large number of these genes (cell adhesion, immune system, organ development) could affect the endometrium and the implantation process.

When compared with the list of key genes that could be relevant for the previously described endometrial receptivity (Horcajadas *et al.*, 2007), we found that the RNA levels of 13 of these 25 genes presented an over-expression in women with high progesterone levels (Table III). Excepting the FOLR1 gene, the other 12 genes are the gene targets included in the endometrial receptivity array, a genomic tool recently published and used for endometrial receptivity evaluation (Díaz-Gimeno *et al.*, 2011). The over-regulation of eight of these genes can be explained directly by high progesterone levels because they contain putative PRE in their regulatory sequences (Table III). However, there are three genes (FXYD2, LIF and CNN1) that were not over-regulated in the high progesterone group, although putative PRE were detected (Table III).

The impact of high progesterone levels on the endometrium can have a double effect: on the one hand, directly induced by the action of progesterone on the gene expression via its own receptor and, on the other hand, the secondary effects that are downstream of the progesterone receptor cascade, such as the activation/repression of other regulatory proteins like the E_2 receptor, which modulates its action in uterine target cells, leading to an heterologous desensitization to E_2 (Bayard *et al.*, 1978).

A recent study showed that in IVF-stimulated cycles, endometrial gene expression on the day of oocyte retrieval discriminates between women with and without histologically advanced endometrial maturation exceeding 3 days (Van Vaerenbergh *et al.*, 2009). Thus, both mechanisms seem to be closely related at that period. Nevertheless, our data reflect that histological endometrial dating is not related to the gene expression profile of the endometrium during the window of implantation as no significant differences in endometrial advancement were observed between the two study groups. One possible explanation for this finding is that Noyes' criteria are less accurate than originally described due to high intersubject, intrasubject and interobserver variability (Murray *et al.*, 2004). This indicates a questionable relationship to endometrial receptivity (Murray *et al.*, 2002) when compared with the gene expression assessment which, according to our present data, seems to be further related to the impact of progesterone in the clinical outcome.

It is of interest to note that when endometrial advancement becomes extreme (>3 days as compared with the expected chronological date) on the day of hCG administration, no clinical pregnancies are obtained when the embryo transfer is performed on Day 3 (Ubaldi *et al.*, 1997; Kolibianakis *et al.*, 2002; Van Vaerenbergh *et al.*, 2009). This is due to an early closure of the window of implantation (Papanikolaou *et al.*, 2005) that disturbs the cross-dialogue between embryo and endometrium. Conversely, these endometrial changes on the hCG day do not exert a detrimental effect when the transfer is delayed until the blastocyst stage. What this implies is that, from the histological viewpoint, the endometrium could recover during the window of implantation period from the violation induced by supraphysiologic steroid concentrations (Papanikolaou *et al.*, 2009). In natural cycles, such an advancement in endometrial maturation is not present (Bourgain *et al.*, 2002), and suggests that high levels of steroid hormones during COS induce secretory changes in the endometrium. It has been postulated that high E_2 levels mediate an earlier expression of the progesterone receptors in the early follicular phase, leading to an advancement of the endometrium in the late follicular phase even with normal progesterone levels at this time (Marchini *et al.*, 1991). Moreover, the higher the E_2 levels are, the higher the progesterone levels are; thus, they usually come together when the ovarian response is greater than expected (Bosch *et al.*, 2003; Venetis *et al.*, 2007; Bosch *et al.*, 2010).

It is difficult to depict between the negative effects of both ovarian steroid hormones when they are at supraphysiological levels during COS (Valbuena *et al.*, 1999; Martínez-Conejero *et al.*, 2007; Chen *et al.*, 2008; van der Gaast *et al.*, 2009). In the present study, E_2 levels on day of rCG administration were not a confounding factor as the levels in both groups did not differ significantly, although there was a huge difference between the groups in terms of progesterone levels. Moreover, we have previously shown that, regardless of the E_2 levels, progesterone exerts a deleterious effect on cycle outcome (Bosch *et al.*, 2010). On the other hand, E_2 levels seem to affect the embryo more negatively than the endometrium (Valbuena *et al.*, 2001), whereas progesterone seems to be more detrimental to the endometrium, according to the present data.

Table III List of altered genes compared with the list of the 25 dysregulated genes related with the window of implantation (Horcajadas *et al.*, 2007) among natural cycle (NC), COS and intrauterine device (IUD).

ACCN	Name	Description	NC	COS	IUD	P4	*P*-value (%)	FDR (%)	Putative PRE (AACAGT)
NM_002084	**GPX3**	**Glutathione peroxidase 3 (plasma)**	25.87	−11.81	−13.4	8.46	2.71	21.03	1
NM_002571	**PAEP**	**Placental protein 14**	81.61	−9.82	−10.18	4.80	3.24	26.29	2
NM_021603	FXYD2	FXYD domain containing ion transport regulator 2	4.07	−4.53	−9.43				5
NM_001935	**DPP4**	**Dipeptidylpeptidase 4**	31.37	−37.14	−8.47	3.92	0.52	1.56	–
NM_002309	LIF	Leukaemia inhibitory factor	36.62	−23.02	−4.57				2
NM_001013398	IGFBP3	Insulin-like growth factor-binding protein 3	4.00	−4.26	−3.76				–
NM_001924	**GADD45A**	**Growth arrest and DNA-damage-inducible, alpha**	8.03	−3.03	−3.52	3.07	0.81	5.92	1
NM_004132	**HABP2**	**Hyaluronan-binding protein 2**	5.90	−6.41	−3.42	3.02	0.52	2.58	1
NM_003991	**EDNRB**	**Endothelin receptor type B**	8.21	−3.20	−3.41	2.11	0.81	4.86	–
NM_012134	LMOD1	Leiomodin 1 (smooth muscle)	29.73	−4.08	−3.10				–
NM_001299	CNN1	Calponin 1, basic, smooth muscle	10.31	−9.26	−2.86				3
NM_203339	CLU	Clusterin	28.78	−7.13	−2.79				–
NM_001001522	TAGLN	Transgelin	8.38	−3.72	−2.73				–
NM_000771	CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	2.48	−4.66	−2.49				–
NM_014214	**IMPA2**	**Inositol(myo)-1(or 4)-monophosphatase 2**	5.23	−5.88	−2.23	2.86	0.00	0.00	1
NM_014289	**CAPN6**	**Calpain 6**	−4.58	10.32	11.20	−3.69	1.60	16.43	1
NM_006528	**TFPI2**	**Tissue factor pathway inhibitor 2**	−3.52	5.42	7.32	−2.77	0.89	8.47	–
NM_002758	**MAP2K6**	**Mitogen-activated protein kinase kinase 6**	−4.87	8.65	6.57	−2.64	1.36	14.78	–
NM_004389	CTNNA2	Catenin (cadherin-associated protein), alpha 2	−8.44	7.32	6.40				–
NM_003104	SORD	Sorbitol dehydrogenase	−2.42	11.56	5.87				–
NM_002120	**HLA-DOB**	**Major histocompatibility complex, class II, DO beta**	−16.48	12.23	4.93	−2.62	2.07	20.17	2
NM_183050	BCKDHB	Branched chain keto acid dehydrogenase E1, beta polyp	−3.33	10.32	4.06				–
NM_000695	ALDH3B2	Aldehyde dehydrogenase 3 family, member B2	−3.12	4.28	3.52				–
NM_201535	**NDRG2**	**NDRG family member 2**	−5.98	5.90	3.35	−2.67	0.76	2.96	–
NM_016725	**FOLR1**	**Folate receptor 1**	−10.49	9.30	2.53	−5.12	0.76	6.95	1

The genes of the present study are in column P4 with their *P*-values and the corrected *P*-value (FDR). Over-expressed genes are highlighted in bold. The last column is the number of progesterone responsible elements (PRE) in the promoter region of the genes.

Women from the study group were significantly younger than the control group. This could explain the higher ovarian response observed in terms of number of oocytes and steroid hormone levels, although these differences were not statistically significant. Age should not be considered as a confounding factor, as endometrial receptivity might depend on the supraphysiological levels of ovarian steroid hormones (Chen *et al.*, 2007, 2008) but not on the age, except for cases of very advanced maternal age (Soares *et al.*, 2005). It is interesting to note that the study group received higher doses of recombinant FSH, although they had similar BMI. The association between doses of FSH and increase of progesterone levels on hCG day has been previously described (Filicori *et al.*, 2002; Bosch *et al.*, 2010).

The oocyte donation model has proved a most useful tool to distinguish between the effects of high progesterone levels on the oocyte from those on the endometrium. A previous clinical study by our group (Melo *et al.*, 2006) compared the outcome of two consecutive oocyte donation cycles in the same donor in accordance with the presence or absence of high serum progesterone levels on the day of hCG administration. No differences were found in terms of embryo

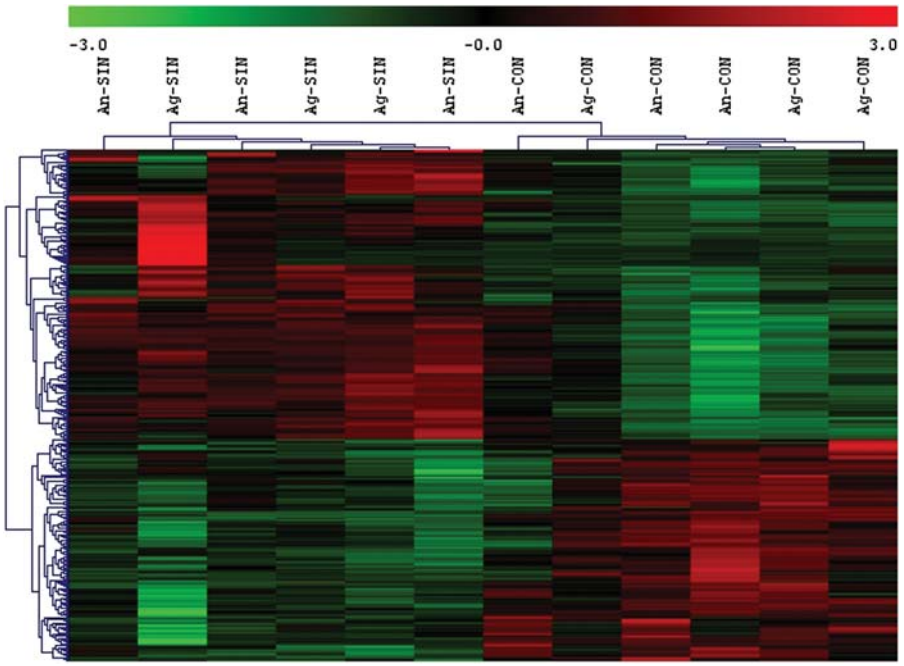

Figure 1 Gene clustering by Pearson's correlation. An: antagonist; ag: agonist, CON: P >1.5 ng/ml; SIN: P <1.5 ng/ml.

Table IV GO terms related to differentially expressed genes.

Category	Term	Genes	%	*P*-value	FDR
BP	Response to wounding	11	8.5	0.0004	0.8
BP	Inflammatory response	9	7.0	0.0007	1.3
BP	Organ development	19	14.7	0.0011	2.2
BP	Anatomical structure development	26	20.2	0.0018	3.3
BP	Response to external stimulus	12	9.3	0.0026	4.9
CC	Extracelullar region	19	14.7	0.0018	2.8
CC	Extracelullar region part	14	10.9	0.0019	2.9

Categories are BP, biological processes; CC, cellular components. The % represents the genes relating to the term by taking into account the total of dysregulated genes. FDR is false discovery rate.

quality between both cycles, which indicates that an increase in progesterone does not affect oocyte quality. Clinical pregnancy rates in oocyte recipients were not affected as the endometrium was not exposed to high progesterone levels.

In the present study, we also used the oocyte donation model as this offers the advantage of performing an endometrial biopsy during the window of implantation. Otherwise, if the study had been carried out in patients undergoing IVF-embryo transfer, a fresh embryo transfer could not have been performed as an endometrium biopsy at the time of the window of implantation could have proved hazardous for embryo implantation. To mimic the endometrial status of a patient undergoing IVF treatment, oocyte donors received the same doses of natural micronized progesterone as patients do in a conventional cycle with their own oocytes. With this approach, we were able to do an in-depth analysis of the events taking place in the receptive phase as they are determining factor for the IVF cycle to be a success. In the current study, no significant differences in terms of the clinical pregnancy rate and the mean number of blastomeres between both study groups were observed. Although the fragmentation rate was significantly higher in the group with $P > 1.5$ ng/ml, it remained below 20% in both groups. In any event, the study design does not allow a comparison to be made of the clinical outcome between both groups given the low number of patients included.

Previously, we focused on several works using microarray technology in terms of gene expression profiling of the human endometrium in the receptive phase in different situations (Riesewijk *et al.*, 2003; Horcajadas *et al.*, 2004a,b; Horcajadas *et al.*, 2005; Horcajadas *et al.*, 2006; Horcajadas *et al.*, 2008b) to show that COS affects the global gene expression profiling across the window of implantation

when compared with the natural cycle. This could explain how, despite advances in IVF procedures, implantation rates remain lower than we desire, suggesting that the endometrium in COS cycles does not reach the receptive status in the same manner as in natural cycles (Horcajadas *et al*., 2008a). In the present study, we demonstrate that the gene expression profiles of the endometrium samples obtained from women with elevated progesterone levels (>1.5 ng/ml) at the end of the follicular phase under a COS treatment differ substantially from those obtained from women with normal progesterone levels, even though both were collected at the window of implantation (rCG + 7).These findings prove that the concept of the poorer outcome of IVF cycles when progesterone is high on the day of rCG administration is due to impaired endometrial receptivity, leading to a lower implantation rate, as previously suggested by other authors (Hofmann *et al*., 1993; Check *et al*., 1994; Shulman *et al*., 1996; Chetkowski *et al*., 1997; Fanchin *et al*., 1997; Smitz *et al*., 2007; Kyrou *et al*., 2009). According to this concept, it is highly valuable to monitor serum progesterone levels in COS for IVF, especially at the end of the follicular phase. In the event of increased values, cryopreservation of all the obtained embryos and their subsequent transfer in a natural cycle is recommended as the clinical guideline.

Authors' roles

E.L designed the study, did the data analysis and interpretation, and wrote the manuscript. She is the corresponding author for the reviewing procedure. J.A.M and J.A.H processed the endometrial samples, and performed the microarray technology, data analysis and manuscript writing. P.A. participated in donors' recruitment and care during the medical treatment, and performed the endometrial biopsy for sample collection. A.P. and C.S. contributed to the review of the manuscript. E.B. participated in the study design and reviewed the intellectual content of the manuscript.

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