The impact of using the combined oral contraceptive pill for cycle scheduling on gene expression related to endometrial receptivity

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STUDY QUESTION: Does the combined oral contraceptive pill (COCP) change endometrial gene expression when used for cycle programming?

SUMMARY ANSWER: COCP used for scheduling purposes does not have a significant impact on endometrial gene expression related to endometrial receptivity.

WHAT IS KNOWN ALREADY: Controversy exists around COCP pretreatment for IVF cycle programming, as some authors claim that it might be detrimental to the live birth rate. Microarray technology applied to the study of tissue gene expression has previously revealed the behavior of genes related to endometrial receptivity under different conditions.


PARTICIPANTS/MATERIALS, SETTING, AND METHODS: Microarray data were obtained from endometrial biopsies from 10 young healthy oocyte donors undergoing COS with GnRH antagonists and recombinant FSH. In group A (n = 5), COCP pretreatment was used for 12–16 days, and stimulation began after a 5-day pill-free interval. Stimulation in group B (n = 5) was initiated on cycle day 3 after a spontaneous menses. Endometrial biopsies were collected 7 days after triggering with hCG.

MAIN RESULTS AND THE ROLE OF CHANCE: No individual genes exhibited increased or decreased expression (fold change (FC) > 2) in patients with prior COCP treatment (group A) compared with controls (group B). However, the results of the functional analysis showed a total of 11 biological processes that were significantly enriched in group A compared with group B (non-COCP).

LIMITATIONS, REASONS FOR CAUTION: The Endometrial Receptivity Array (ERA) has only been validated on endometrial samples obtained in natural cycles and after hormonal replacement treatment (HRT). Therefore, it was not possible in this study to classify the endometrial samples as receptive or non-receptive. We used the ERA to focus on 238 genes that are intimately related to endometrial receptivity, thus simplifying the analysis and understanding of the data.

WIDER IMPLICATIONS OF THE FINDINGS: Cycle scheduling is common in IVF units and is used to avoid weekend retrievals and/or to distribute evenly the workload for better efficiency. Our failure to detect any relevant changes in the genes related to the window of implantation when cycles were programmed with COCP pretreatment suggests that, despite controversial clinical results in previous studies, the use of COCPs in this way does not affect uterine receptivity adversely.

STUDY FUNDING/COMPETING INTEREST(S): Funding for this study was provided by an unrestricted grant from Merck Sharp & Dohme. C.S. and A.P. are co-inventors (with Patricia Diaz-Gimeno) of the Endometrial Receptivity Array and hold the patent. The other authors have no conflicts of interest to declare.
Introduction

For IVF applications, cycle planning provides improved organization and distribution of laboratory work at assisted reproduction centers. Cycle planning evenly distributes the number of oocyte retrievals throughout the week, which leads to cost savings by reducing the requirement for weekend personnel (Zorn et al., 1987).

Protocols that include GnRH antagonists (GnRHant) have been associated with a reduction in ovarian hyperstimulation syndrome, shorter stimulation times and lower gonadotrophin dosages. The GnRHant protocols have achieved pregnancy rates similar to those achieved with longer protocols (Devroye et al., 2009; Al-Imam et al., 2011). However, GnRHant protocols have the drawback that the stimulation cycle must begin with spontaneous menstruation. In contrast, the long protocol allows one to delay the onset of stimulation once pituitary desensitization has been achieved. To plan the start of a GnRHant protocol, different approaches have been described: a flexible start on Days 2 or 3, a delay or advance of the hCG trigger or steroid pretreatment (Barmat et al., 2005; Tremellen and Lane, 2010; Cédrid-Durnerin et al., 2012).

Over the last few years, controversy has arisen regarding whether pretreatment with a combined oral contraceptive pill (COCOP) may have an impact on the IVF cycle outcome (Kolibianakis et al., 2006; Rombaets et al., 2006; Bellver et al., 2007). A recent meta-analysis suggested that COCPs had a negative impact on IVF results (Griesinger et al., 2010); however, not all authors agree (García-Velasco et al., 2011). This discrepancy prompted us to further evaluate endometrial receptivity in IVF cycles where COCPs were administered as a pretreatment.

Over the last decade, with the arrival of microarray technology, investigations have focused on the genes related to endometrial receptivity during pretreatment applied prior to the start of the cycle might have a molecular expression standpoint. This information can be linked to whether steroid pretreatment applied prior to the start of the cycle might have a molecular effect on endometrial receptivity and whether steroid pretreatment might influence the clinical results of IVF. In the present study, we compared endometrial gene expression between women treated with COCP and those allowed to undergo spontaneous menstruation without COCP.

Materials and Methods

Study design

This was a single-center, proof-of-concept study conducted in a university-affiliated private infertility clinic between June 2012 and February 2013. The study was designed to compare the gene expression profile in the endometrium between two groups: A, the study group (five women) who received a COCP during the cycle prior to stimulation to schedule the cycle starting on Day 1 (D1) of menses, and B, the control group (five women) who began stimulation directly with a spontaneous menses without prior COCP priming.

Study population and randomization

A total of 10 volunteers from our egg donor program were included in the study. The inclusion criteria were as follows: Caucasian; age 18–35 years; a regular menstrual cycle (25–35 days); normal basal serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH; both < 10 IU/ml) and estradiol (E2; < 60 pg/ml); normal karyotype; body mass index 18–25 kg/m²; normal cervical smear from the past year; and a normal vaginal ultrasound. The exclusion criteria were the presence of the following: endometriosis (AFS classification stage > 2); polycystic ovary syndrome (according to the revised Rotterdam criteria); or an intrauterine device within the last 3 months.

All patients agreed to participate in the study and signed the informed consent form on the hCG triggering day. The Institutional Review Board and the Institutional Ethics Committee approved the study. The EudraCT registration number is 2011-003250-34.

Study groups and stimulation protocol

Patients from group A received a COCP (Microgynon30, Bayer, Berlin, Germany) during the cycle prior to stimulation. The dosing consisted of 1 tablet per day for 12–16 days to schedule the cycle starting on D1 of menses. In group A, stimulation began after a 5-day pill-free interval. Patients from group B began stimulation directly on D2/D3 of the spontaneous menstrual cycle without prior COCP priming.

The stimulation started with a fixed dose of 150 IU recombinant FSH administered subcutaneously (sc; Puregon, MSD, Madrid, Spain) for 4 days. After that, doses were adjusted according to the follicular response, as visualized on ultrasound. To avoid a premature increase in LH, a daily dose of 0.25 mg GnRHant (Orgalutran, MSD, Madrid, Spain) was administered sc on fixed DS until the day of triggering.

Final oocyte maturation was induced as soon as three follicles with a mean diameter > 17 mm were observed on ultrasound. This was designated day LH+0. For triggering, patients received 250 μg recombinant HCG sc (Ovitrelle, Merck-Serono, Geneva, Switzerland). Follicle aspiration was conducted 36 h after triggering.

For luteal phase support, patients in both groups applied 200 mg vaginal micronized progesterone (Progeffik, Effik, Madrid, Spain) every 12 h. This medication was continued until the day of the biopsies in all patients (LH+7).
Hormonal and statistical analysis

Donors were tested for progesterone (P4), LH and estradiol (E2) concentrations on the day of recombinant HCG administration (LH+0) and on days LH+1–2, LH+4–5 and LH+6. Serum E2, P4 and LH were evaluated using a commercially available, microparticle enzyme immunoassay kit (Abbott Laboratories, Abbott Park, IL, USA). Plasma samples from all donors were centrifuged at 200 g for 5 min, and the supernatant was stored at −80 °C until assayed.

The mean hormonal concentrations were compared with a one-way analysis of variance or Kruskal–Wallis test based on the normality of the distribution. Significance was set at $P < 0.05$. The Mann–Whitney U-test was used for pairwise comparisons of values that were not normally distributed, which yields results identical to the Kruskal–Wallis test for two independent samples. If multiple comparisons were performed, Bonferroni correction for multiple comparisons was used to analyze the differences between the protocols. All statistical analyses were performed with the SPSS (Statistical Package for the Social Sciences) 18.0 software package (SPSS, Inc.).

Sample attainment

At 7 days after triggering (LH+7), an endometrial biopsy was obtained under sterile conditions with a Pipelle catheter (Pipelle de Cornier; Prodimed, Neuilly-en-Thelle, France) from the uterine fundus of each woman. Samples were immediately placed in cryovials, homogenized in 1.5 ml RNA Later solution (QIAGEN, Barcelona, Spain), vigorously shaken for a few seconds and stored at room temperature for subsequent ERA analysis. All samples were processed within 48–72 h.

Sample labeling and microarray hybridization

Total RNA was extracted with the QIAGEN RNeasy mini Kit (QIAGEN, Chatsworth, CA, USA). Approximately 1–2 µg of total RNA per mg of endometrial tissue was obtained. The RNA quality was assessed by loading 300 ng of total RNA onto an RNA LabChip and running the chip on an Agilent A2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Good-quality RNA samples, with an RNA Integrity Number equal to or greater than seven, were a prerequisite for ERA analysis. A total of 10 separate hybridizations were performed. Sample preparation and hybridization were adapted from the Agilent technical manual (one-color). In short, first-strand cDNA was reverse transcribed from 200 ng of total RNA with T7-Oligo(dT) Promoter Primers. Samples were transcribed into cDNA and labeled with Cy-3 with the Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The labeling reaction typically yielded 4–5 µg of complementary RNA (cRNA) with a specific activity greater than six. Fragmented cRNA samples were hybridized onto the customized ERA array by incubating at 65 °C for 17 h with constant rotation. The microarray was then washed in two 1-min steps and two washing buffers (Agilent Technologies, Inc., Santa Clara, CA, USA). The microarrays with hybridized cRNAs were scanned with an Axon 4100A scanner (Molecular Devices, Sunnyvale, CA, USA), and the data were extracted using the GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA).

Endometrial receptivity array analysis

ERA gene expression values were pre-processed and normalized, and the endometrial receptivity status was diagnosed with the ERA computational predictor (Díaz-Gimeno et al., 2011). The ERA test indicated whether the endometrial samples were Receptive (R) or Non-receptive (NR), with an associated diagnostic probability. However, the ERA test has only been validated on endometrial samples obtained in natural cycles and after hormonal replacement treatment (HRT). Therefore, in this study, we used the ERA to focus on 238 genes that are intimately related to endometrial receptivity. The accuracy and consistency of the ERA diagnostic tool has been demonstrated to be superior to endometrial histology. The results were completely reproducible in a second ERA test that was applied 29–40 months after the first ERA test (Díaz-Gimeno et al., 2013).

Data processing and data analysis

All analyses were performed using the Babelomics web-based suite (Medina et al., 2010). The Agilent array background was corrected with robust multi-array averaging (RMA) methodology. The intensity signal was standardized across arrays with the quantile normalization algorithm.

The data obtained from group A were compared with the data from group B to evaluate the effects of COCP pretreatment. For all comparisons, we assessed the differential gene expression with limma moderated t-statistics. Standard microarray analysis techniques were used to perform one test for each gene (or a probe-set) in the microarray and for comparisons. Thus, for each gene, a t-test statistic is reported together with its corresponding P-value.

The raw P-values had to be corrected for multiple testing to minimize the amount of false positives in the study. In this analysis, we used the conventional multiple testing P-value correction procedures proposed by Benjamini–Hochberg (Benjamini et al., 2001) to derive adjusted P-values.

The gene Set Analysis was performed for each of the comparisons explored in the study. We used the logistic regression models described in previous studies (Montaner and Dopazo, 2010; Sartor et al., 2010) to identify functional blocks that were enriched in either of the conditions. In this study, we used the functional blocks described in the GO Biological Process database (Dennis et al., 2003). The conventional multiple testing $P$-value correction procedure proposed by Benjamini–Yekutieli (Benjamini and Yekutieli, 2001) was used to derive the adjusted P-values.

When performing the array analysis, we opted for a two-way approach to overcome type II error: (a) instead of using triplicates as usual, we chose five samples per group to minimize inter-patient variation and obtain a homogeneous group, and (b) to obtain as much information as possible from each sample, we decided that they should be tested individually.

Results

Epidemiological characteristics and the baseline ovarian stimulation parameters for each group are summarized in Table I. There were no significant differences in age, body mass index or the number of prior donations. With respect to ovarian stimulation, there were no significant differences in the gonadotrophin doses received, the number of mature oocytes retrieved, or the endometrial biopsies. The mean hormonal concentrations were compared with a one-way analysis of variance or Kruskal–Wallis test based on the normality of the distribution. Significance was set at $P < 0.05$. The Mann–Whitney U-test was used for pairwise comparisons of values that were not normally distributed, which yields results identical to the Kruskal–Wallis test for two independent samples. If multiple comparisons were performed, Bonferroni correction for multiple comparisons was used to analyze the differences between the protocols. All statistical analyses were performed with the SPSS (Statistical Package for the Social Sciences) 18.0 software package (SPSS, Inc.).

<table>
<thead>
<tr>
<th>Table I</th>
<th>Baseline characteristics and parameters of ovarian stimulation.</th>
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<tbody>
<tr>
<td></td>
<td>A (COCP, n = 5)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.0 ± 1.4</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>20.9 ± 0.9</td>
</tr>
<tr>
<td>Total dose FSH (IU)</td>
<td>1485 ± 314</td>
</tr>
<tr>
<td>Follicles &gt;15 mm triggering day</td>
<td>12.6 ± 2.4</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>20.8 ± 4.6</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>10.4 ± 0.7</td>
</tr>
<tr>
<td>E2 on day of triggering (pg/ml)</td>
<td>2110 ± 350</td>
</tr>
</tbody>
</table>

Values represent means ± SD. COCP, combined oral contraceptive pill; BMI, body mass index; FSH, follicle stimulating hormone; IU, international units; E2, plasma estradiol; ns, not significant.
oocytes retrieved, the pre-puncture estradiol levels or the number of days of stimulation.

The serum levels of estradiol, LH and progesterone are summarized in Fig. 1 for each of the three donor sampling points: the day of oocyte retrieval (LH + 2), 48 h later (LH + 4) and prior to endometrial biopsy (LH + 7). No significant differences were found between group A and the non-COCP group (B).

**Differential gene expression**

All samples were adequately hybridized and pre-analyzed. Data normalization reduced the technical artifacts and boosted detection of the biological signal.

We analyzed 238 genes in the ERA and compared genetic expression levels between the two groups. Only one gene, tyrosine-3 monooxygenase (TH), exhibited increased differential expression (fold change (FC) > 2) in patients with prior COCP treatment (group A) compared with the patients without prior cycle planning (group B). However, the difference was not statistically significant (adjusted P-value > 0.05). The heat map plot (Fig. 2) showed a similar gene profile for samples within groups A (COCP) and B (non-COCP). The principal component analysis (PCA) plot is shown in Fig. 3.

The results obtained from the ERA bioinformatic predictor do not have clinical value in our study because the results and predictions have only been validated in natural or hormone replacement therapy cycles. Assuming this limitation, all analyzed samples were classified as receptive by the ERA predictor.

**Functional analysis of gene expression**

Functional blocks from the GO Biological Process database were used in this analysis (Dennis *et al.*, 2003). The GO database is unique in that the functions or blocks of genes are organized into a Directed Acyclic Graph (DAG) structure. However, this procedure makes many of the significantly enriched functions redundant. We displayed only the significant, non-redundant terms (functions); that is, among the significant terms, we selected the more specific terms.

The results showed that 11 biological processes were significantly enriched samples from group A (COCP) compared with those from group B (non-COCP). These processes were defined as follows: ‘response to metal ion’ (regulation of changes or cellular activities in response to stimulation with a metal ion); ‘organ morphogenesis’ (the development of different organs upon stimulation from specific

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**Figure 1** Mean (n = 5) serum levels of luteinizing hormone (LH, A), estradiol (E2, B) and progesterone (P4, C) at each of the three sampling points (LH + 2, LH + 4 and LH + 7, considering day of triggering as day LH + 0). Blue and red lines represent group A (combined oral contraceptive pill, COCP) and B (controls, non-COCP) respectively.

**Figure 2** Gene clustering by Pearson’s correlation. A1 – A5 and B1 – B5 represent samples from group A (combined oral contraceptive pill, COCP) and group B (controls, non-COCP), respectively. On the left of the heat map is the color-coding of gene transcripts: red signifies up-regulation, blue down-regulation and white unchanged.
activities); ‘response to oxygen-containing compounds’ (regulation of cellular activity in response to an oxygenated compound); ‘tissue development’ (regulation of tissue development until the definitive structure is achieved); ‘cellular response to organic substance’ (regulation of the cellular response to certain molecular stimuli); ‘single organism signaling’ (cell signaling processes); ‘cell communication’ (mediation of intercellular or extracellular matrix communications); ‘primary metabolic process’ (normal cellular catabolic or anabolic processes); ‘cellular metabolic process’ (chemical processes by which a cell transforms substances); ‘organic substance metabolic process’ (chemical reactions for processing molecules with a carbon backbone); and ‘regulation of biological processes’ (control of genetic expression, protein changes and molecular interactions). The affected biological processes, the implicated genes and their respective P-values are summarized in Table II.

Table II  GO terms related to differentially expressed genes.

<table>
<thead>
<tr>
<th>GO term</th>
<th>Name</th>
<th>Genes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:010038</td>
<td>Response to metal ion</td>
<td>11</td>
<td>0.033</td>
</tr>
<tr>
<td>GO:009887</td>
<td>Organ morphogenesis</td>
<td>23</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:1901700</td>
<td>Response to oxygen-containing compound</td>
<td>29</td>
<td>0.024</td>
</tr>
<tr>
<td>GO:009888</td>
<td>Tissue development</td>
<td>37</td>
<td>0.011</td>
</tr>
<tr>
<td>GO:001310</td>
<td>Cellular response to organic substance</td>
<td>40</td>
<td>0.019</td>
</tr>
<tr>
<td>GO:004700</td>
<td>Single organism signaling</td>
<td>110</td>
<td>0.024</td>
</tr>
<tr>
<td>GO:0007154</td>
<td>Cell communication</td>
<td>114</td>
<td>0.032</td>
</tr>
<tr>
<td>GO:004238</td>
<td>Primary metabolic process</td>
<td>131</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:004237</td>
<td>Cellular metabolic process</td>
<td>127</td>
<td>0.033</td>
</tr>
<tr>
<td>GO:0071704</td>
<td>Organic substance metabolic process</td>
<td>137</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:0050789</td>
<td>Regulation of biological process</td>
<td>158</td>
<td>0.023</td>
</tr>
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</table>

Figure 3  Normalized data from Principal Component Analysis (PCA) plots. PCA was done on the 10 samples from the 2 groups of this study. The samples are represented spatially according to the gene expression value for the 238 Endometrial Receptivity Array (ERA) genes. The groups are represented by different colors: Group A (combined oral contraceptive pill, COCP), red; Group B (non-COCP), blue. PC1 and PC2 are first and second principal components that explain the highest variability that separates samples in the space.

Discussion

In the past few years, the use of COCPs for GnR-Hant cycle planning has elicited substantial controversy (Kolbianakis et al., 2006; Rombauts et al., 2006; Bellver et al., 2007). It has been proposed that COCPs could negatively affect clinical results due to the possible effect of progestogen on endometrial receptivity (Griesinger et al., 2010). Additionally, it was suggested that COCP pretreatment may strongly suppress LH secretion and impair folliculogenesis in subsequent cycles when only rFSH was used. In the present study, we examined the expression of genes responsible for endometrial receptivity in patients subjected to controlled ovarian stimulation (COS) under a GnR-Hant protocol in the presence or absence of COCP treatment. The take home message of this study was that we did not observe significant differences in endometrial gene expression related to endometrial receptivity in these patients, regardless of previous COCP treatment.

When analyzing our results, only one gene, TH, showed an increased expression in the COCP group compared with patients without prior cycle scheduling, but this increase was not significant (adjusted P-value >0.05). This enzyme catabolizes tyrosine conversion to phenylalanine, an essential amino acid that acts as a dopamine precursor. TH gene up-regulation within the window of implantation has been described previously by other authors (Díaz-Gimeno et al., 2011) under natural conditions.

As expected, the secretory phase is marked by more gene expression changes due to the presence of the progesterone peak. These changes coincide with the time that the endometrium becomes receptive. The genes that are overexpressed in the mid-secretory versus the early secretory phase have known functions that are involved in implantation preparation, and they are implicated in processes related to cell adhesion, metabolism, response to external stimuli, signaling, immune response, cell communication and negative regulation of proliferation and development (Talbi et al., 2006).

Eleven biological processes were significantly up-regulated in women pretreated with COCP compared with non-COCP. Three of them are related to metabolic processes (involving primary, cellular and organic substances), and one (single organism signaling) was related to signaling functions. All of these processes are enriched, which occurs in a natural cycle during the window of implantation (Ruiz-Alonso et al., 2012). Other key processes for embryo implantation, such as cell adhesion, innate immune response and the response to stress or wounding, were not modified in the endometrial samples from the COCP group. Functional analysis of the included gene in the ERA revealed that the most significant annotation is the presence of 19 processes including ones relating to the immune response, cytoskeleton proteins needed for remodeling the endometrium, oxidoreductase activity, carbohydrate binding and receptor binding (Díaz-Gimeno et al., 2011). None of these 19 biological
processes that characterize the receptive endometrium were affected in the study group (Table II).

The estrogen and progestogen in COCP have been shown to affect FSH and LH secretion, respectively (Tsaï and Yen, 1971; Anderson et al., 1990). However, the estrogen doses in the COCPs used in different studies have greatly varied from 15 to 50 μg. In addition, different studies have varied the COCP-free interval prior to the start of stimulation between 2 and 5 days, with variation of prior treatment duration between 10 and 21 days (Cédzin-Durnerin et al., 2007; Smulders et al., 2010). These variables could have an impact on clinical results. Previous studies have demonstrated that after a 5-day pill-free interval, the endocrine profile of women pretreated with a COCP resembled that of women allowed to undergo spontaneous menstruation (Cédzin-Durnerin et al., 2007).

The first studies to assess the effects of the use of COCP in cycle planning with GnRHa suggested that COCP had a possible beneficial effect on clinical results (Biljan et al., 1998; Fukuda et al., 2000). This result contrasted with later studies, which suggested that COCP had a negative effect on pregnancy rates (Pinkas et al., 2008; Meldrum et al., 2009). However, in some studies, the washout period was only 2 days. A short washout interval has been linked to higher early pregnancy loss, due to the continued, strong COCP-mediated suppression of endogenous gonadotrophin secretion during the early washout period (Griesinger et al., 2010). The variability in the type of COCP, the duration of treatment and the duration of the pill-free interval has generated heterogeneous results and confusion about the effects of COCP on IVF outcome.

A turning point occurred with the meta-analysis published in 2010 (Griesinger et al., 2010). That meta-analysis included six previous studies and reported that the group that received prior COCP treatment showed a significant decrease in the rate of ongoing pregnancy, an increased duration of stimulation, and greater consumption of gonadotrophins compared with those that did not receive COCP. Thus, it was proposed that the progesterone in COCPs might have a negative effect on endometrial receptivity.

A randomized controlled study from our group (García-Velasco et al., 2011) evaluated the clinical results from 115 women who had received prior treatment with COCP before starting a GnRHa protocol as part of their IVF/ICSI cycle. These results were compared with the results from 113 patients who received stimulation under a long protocol without prior COCP treatment. We found no significant differences in the pregnancy, implantation or live birth rates between the groups.

The number of days of COCP administration is relevant because COCP suppresses pituitary and ovarian activity. Prolonged COCP administration was associated with a greater suppression of activity (van Heusden et al., 2002). Therefore, more stimulation days and higher FSH doses would be required throughout the COS cycle, which would have a negative economic impact on the cost of the cycle. In the present study, we administered COCP for between 12 and 16 days, which we considered sufficient to restrain FSH levels without excessive inhibition. In this series and in our previous study (García-Velasco et al., 2011), COCP pretreatment for 12–16 days did not increase the total dose of gonadotrophins required compared with non-COCP patients.

High progesterone levels at the beginning of the cycle, which could arise from the progestogen content of the COCP, have been linked to a decreased probability of pregnancy; however, this effect remains controversial because recent publications have not confirmed those findings (Fatemi et al., 2013). Interestingly, in addition to the deleterious effects of progesterone at the beginning of the cycle, elevated progesterone levels at the end of the stimulation may have a negative effect on endometrial receptivity; this was recently studied using microarray technology. Important differences were observed in the genetic expression patterns of patients with progesterone concentrations above or below 1.5 ng/ml on the day of follicular maturation (Labarta et al., 2011).

In the present study, we used a 30-μg dose of COCP and implemented a 12- to 16-day treatment period with a 5-day washout period. We did not observe differences in the gene expression patterns at the endometrial level that could affect endometrial receptivity. Thus, we concluded that the 10 samples were nearly identical in this respect regarding the endometrial gene expression of the genes related to the window of implantation.

All participants received the same protocol to avoid problems with reproducibility or cycle-to-cycle variations and to increase the consistency of the gene expression patterns for assessing endometrial receptivity. A recent report has demonstrated the safety and reproducibility of ERA endometrial microarrays and compared the results with classical histological analyses. Duplicate biopsies were obtained under the same conditions from seven patients, with intervals between biopsies ranging from 29 to 40 months. They demonstrated 100% reproducibility between the two samples (Díaz-Gimeno et al., 2013).

We can conclude that the information we discovered regarding endometrial gene expression using microarray analysis suggests that the use of COCP as a pretreatment did not negatively impact the profile of endometrial gene expression for women undergoing IVF with the GnRHa protocol. When COCPs were used for a maximum of 16 days and a pill-free interval was allowed for 5 days, endometrial gene expression was not modified.

Authors’ roles
A.B. designed the study, conducted the data analysis and interpretation and wrote the manuscript. He is the corresponding author for the reviewing procedure. M.R.-A. and D.B. processed the endometrial samples and performed the microarray technology, data analysis and manuscript writing. C.I. participated in donor recruitment and care during the medical treatment and performed the endometrial biopsies for sample collection. A.P. and C.S. contributed to the review of the manuscript. J.G.-V. participated in the study design and reviewed the intellectual content of the manuscript.

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Conflict of interest
C.S. and A.P. are co-inventors (with Patricia Díaz-Gimeno) of the Endometrial Receptivity Array and hold the patent. None of the other authors has any conflicts of interest to declare.
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