Effect of single post-ovulatory administration of levonorgestrel on gene expression profile during the receptive period of the human endometrium

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Abstract

The hypothesis that levonorgestrel (LNG) used as an emergency contraceptive interferes with endometrial receptivity remains unproven. We compared the endometrial gene expression profile during the receptive period after administering a single dose of LNG 1·5 mg or placebo on day 1 of the luteal phase. An endometrial biopsy was done on day LH + 7 or LH + 8 and samples were taken from seven volunteers, each one contributing with one cycle treated with placebo and another with LNG. The expression of 20 383 genes was determined using cDNA microarrays. Real-time RT-PCR was used 1) to confirm the differences found in DNA microarray analysis and 2) to determine the effect of LNG on transcript levels of *C3*, *C4BPα*, *COX2*, *MAOA*, *S100A4*, and *SERPINB9*, known to be upregulated during receptivity, and on *cPLA2α*, *JAK1*, *JNK1*, *CTSL1*, and *GSTP1*, known to respond to mifepristone. Additional endometrial biopsies were done during the pre-receptive (LH + 3) and receptive (LH + 7) period and samples were taken from eight untreated volunteers in order to determine the changes associated with acquisition of receptivity of 14 genes. Mean levels of *PAEP*, *TGM2*, *CLU*, *IGF2*, and *IL6ST* mRNAs increased after administering LNG while those of *HGD*, *SAT1*, *EVA1*, *LOC90133*, *ANXA1*, *SLC25A29*, *CYB5A*, *CRIP1*, and *SLC39A14* decreased. Except for the level of *ANXA1* transcript, all changes remained within the range observed in untreated controls, and none of the transcripts responding to mifepristone changed in response to LNG. Post-ovulatory administration of LNG caused minimal changes in gene expression profiling during the receptive period. Neither the magnitude nor the nature or direction of the changes endorses the hypothesis that LNG interferes with endometrial receptivity.

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Introduction

Implantation requires the development of a receptive endometrium able to respond to signals from the blastocyst (Pope 1988). The action of progesterone on an estrogen-primed endometrium results in a particular gene expression profile that renders the endometrium receptive to embryo implantation (Martin *et al.* 2002).

Levonorgestrel (LNG) is a progestin widely used for regular hormonal contraception, and more recently for emergency contraception. Scientific evidence on the mode of action of LNG as an emergency contraception began to unfold after the method had been introduced in many countries. This situation fostered much debate when this method was introduced in more conservative societies due to the belief that LNG can alter endometrial receptivity and prevent implantation. Administration of LNG during the follicular phase interferes with ovulation in 93% of cycles treated before

the onset of the LH surge (Croxatto *et al.* 2004), implying that most of the contraceptive efficacy of LNG may be accounted by its interference with ovulation. This is supported by a recent study reporting that none of the 34 women at risk who took LNG before ovulation became pregnant while three pregnancies were recorded among 17 women who took LNG after ovulation (Novikova *et al.* 2007).

Experiments in monkeys, *Cebus apella*, and rats have shown that LNG interferes with ovulation but not with implantation (Müller *et al.* 2003, Ortiz *et al.* 2004). Although extrapolation of these results to humans has limitations, they give no support to the notion that LNG interferes with endometrial receptivity. Ethical considerations prevent from reproducing those experiments in humans, so alternative approaches need to be used to tackle with this issue. An indirect approach to assess the possible interference of LNG with endometrial receptivity in humans is to determine its

effects on endometrial morphological or molecular parameters during the period of receptivity. Treatment on day LH–2 had no effect on endometrial morphology assessed at the expected time of endometrial receptivity (Marions *et al.* 2002) and post-ovulatory treatment administered on day LH+2 had no consistent effect on endometrial morphology or markers of receptivity (Durand *et al.* 2001, 2005, Marions *et al.* 2002).

The gene expression profile temporally associated with the acquisition of endometrial receptivity has been established in untreated normal women utilizing microarrays (Carson *et al.* 2002, Kao *et al.* 2002, Borthwick *et al.* 2003, Riesewijk *et al.* 2003, Mirkin *et al.* 2005, Haouzi *et al.* 2009, Talbi *et al.* 2006), but the effect of LNG on global gene expression profile in the human endometrium has not been reported.

Here, we report the effect of post-ovulatory administration of LNG on endometrial gene expression profile during the period of receptivity in normal healthy women unexposed to the risk of pregnancy.

Materials and methods

Participants

The study protocols were approved by the ethics and scientific review committee that serves Instituto Chileno de Medicina Reproductiva (ICMER) in accordance with the Declaration of Helsinki. Two groups of eight women were recruited for the study. Each volunteer signed an informed consent form before participating. All were in good health as determined by medical history, physical and gynecological examination. The mean body mass index was 26.8 kg/m² (range 23.4–30), mean hemoglobin 14.3 g/dl (range 12.7–15.6), and mean age 36 years (range 32–39). A Papanicolaou smear was done as required. All volunteers had been surgically sterilized at least 1 year before, for reasons unrelated to this study, and had regular menstrual cycles within the range 24–35 days. None was under a chronic treatment or had used hormones or drugs able to modify the metabolism of steroid hormones in the preceding months.

Study design

The first part of the study was a placebo-controlled, crossover, double-blind, randomized trial done with the participation of eight volunteers. Each subject contributed with one placebo-treated cycle (control) and one LNG-treated cycle (experimental) in a randomized order with two resting cycles between them. Four started their participation with the control cycle and the other four with the experimental cycle. They received two placebo pills in the control cycle and two LNG pills of 0.75 mg in the experimental cycle. Placebo pills

identical in appearance to LNG pills were provided by Laboratorios Silesia S.A., Santiago, Chile. Treatment was given at the clinic under supervision of a nurse in all cases.

When ovulation is suppressed or postponed by LNG, endometrial effects may result from insufficient progesterone secretion rather than from a direct action of the drug. In addition, when LNG interferes with ovulation, its effects on the endometrium are irrelevant for the mechanism of action as there will be no embryo to implant. For these reasons, we choose to administer LNG in the immediate post-ovulatory period, i.e. on the same day the follicular rupture was diagnosed by transvaginal ultrasound (TVU). In order to control the timing of the interventions and insure normality of the cycle under study, beginning in the mid-follicular phase, a venous blood sample for the assay of LH was taken every day and follicular growth was monitored by TVU. Blood sampling and TVU were discontinued once ovulation was detected. Serum LH was measured by enzyme immunometric assays provided by Immunometrics UK (London, UK). When the echo-image of the leading follicle was found to have disappeared, treatments were given immediately. This ensured that LNG was administered within 24 h of ovulation.

Endometrial samples were collected on LH+7 or LH+8 for convenience to avoid weekends. They were obtained from the uterine fundus, using pipelle catheters (CCD Laboratories, Paris, France). An additional blood sample was taken on that day to determine serum progesterone level. Progesterone was measured by RIA using Coat-ACount Progesterone from Siemens Medical Solutions Diagnostics Products (Los Angeles, CA, USA).

The second part of the study was a straightforward follow-up of the menstrual cycle of another group of eight volunteers selected with the same criteria. Blood samples were drawn daily and TVU was performed as described in each volunteer for a single menstrual cycle in which no treatment was given. Two endometrial biopsies were obtained within that menstrual cycle, one during the pre-receptive (LH+3) and another in the receptive (LH+7) phase. For both parts of the study, one piece of biopsy was processed for histological assessment by an independent pathologist, under blind conditions, using the criteria described by Noyes *et al.* (1950). The rest was immediately frozen in liquid nitrogen for subsequent RNA isolation for its use in microarray and real-time RT-PCR analysis.

Isolation of RNA

Total RNA was isolated from tissue samples according to a modification of the method of Chomczynski & Sacchi (1987), using Trizol reagent (Invitrogen) using the protocol provided by the manufacturer. The quality of RNA was checked using Agilent's Lab-on-a-Chip

total RNA nano biosizing assay by examining the 18s and 28s ribosomal bands. Protein contamination of the isolated RNAs was quantified spectrophotometrically by the A_{260}/A_{280} ratio; this varied between 1.8 and 1.9.

Microarray analysis

Production of microarray slides

Oligonucleotide microarrays were produced in the Laboratory of Molecular Technology (Frederick, MD, USA). Oligonucleotides from the Oligator Human RefSet oligos comprising 70mer probes (Operon Biotechnologies, Huntsville, AL, USA) were spotted on poly-L-lysine (Sigma) coated glass slides with a MicroGrid II microarrayer (Biorobotics, Cambridgeshire, UK). The oligonucleotide set comprised 20 736 genes included in the National Center for Biotechnology Information (NCBI) Human Reference Sequence database. Control staining with SYBR 555 (Paragon, Molecular Probes, Eugene, OR, USA) was performed for quality control.

Synthesis and labeling of cDNA

Fifteen micrograms of total RNA from each sample obtained from treatment (LNG) and control (placebo) cycles were processed separately as individual samples in order to generate a labeled cDNA. All samples were compared against a Human Universal RNA (Stratagene, La Jolla, CA, USA) composed of total RNA from ten human cell lines. Cyanine 3-dUTP or cyanine 5-dUTP (Perkin Elmer, Boston, MA, USA) was enzymatically incorporated into the reverse transcription reaction, using the LabelStar kit according to the recommended procedure of the manufacturers (Qiagen). Five micrograms of cyanine 5-dUTP-labeled cDNA of each endometrial sample were combined with 5 µg cyanine 3-dUTP-labeled cDNA from Human Universal RNA. This was done in duplicate and with one dye swap (three mixtures total per sample).

Hybridization of glass oligonucleotide microarrays

Oligonucleotide microarrays were hybridized as described for cDNA arrays (DeRisi *et al.* 1997). Each mixture of labeled cDNA (sample and reference) was adjusted to 29 µl with elution buffer provided in the LabelStar kit and subsequent addition of 2 µl human *COT1* DNA (Invitrogen) and 2 µl poly-d(A)_{40–60} DNA (Amersham Biosciences Corp.) (both 10 mg/ml), 6.3 µl of 20× SSC, and 1.06 µl of 10% (w/v) SDS. The hybridization solution was heated for 2 min at 99 °C and centrifuged for 5 min at 16 000 g. Slides were hybridized in a water bath at 57 °C for 14–16 h.

After hybridization, slides were washed in 2× SSC-0.1% SDS, 1× SSC, 0.2× SSC, and 0.05× SSC for 1 min each time and finally washed in ultrapure water for 10 s. Before scanning, slides were dried by spinning at 30 g for 5 min.

Scanning, feature extraction, and analysis

Fluorescent images from microarray slides were captured using the Genepix 4000 Scanner (Axon Instruments, Inc., Foster City, CA, USA). Feature extraction was done using GenePix Pro 4.0 software (Axon Instruments, Inc.). Spots with high local background or aberrant spot shape were flagged by the software and checked manually. For each slide, the average foreground signal intensity adjusted for local channel-specific background was calculated. Spots with signal intensities in both channels <100 were excluded. If at least one channel had intensity above 100, the intensity under 100 was set at 100.

Analysis of microarray data

The images and signal intensity data were stored in the NCI Microarray Data Base supported by the Center for Information Technology of NIH (<http://nciarray.nci.nih.gov>). All signal intensities from data sets used for the analysis ranged from 0.7 to 1.25. The distribution ratio extracted from microarray images exhibited normal distribution, constant coefficient of variation, and high positive signal. We used twofold above background as the intensity cutoff. Genes selected for further analyses were only those for which data was available in the eight control and eight experimental cycles and showed a statistically significant difference of at least 95% between the two sets of cycles. Using this analysis, we selected 245 genes, and with this new subset of genes, we performed an unsupervised hierarchical clustering to determine the diversity of the samples and groups according to their gene expression pattern (not shown). From this analysis, we eliminated the data obtained from one volunteer because her profile was the reverse of her assigned group. Hence, we performed a new statistical analysis, excluding the data of the outlier pair of samples, on those genes whose transcript level changed at least twofold in the experimental cycle with respect to the control cycle. Differences between the two sets of cycles were analyzed by paired *t*-test.

Real-time RT-PCR

One microgram of total RNA from each endometrial sample used for the cDNA microarray analysis and from LH+3 and LH+7 of untreated cycles was treated with DNase I Amplification grade (Invitrogen) and the

first-strand cDNA was synthesized by reverse transcription using the Superscript III Reverse Transcriptase First-Strand System for RT-PCR (Invitrogen), according to the recommended procedure of the manufacturers. Levels of all genes were measured from each cDNA sample relative to Human Universal RNA reference used for the cDNA microarrays and the expression values were corrected against GAPDH to account for differing amounts of starting material.

Real-time RT-PCR was performed using an ABI PRISM 7900HT sequence detection system (TaqMan) according to the instructions of manufacturers. Pre-validated primers and probes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were designed by Applied Biosystems (Table 1; PE Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed in duplicate using the conditions recommended by the manufacturer. The thermal cycling conditions included an initial activation step at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturing and annealing amplification (95 °C for 15 s, 60 °C for 1 min).

Quantitative analysis was based on the relative quantification of each gene of interest in the samples of each group using a variation of the ‘delta–delta method’ ($2^{-\Delta\Delta C_T}$) developed by PE Applied Biosystems (Livak & Schmittgen 2001):

$$2^{-(C_T(\text{Gene Universal RNA} - \text{Gene RNA samples}) - [C_T(\text{GAPDH Universal RNA} - \text{GAPDH RNA samples})])}$$

where cycle threshold (C_T) is the cycle in the amplification reaction in which fluorescence begins to be exponential above the background baseline. The fold change in relative level of each transcript was obtained from the ratio between the median of the control and experimental cycles or between LH+7 and LH+3. A paired *t*-test was done to determine statistically significant differences in fold change between sets of cycles.

Table 1 IDs from Applied Biosystems assay used for gene expression analysis

Genes	AB ID	Genes	AB ID
PAEP	Hs00171462_m1	C4BPα	Hs00426339_m1
TGM2	Hs00190278_m1	MAOA	Hs00165140_m1
CLU	Hs00156548_m1	C3	Hs00163811_m1
IGF2	Hs00171254_m1	SERPINB9	Hs00244603_m1
IL6ST	Hs00174360_m1	S100A4	Hs00243201_m1
HGD	Hs00164847_m1	COX2	Hs00153133_m1
EVA1	Hs00170684_m1	MAPK8	Hs00177083_m1
LOC90133	Hs00163781_m1	JAK1	Hs01026983_m1
SAT1	Hs00161511_m1	CTSL1	Hs00377632_m1
ANXA1	Hs00167549_m1	PLA2G4A	Hs00996915_m1
SLC25A29	Hs00402742_m1	GSTP1	Hs00168310_m1
CRIP1	Hs00832816_g1		
SLC39A14	Hs00299262_m1	GAPDH	Hs02758991_g1

Results

Menstrual cycle features, histological dating, and progesterone levels in placebo- and LNG-treated cycles

There were no remarkable differences between placebo- and LNG-treated cycles with respect to cycle length and bleeding. Neither histological features of the endometrium nor progesterone levels displayed differences between control and experimental cycles (mean±s.d.: control cycle 41.8±14.4 nmol/l; LNG cycle 38.6±14.4 nmol/l).

cDNA microarray analysis

Complete data were obtained and analyzed from 20 383 out of the 20 736 genes spotted in the microarray slides. A total of 15 transcripts (0.07%) displayed a statistically significant change ($P<0.05$) in the expression of at least twofold between LNG and placebo cycles, of which five increased and ten decreased (Table 2). The Multicriteria analysis (MCA) confirmed that there were no remarkable differences between placebo- and LNG-treated cycles with respect to the global gene expression profile (Fig. 1).

Real-time RT-PCR

Validation of differences in transcript levels found in microarray analysis

In order to validate the microarray-positive findings, total RNA from each of the 14 samples was subjected to real-time RT-PCR, using the TaqMan system, with appropriate starters for the 15 transcripts shown in Table 3. All values were corrected for differences in loading relative to GADPH.

The transcript levels found in real-time RT-PCR were similar to those found in the microarray analysis, with minor differences that can be attributed to the higher sensitivity of the former technique (Table 3). All differences were statistically significant in the seven LNG-treated samples. The transcript level corresponding to progestagen-associated endometrial protein (PAEP), transglutaminase 2 (TGM2), HGD, spermidine/spermine N-acetyltransferase (SAT1), ANXA1, EVA1, and SLC25A29 genes showed a larger difference between LNG- and placebo-treated cycles with real-time RT-PCR than with microarray analysis, whereas those of clusterin (CLU), insulin-like growth factor 2 (IGF2), cysteine-rich intestinal protein 1 (CRIP1), and SLC39A14 kept the same level of difference and those of CYB5A, LOC90133, and FAU showed a smaller difference than the one found in the microarray

Table 2 Transcripts showing a mean fold change ≥ 2 in human endometrium after post-ovulatory administration of LNG, determined by microarrays

	UniGene ID	Microarrays	
		Fold change	P value
Genes			
<i>PAEP</i>	Hs.532325	3.7	0.018
<i>TGM2</i>	Hs.517033	2.5	0.004
<i>CLU</i>	Hs.436657	2.2	0.002
<i>IGF2</i>	Hs.272259	2.0	0.008
<i>IL6ST</i>	Hs.532082	2.0	0.007
<i>HGD</i>	Hs.368254	-2.8	0.009
<i>EVA1</i>	Hs.116651	-2.5	<0.001
<i>LOC90133</i>	Hs.101651	-2.5	0.004
<i>SAT1</i>	Hs.28491	-2.3	0.005
<i>ANXA1</i>	Hs.494173	-2.2	<0.001
<i>SLC25A29</i>	Hs.578109	-2.1	<0.001
<i>CYB5A</i>	Hs.465413	-2.1	<0.001
<i>FAU</i>	Hs.387208	-2.1	0.017
<i>CRIP1</i>	Hs.70327	-2.0	0.002
<i>SLC39A14</i>	Hs.491232	-2.0	0.002

analysis. Therefore, differences found by microarray analysis correspond to actual changes caused by treatment with LNG.

Analysis of transcripts responding to LNG in samples taken in the pre-receptive and receptive phase of untreated menstrual cycles

TaqMan system was used to determine the direction and magnitude of change from the pre-receptive to the receptive phase of untreated menstrual cycles of those transcripts that changed after treatment with LNG. As shown in Table 3, all transcripts whose median level increased after post-ovulatory LNG administration increased significantly from LH+3 to LH+7. On the other hand, all genes that decreased in LNG-treated cycles did not change significantly from pre-receptive to receptive phenotype. *ANXA1* was the exception, as its median value increased less during the receptive period after LNG than in untreated cycles.

Effect of LNG on selected transcripts that changes from the pre-receptive to receptive period in untreated menstrual cycles

Selected transcripts that had previously been found by microarrays to increase during the receptive phase but had not been confirmed by an independent technique were subjected to TaqMan analysis in endometrial biopsies obtained on LH+3 and LH+7 from eight normal fertile women receiving no treatment. We selected these targets from a large set of genes that changed their expression level during the period of endometrial receptivity because we had the primers

and probes for analysis. In addition, we studied *MAOA*, *SERPINB9*, and *C4BP α* because we previously reported that these genes decrease their expression in infertile women with endometrial origin (Henriquez *et al.* 2006, Tapia *et al.* 2008).

All transcripts analyzed increased during the receptive period confirming previous findings with microarrays (Table 4). The same transcripts were analyzed by TaqMan in samples taken after LNG or placebo. LNG caused statistically significant changes in four of six transcripts in the range 1.4–1.8 times and no statistically significant changes in the other 2 (Table 4).

Effect of LNG on genes that respond to mifepristone

The effect of LNG on five transcripts that have been reported to respond to mifepristone in endometrial explants was determined. None of these transcripts responded with a statistically significant change to LNG (Table 5).

Discussion

The current study tested the effect of single post-ovulatory administration of LNG on the level of thousands of transcripts in order to assess the likelihood it may impair endometrial receptivity.

Figure 1 Multicriteria analysis (MCA) of samples obtained during placebo- and LNG-treated cycles showing there were no remarkable differences between placebo- and LNG-treated cycles with respect to the global gene expression profile. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0094>.

Table 3 Validation by real-time RT-PCR of changes in transcript levels detected by microarrays in human endometrium after post-ovulatory administration of LNG and their level of expression in the pre-receptive (LH+3) and receptive (LH+7) phases of the untreated menstrual cycles. The fold change in transcript level determined by real-time RT-PCR derived from the ratio between the median of the placebo- and LNG-treated cycles of all seven volunteers (A) or the ratio between the median of the receptive and pre-receptive phase of eight untreated volunteers (B). The *P* values are derived from the Wilcoxon matched pairs test. Fold changes marked NS were not calculated because the difference was not significant ($P > 0.05$). Note the wide variations in the range of changes between the samples obtained from different women, especially during the transition from pre-receptive to receptive period where the same gene, in some women increased and in others decreased, indicating that probably the transcript level of these genes is not crucial for the acquisition of receptivity. The genes *CYB5* and *FAU* were not analyzed in control cycles

Genes	(A) Treated cycles* (n=7)		(B) Untreated cycles** (n=8)	
	Mean fold change (range)	<i>P</i> value	Mean fold change (range)	<i>P</i> value
<i>PAEP</i>	4.4 (+2.0 to +31.5)	0.018	87 (+8.0 to +1266)	0.012
<i>TGM2</i>	3.3 (+1.0 to +7.4)	0.018	7.9 (+2.2 to +26.3)	0.012
<i>CLU</i>	2.3 (+1.3 to +10.4)	0.018	18 (+7.1 to +49.7)	0.012
<i>IGF2</i>	2.0 (+1.2 to +7.5)	0.018	5.6 (+1.4 to +13.3)	0.012
<i>IL6ST</i>	1.7 (+1.0 to +3.5)	0.027	2.5 (−2.1 to +7.6)	0.012
<i>HGD</i>	−2.7 (−5.6 to +1.0)	0.018	NS (−4.4 to +7.1)	0.58
<i>EVA1</i>	−3.0 (−3.9 to −1.5)	0.018	1.9 (−4.8 to +29.5)	0.40
<i>LOC90133</i>	−1.3 (−2.6 to −1.1)	0.04	NS (−19.6 to +3.9)	0.48
<i>SAT1</i>	−3.8 (−5.1 to −2.0)	0.018	NS (−2.7 to +4.3)	0.09
<i>ANXA1</i>	−3.5 (−4.9 to −2.7)	0.028	4.7 (−2.3 to +11.1)	0.04
<i>SLC25A29</i>	−2.4 (−2.7 to −1.4)	0.018	NS (−2.3 to +9.4)	0.07
<i>CYB5A</i>	−1.8 (−3.5 to −1.1)	0.018	Not analyzed	
<i>FAU</i>	−1.3 (−2.5 to +1.1)	0.028	Not analyzed	
<i>CRIP1</i>	−2.0 (−3.0 to −1.3)	0.018	NS (−9.0 to +29.0)	0.21
<i>SLC39A14</i>	−2.4 (−3.6 to −1.4)	0.018	NS (−1.2 to +148.0)	0.21

*LNG vs placebo; **receptive vs pre-receptive.

The level of 15 transcripts was found to change in a statistically significant manner after treatment with LNG in the microarray analysis. Those transcripts represent 0.07% of the genes present in the microarrays.

Previous studies on endometrial gene expression profiling during the window of implantation (Popovici *et al.* 2000, Carson *et al.* 2002, Kao *et al.* 2002, Borthwick

et al. 2003, Riesewijk *et al.* 2003, Mirkin *et al.* 2005, Talbi *et al.* 2006, Haouzi *et al.* 2009) have found a higher number of genes whose expression changed significantly (1.2–9.6%). The lower number of transcripts influenced by LNG may be explained by the concomitant rise in serum progesterone levels and decrease in plasma LNG concentration during the days preceding the biopsy. Presumably, under those

Table 4 Effect in human endometrium of post-ovulatory administration of LNG on the endometrial level of selected transcripts that increase from pre-receptive to receptive phase, determined by real-time RT-PCR. The fold change determined by real-time RT-PCR derived from the ratio between the median of the receptive and pre-receptive phase of eight volunteers (A) or the ratio between the median of the LNG- and placebo-treated cycles of all seven volunteers (B). The *P* values are derived from the Wilcoxon matched pairs test. Fold changes marked NS were not calculated because the difference was not significant ($P > 0.05$). All transcripts changed significantly by LNG experienced a change smaller than twofold

UniGene ID	(A) Untreated cycles* (n=8)		(B) Treated cycles** (n=7)	
	Mean fold change (range)	<i>P</i> value	Mean fold change (range)	<i>P</i> value
Genes				
<i>C4BPα</i> or <i>C4BPA</i>	Hs.1012 284 (+3.2 to +973)	0.012	1.7 (+1.2 to +7.0)	0.028
<i>MAOA</i>	Hs.183109 25 (+8.5 to +142)	0.012	NS (−1.3 to +3.6)	0.18
<i>C3</i>	Hs.529053 25 (+9.6 to +31.5)	0.012	1.8 (−1.3 to +7.5)	0.018
<i>SERPINB9</i>	Hs.104879 13 (+4.4 to +55.1)	0.012	1.7 (+1.2 to +3.8)	0.018
<i>S100A4</i>	Hs.654444 5.1 (+1.6 to +41.4)	0.012	1.4 (+1.3 to +2.4)	0.018
<i>COX2</i>	Hs.196384 3.4 (1.0 to +10.7)	0.017	NS (−2.2 to +15.4)	0.24

*receptive vs pre-receptive; **LNG vs placebo.

Table 5 Effect in human endometrium of post-ovulatory LNG administration on transcripts that respond to mifepristone, determined by microarrays or real-time PCR. The fold changes from real-time RT-PCR derived from the ratio between the median of the LNG- and placebo-treated cycles of all seven volunteers. The *P* values are derived from the Wilcoxon matched pairs test; Fold changes marked NS were not calculated because the difference was not significant ($P > 0.05$). None of the genes that responded to mifepristone responded to LNG

Gene	UniGene ID	Mifepristone*	Treated cycles** (n=7)	
			Fold change (range)	<i>P</i> value
<i>MAPK8</i> or <i>JNK1</i>	Hs.138211	Decreased	NS (+1.0 to +1.4)	0.18
<i>JAK1</i>	Hs.207538	Decreased	NS (+1.0 to +1.5)	0.74
<i>CTSL1</i>	Hs.418123	Decreased	NS (−1.7 to +3.2)	0.18
<i>PLA2G4A</i> or <i>cPLA2α</i>	Hs.497200	Increased	NS (−4.6 to +2.5)	0.18
<i>GSTP1</i>	Hs.523836	Increased	NS (−1.4 to +3.0)	0.18

*Catalano *et al.* 2003; **LNG vs placebo.

circumstances, both hormones are acting through the same receptor that is likely to be saturated considering it is downregulated in the first half of the luteal phase and the natural ligand concentration is raising. Therefore, it is not surprising that the transcript profile after LNG approximates the one found after placebo. The results of the real-time RT-PCR support our microarray analysis, confirming our findings although false negatives in the microarrays cannot be ruled out, as those were not tested due to practical reasons. Our data showed that all transcripts that increased upon LNG displayed the same regulation in spontaneous cycles in the transition from pre-receptive to receptive state (Table 3). Previous microarray studies have shown that the three transcripts most upregulated in our analysis, *PAEP*, *TGM2*, and *CLU*, also increased during the window of implantation, supporting our findings. The transcripts of *IGF2* and signal transducer of IL6 (*IL6ST*) displayed smaller changes from pre-receptive to receptive state, where *IL6ST* has been reported to increase from early to mid-secretory endometrium (Talbi *et al.* 2006). None of the transcripts downregulated by LNG, except *ANXA1*, displayed statistically significant changes during the window of implantation. Of these, only *CYB5A*, *SAT1*, *HGD*, and *EVA1* have been described previously to change with acquisition of receptivity. However, we found no changes in transcript levels of *SAT1* and *HGD* most likely because they have been described to be regulated from the late proliferative to mid-secretory phase, reflecting changes from an estrogen to a progesterone-dominated endometrium (Kao *et al.* 2002, Borthwick *et al.* 2003). *EVA1* and *CYB5A* were reported to decrease from early to mid-secretory phase as in this study (Talbi *et al.* 2006). *EVA1* was also reported to increase during the same period (Carson *et al.* 2002, Riesewijk *et al.* 2003). Such discordant results have been described by several endometrial gene expression profile studies that are not readily explained by differences in methodology (Tapia *et al.* 2011).

Similarly, several transcripts that did not change significantly in our study exhibited high variability between subjects; in some subjects, they appear upregulated while in others they appear downregulated (see range of Table 2). As all women participating in this study were fertile, we speculate that the level of those transcripts is not critical for acquisition of receptivity. The main question remaining is whether or not, at least in theory, the changes found in this study could adversely affect endometrial receptivity. None of the proteins coded by the transcripts affected by LNG have been shown yet to be directly involved in endometrial receptivity; therefore, one can only speculate regarding their potential impact on embryo implantation and clearly functional studies are needed to settle this issue.

Four genes that respond to LNG could be related to the immune response. The first is the gene that codes for *PAEP*, also known as glycodelin, placental protein 14, or placental $\alpha 2$ -macroglobulin (Seppala *et al.* 1998, 2002). The direct action of progesterone brings this protein to its highest expression during the human endometrial receptivity period (Taylor *et al.* 2000). In women who took LNG as an emergency contraceptive 2 days before the LH surge, the concentration of *PAEP* in uterine flushes measured at LH+1 was significantly elevated when compared with control cycles (Durand *et al.* 2010). This provides a possible new explanation of the contraceptive effect of LNG around the fertilization window, as it has also been reported that LNG can inhibit sperm–oocyte interaction. *PAEP* exhibits a potent immunosuppressive activity (Bolton *et al.* 1987) that has been proposed to help avoid a maternal immune rejection against the embryo at the time of implantation. It is possible that increased endometrial *PAEP* expression during the receptive period in response to LNG would create a stronger immunosuppressive microenvironment to protect the embryo against immune system response.

The *IL6ST* or glycoprotein (gp)-130 (Kamimura *et al.* 2003) is a molecule that participates in LIF and IL11 signaling. Both bind to their specific receptors IL11R α and LIFR β , respectively, which heterodimerize with IL6ST for subsequent signaling through the JAK/STAT pathway. Transgenic mice that do not express LIF or IL11 or without the carboxyl-terminal region of gp-130 are infertile due to implantation failure (Ernst *et al.* 2001, Robb *et al.* 2002), indicating that the gp-130 signaling cascade is critical to this process. As in this study, *IL6ST* was reported to increase from early to mid-secretory phase (Talbi *et al.* 2006).

CLU is another gene related to the immune system that displays increased expression in response to LNG. *CLU* inhibits the final step of the complement cascade, preventing cell lysis by stopping the insertion of the attack complex into cell membranes (Murphy *et al.* 1998, Jones & Jomary 2002) and interacts with IgG to increase the rate of formation of insoluble immune complexes (Wilson *et al.* 1991) implying that *CLU* could modulate the immune system in the endometrium during the receptive period. In addition, *CLU* has been reported to be upregulated during the window of implantation (Riesewijk *et al.* 2003) and downregulated in women with implantation failure (Tapia *et al.* 2008), suggesting that it is important for the endometrial receptivity acquisition.

Another gene that responds to LNG and could modulate the immune response is *CRIPI*. The function of this gene in humans has not been described, but it is known to be preferentially expressed in immune system cells (Khoo *et al.* 1997). Its expression has been described in human endometrium (Talbi *et al.* 2006). In transgenic mice, overexpression of *CRIPI* alters the cytokine pattern and thus the immune response (Lanningham-Foster *et al.* 2002). It appears that an overexpression of *CRIPI* in this model favors Th2 response over Th1, making it possible to propose a similar role in human endometrium. The expression of this gene decreased twofold in response to LNG and did not change between the pre-receptive and receptive periods, which means it may not have a critical role in the establishment of receptivity.

Another process considered important for endometrial receptivity is the remodeling of the extracellular matrix (ECM) and cell adhesion molecules. One of the molecules we found that had an expression change after post-ovulatory LNG administration that could be related to the remodeling and organization of the ECM in the endometrium is TGM2. This enzyme catalyzes the covalent attachment of proteins and polyamines, both of which contribute to ECM stability in a variety of tissues by interacting with ECM components such as fibronectin and integrins (Fesus & Piacentini 2002). TGM2 plays an important role in the adhesion and migration of many cell types (Mohan *et al.* 2003),

including the trans-endothelial migration of blood cells, which may mimic the initial step in trophoblast invasion (Fazleabas *et al.* 2004). While it has been observed that progesterone increases *TGM2* transcript expression in Ishikawa endometrial cells (Li *et al.* 2006), the activity of this enzyme is ten times higher in the secretory endometrium than the proliferative endometrium (Hager *et al.* 1997). *TGM2* mRNA also increases during the period of receptivity (Riesewijk *et al.* 2003), suggesting that progesterone regulates the expression of *TGM2* *in vivo*. In addition, *TGM2* is expressed in pinopodes and co-localizes with the integrin $\alpha v \beta 3$ in the maternal-embryonic interface (Kabir-Salmani *et al.* 2005). A recent study showed increased protein expression of TGM2 in decidualized vs control endometrial stromal cells (Garrido-Gomez *et al.* 2011), suggesting a role for TGM2 during implantation. Because the administration of LNG produced a greater increase than normally occurs during the period of receptivity in a spontaneous cycle, LNG is not expected to have a deleterious effect on implantation.

The actions of estradiol and progesterone on the endometrium are controlled by paracrine mediators such as the IGF1 and IGF2. The expression of *IGF1* and its receptor are stimulated by estrogen, so it is thought that the actions of estrogen-induced cell growth are mediated, at least in part, by IGF1 (Murphy & Ghahary 1990). Meanwhile, IGF2 has been associated with the characteristic cellular differentiation of the secretory phase (Lopez *et al.* 1996) with an increase in mRNA levels in the decidualization of ESC (Ganef *et al.* 2009). LNG continuously released into the endometrial cavity causes a downregulation of *IGF1* mRNA and an increase in *IGF2* (Rutanen *et al.* 1997). This is consistent with the effect of intrauterine LNG release on endometrial morphology, the dominant feature of which is epithelial atrophy and stromal decidualization (Nilsson *et al.* 1978). Unlike the observation by Rutanen *et al.* (1997), who reported no difference in *IGF2* throughout the menstrual cycle, here we found a 5-6-fold increase in the *IGF2* transcript in the receptive period relative to the pre-receptive period. We were probably able to detect this difference because we used real-time RT-PCR, a more sensitive method. We also found that the post-ovulatory administration of LNG causes a twofold increase of this transcript.

Another gene that decreases its expression after LNG administration and could have a role in the endometrium is *SAT1*. This enzyme limits the bio-availability of the polyamines spermine and spermidine by mediating their mono- or di-acetylation and their subsequent conversion to putrescine. Consequently, the intracellular concentration of polyamines is crucial in cell growth and proliferation, where SAT1 typically functions as a cell growth inhibitor by decreasing the supply of polyamines. Accordingly, transgenic mice

overexpressing *SAT1* have infertility associated with uterine hypoplasia and ovarian hypofunction (Pietila *et al.* 1997). We found no statistically significant difference in the expression of *SAT1* between the pre-receptive and receptive state. However, as mentioned above, Borthwick *et al.* (2003) reported an increased expression of *SAT1* during the period of receptivity, though this was not confirmed by real-time RT-PCR. From our results, we speculate that the acquisition of receptivity does not require changes in the expression of *SAT1*.

ANXA1 was the only transcript presenting a statistically significant decrease in mean level after LNG while it showed a statistically significant increment from pre-receptive to receptive state. The mean level of *ANXA1* was 3.5-fold lower with LNG (range: -4.9 to -2.7 , $P=0.028$) than in the normal receptive state. Its mean level was 4.7-fold higher in the receptive compared with the pre-receptive state (range: -2.3 to $+11.1$, $P=0.04$) (Table 3). Thus, post-ovulatory administration of LNG partially prevented the increase in *ANXA1* level that takes place at the onset of receptivity. *ANXA1* inhibits phospholipases A2 (cPLA2 α or PLA2G4A; Wallner *et al.* 1986). It has been described that the inhibition of PLA2 decreases the release of arachidonic acid needed for the synthesis of prostaglandins (PGs) and leukotrienes by cyclooxygenases (COX) 1 and 2. In murine models, PGs are implicated in ovulation and implantation, and mice defective in *COX2* show multiple female reproductive failures, such as defective ovulation, fertilization, implantation, and decidualization (Lim *et al.* 1997). Specific COX2 inhibitors also prevent implantation (Shafiq *et al.* 2004). In addition, mice defective in cPLA2 α have an abnormal distribution of uterine embryo (Song *et al.* 2002), suggesting that these enzymes and PG production have a critical role in implantation. High levels of COX2 have been found in human endometrium during the receptive period and decreased expression was reported after post-ovulatory administration of mifepristone (Marions & Danielsson 1999) but not after the post-ovulatory administration of LNG (Marions *et al.* 2002). Microarray studies have established that PLA2 increases during the receptive period (Kao *et al.* 2002, Riesewijk *et al.* 2003) and also after the administration of mifepristone to explants of endometrium (Catalano *et al.* 2003). We speculate that following post-ovulatory administration of LNG, the increased level of *ANXA1* transcripts that normally occurs in the receptive period was still present, albeit less pronounced, and was associated with a 3.4-fold increase in the transcript level of *COX2* (Table 4) with no change in cPLA2 α (Table 5).

Additionally, we examined another group of six genes, *C4BP α* , *MAOA*, *C3*, *SERPINB9*, *S100A4*, and *COX2*, previously found in microarray analyses

mentioned above to be upregulated during the receptive period. To our knowledge, increased expression of *C4BP α* , *SERPINB9*, and *S100A4* had not been submitted to confirmation by an independent technique; real-time RT-PCR analysis of biopsies performed in LH+3 and LH+7 confirmed that the level of all these transcripts increase during the transition from non-receptive to receptive state. Our limited resources did not allow us to examine all transcripts that have been reported in previous studies to change at the onset of receptivity. In a separate study, we found that *PAEP*, *CLU*, *MAOA*, *C4BPA*, and *SERPINB9* have markedly decreased expression in women diagnosed with implantation failure of endometrial origin (Henriquez *et al.* 2006, Tapia *et al.* 2008, Tapia *et al.* 2011). *PAEP* and *C4BPA* expression is also diminished in women with impaired fertility associated with endometriosis (Kao *et al.* 2003). In this study, we found only minimal or no differences in the expression of these genes in the endometrium after post-ovulatory administration of LNG.

Finally, we found no change in five genes (Table 5) that previously have been shown to respond to mifepristone in the human endometrium (Catalano *et al.* 2003) and none of the genes that we found that change with LNG had been reported to change their expression after systemic mifepristone administration in mice (Bagchi *et al.* 2005). This may seem paradoxical considering that both LNG and mifepristone act through the same receptors, but eliciting opposite effects. Nevertheless, we think that in the experimental settings tested, mifepristone prevents the genomic actions of progesterone through its receptors, while decreasing serum levels of LNG in the early luteal phase, were probably overcome by concomitant increasing levels of endogenous progesterone. It is possible that progesterone receptors whose expression is greatly decreased during the luteal phase are saturated by the high concentration of circulating progesterone causing maximum response, while this situation is ideal to put in evidence the action of an antagonist, it is the least suitable in order to demonstrate agonistic activity. In addition, low doses of mifepristone administered in the luteal phase alter endometrial maturation (Greene *et al.* 1992, Marions *et al.* 1999) and other antiprogestins, such as onapristone produce the same pattern of endometrial alterations (Katkam *et al.* 1995) that will inhibit endometrial receptivity providing a basis of their development for endometrial contraception (Cameron *et al.* 1997) whereas no effects of post-ovulatory LNG administration on the histological features of the endometrium examined during the receptive phase were found (Durand *et al.* 2001, 2005, Marions *et al.* 2002). Furthermore, two independent studies have shown that emergency contraception with LNG given before ovulation prevents pregnancy in all treated

women and that it fails completely to prevent pregnancy when it is given after ovulation (Novikova *et al.* 2007, Noe *et al.* 2010), confirming the use of LNG as an effective contraceptive method only if it is taken before ovulation. Those two studies provide functional evidence that the few changes in gene expression described here are irrelevant for endometrial receptivity.

In summary, under the conditions studied, LNG caused minimal changes in transcripts levels and, considering their nature and magnitude, it is unlikely they would interfere with endometrial receptivity an assumption confirmed by the studies of Novikova *et al.* (2007) and Noe *et al.* (2010) cited above.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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