

# Similar endometrial development in oocyte donors treated with either high- or standard-dose GnRH antagonist compared to treatment with a GnRH agonist or in natural cycles

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**BACKGROUND:** This descriptive study evaluates the impact on endometrial development of standard and high doses of a GnRH antagonist in stimulated cycles compared with GnRH agonist and natural cycles. **METHODS:** Thirty-one oocyte donors were treated with a combination of rFSH and 0.25 mg/day ganirelix (standard dose), 2 mg/day ganirelix (high dose) or 0.6 mg/day buserelin (long protocol). Vaginal progesterone (200 mg/day) was administered in the luteal phase. Endometrial biopsies were performed 2 and 7 days after HCG administration. Additional biopsies were carried out in a subset of 12 subjects, 2 and 7 days following the LH peak of their previous natural cycle. Biopsies were evaluated histologically and by scanning electron microscopy. Gene expression profiles were also studied. **RESULTS:** At HCG +2, all the parameters studied were similar in all the groups and comparable to those observed in the natural cycle. At HCG +7, endometrial dating, steroid receptors and the presence of pinopodes were comparable in both GnRH antagonist groups and in the natural cycle. In buserelin group, endometrial dating and pinopode expression suggested an arrested endometrial development. For window of implantation genes, expression patterns were closer to those in the natural cycle following standard- or high-dose ganirelix than after buserelin administration. **CONCLUSION:** No relevant alteration was observed in the endometrial development in the early and mid-luteal phases in women undergoing controlled ovarian stimulation for oocyte donation following daily treatment with a standard- or high-dose GnRH antagonist. In addition, the endometrial development after GnRH antagonist mimics the natural endometrium more closely than after GnRH agonist.

*Key words:* endometrial receptivity/GnRH agonist/GnRH antagonist/natural cycles

## Introduction

The GnRH antagonists ganirelix and cetrorelix are now widely used for the prevention of a premature LH surge in women undergoing controlled ovarian stimulation (COS) for assisted reproduction techniques. During the initial studies it became evident that a comparable number of good quality embryos could be obtained if COS is combined with a short GnRH antagonist treatment instead of the traditional long protocol of GnRH agonist (Out and Mannaerts, 2002). However, a Cochrane review of the initial five randomized studies indicated a trend towards slightly lower implantation and pregnancy rates for the GnRH antagonist treatment group compared to those in the GnRH agonist group (Al-Inany and Aboulghar, 2002). Various theories have been put forth to explain the results but consensus has not yet been reached.

The dose-finding study (Devroey *et al.*, 1998), investigating the effects of six different dosages of ganirelix, showed a

dose-dependent decrease in implantation, with a complete absence of pregnancies at doses >1 mg/day, whereas no impact on the number of oocytes and good quality embryos was seen with higher doses. Follow-up of the frozen embryos obtained in this dose-finding study revealed that ongoing pregnancies were subsequently achieved in 11 patients, of which six were treated with a high ganirelix dose of 1.0 or 2.0 mg (Kol *et al.*, 1999). These data indicate that high GnRH antagonist dosages do not affect the potential of embryos to establish pregnancy and consequently suggest that the lower implantation and pregnancy rates in cycles in which the GnRH antagonist is started at fixed doses, on day 6 of stimulation, may originate from differences in endometrial receptivity.

Therefore, the present descriptive study was designed to evaluate the effect of both low- (0.25 mg) and high-dose (2.0 mg) GnRH antagonist as well as a GnRH agonist treatment on the

endometrial development in women undergoing COS for oocyte donation. For a subset of patients, endometrial development in a previous natural cycle was studied as a reference. Parameters used in this study as markers of endometrial receptivity were evaluation of endometrial thickness and pattern by ultrasound and endometrial biopsy assessments in terms of endometrial dating, estrogen and progesterone receptor expression and surface structure (pinopodes). In addition, gene expression profiles were investigated.

## Materials and methods

### Subjects

All subjects were selected and treated at the Instituto Valenciano de Infertilidad in Valencia, Spain. A total of 42 healthy women were screened and randomly assigned to one of three treatment groups (i.e. 14 subjects per treatment group) by means of a computer-generated randomization list. Eligible subjects were healthy women (age 18–35 years, body mass index 18–29 kg/m<sup>2</sup>) who had a regular menstrual cycle (range 24–35 days) and were undergoing COS for oocyte donation. Women with any endocrine abnormality or abnormal early follicular gonadotrophin values were not eligible.

Of the 42 subjects randomized, 31 subjects were considered evaluable (12 in the standard-dose ganirelix group, nine in the high-dose ganirelix group, and 10 in the buserelin group). Three patients did not start treatment for personal reasons; one stopped treatment prematurely because an exclusion criterion was violated; one stopped because of reasons unrelated to treatment outcome; four patients discontinued because of protocol violations; and one was not evaluable because only one endometrial biopsy was performed. For evaluation of the natural cycle, data on a subgroup of 12 subjects were available. Of these 12 patients, five were enrolled in the standard-dose ganirelix group, four in the high-dose ganirelix group, and three in the buserelin group in the subsequent cycle.

### Study design

This open-label, randomized, single-centre study was designed to evaluate the effect of two dose regimens of GnRH antagonist (standard dose and high dose) on the endometrial receptivity in women undergoing COS for oocyte donation; a long protocol of a GnRH agonist was used as a reference treatment. Ganirelix treatment (Orgalutran<sup>®</sup>; NV Organon, The Netherlands) was started on day 6 of recombinant FSH (rFSH) treatment and continued until and including the day of HCG administration. The ganirelix doses (0.25 or 2 mg) were administered s.c. in the thigh, once daily in the morning. The buserelin (Suprecur<sup>®</sup>; Hoechst, Germany) protocol started on day 21–24 of the preceding cycle. Buserelin was administered intranasally at a daily initial dosage of 0.6 mg/day (0.15 mg dosage, four times per day) until and including the day of HCG administration. If pituitary down-regulation was not achieved after 2 weeks (i.e. serum estradiol was <50 pg/ml or <200 pmol/l), the daily dose of buserelin was doubled to 1.2 mg (0.30 mg dosages, four times a day).

In all three groups, ovarian stimulation was initiated with a fixed dose of 150 IU rFSH (Puregon<sup>®</sup>; NV Organon, The Netherlands), injected s.c. once daily in the morning. After 5 days, this dosage of rFSH could be adjusted depending on the ovarian response as assessed by ultrasound. Stimulation with rFSH was started on day 2–3 of the menstrual cycle (ganirelix groups) or after achievement of pituitary down-regulation in the buserelin group (after 2–4 weeks of buserelin treatment). Treatment with rFSH was continued until the day that the criterion for giving HCG was reached. Administration of rFSH on the day of HCG was optional.

On the first day that three follicles  $\geq 17$  mm were measured by ultrasound, 10 000 IU of HCG (Pregnyl<sup>®</sup>; NV Organon, The Netherlands) was administered s.c. or i.m. Oocyte retrieval was performed by follicle puncture 30–36 h after HCG administration. Luteal phase support was given by daily progesterone (200 mg, vaginally), from day 2 to day 7 after HCG administration (day HCG +2 to day HCG +7).

All subjects gave written informed consent. The study, approved by the Ethics Committee of the study centre, was performed according to the Declaration of Helsinki, the International Conference on Harmonisation (ICH) guidelines and Good Clinical Practice.

### Assessments

Baseline characteristics were evaluated prior to the start of treatment, to exclude any abnormality. Endometrial development was assessed at day HCG +2 and day HCG +7 by vaginal ultrasonography (non-blinded) and by endometrial biopsy (blinded). Endometrial biopsy specimens were taken from the pars functionalis of the uterine fundus at day HCG +2 and day HCG +7 with a Pipelle<sup>®</sup> catheter (Genetics, Namont-Achel, Belgium) under sterile conditions. In some of the subjects, additional endometrial biopsy had been performed on day 2 and day 7 after the LH peak (day LH +2 and LH +7) of the previous (natural) cycle. Daily assessment of the urinary LH levels beginning cycle day 10 was performed in the clinic using a commercially available ovulation predictor kit (Donacheck ovulación; Novalab Ibérica, S.A.L., Coslada, Madrid, Spain) and the day of the urinary LH surge was considered as LH = 0. If sufficient tissue was available, the endometrial samples were divided into three parts. One part was fixed in formalin, embedded in paraffin, and used for the histological assessments (endometrial dating, steroid receptor expression). The second part was processed for scanning electron microscopy. The third part was frozen at  $-80^{\circ}\text{C}$  for RNA isolation and subsequent microarray hybridization.

The following parameters of endometrial development were evaluated.

#### *Endometrial thickness and pattern (ultrasound)*

All measurements were done at the fundus in the longitudinal plane, performed by the same gynaecologist. Endometrial pattern was evaluated and scored as 'multilayered', 'non-multilayered' or 'non-multilayered solid pattern'.

#### *Endometrial dating (histology)*

Microscopic sections were evaluated according to the criteria described by Noyes *et al.* (1950) by two assessors (CYTOPAT, Valencia, Spain). In cases in which the endometrium appeared as if the sample had been taken before day 2 post-ovulation, criteria according to Hendrickson and Kempson (1994) were used. If the two assessors produced discordant results, a meeting was organized in which the assessors reviewed the biopsies and discussed the outcome in order to reach consensus on the dating. Assessors were blinded for patient treatment and assessment number.

#### *Expression of estrogen and progesterone receptor protein (histology)*

Estrogen receptor ( $\alpha$ ) and progesterone receptor (A and B) proteins were stained by immunohistochemical staining methods (performed at the Pharmacology Department of NV Organon, Oss, The Netherlands). For each tissue compartment (i.e. glands, luminal epithelium, and stromal cells), the percentage of stained cells and the intensity of staining were scored by two assessors according to a semiquantitative scoring system: the percentage of stained cells was scored 0 (0–9%), 1 (10–39%), 2 (40–69%), 3 (70–89%) or 4 (90–100%) and the staining intensity was scored 0 (no staining), 1 (weak but definite staining), 2 (moderate staining), 3 (pronounced staining) or 4 (intense staining).

From these two parameters, a histological score (H-score) was calculated per tissue compartment according to the following formula:

$$H = \frac{\% \text{ staining (0 to 4)} * \text{intensity (0 to 4)}}{4}$$

The final score was calculated by taking the mean score of the two observers. If the two scores differed by >1.5, the observers re-evaluated the sample in order to reach consensus.

#### Endometrial pinopodes (scanning electron microscopy)

The samples were evaluated by a single assessor (Dr S.Adams) at the University of Sydney (Australia), using a final magnification of  $\times 5000$ . Ten random areas of endometrial surface were photographed and examined. Each area was assessed for the percentage of cells with developing, fully developed, and regressing pinopodes (Nikas, 1999). The assessor of electron microscopic sections was blinded for patient, treatment, and assessment number.

For evaluation of serum LH, FSH, estradiol ( $E_2$ ) and progesterone values, blood samples were taken during stimulation treatment (just before the drug administration of that particular day) and on the days that endometrial biopsies were performed. These samples were analysed by a central laboratory using a time-resolved fluoroimmunoassay (DELFLIA; Wallac Oy, Turku, Finland).

Other parameters assessed were number of rFSH, buserelin, and ganirelix treatment days; total dose of rFSH; number of serum LH and progesterone rises ( $LH \geq 10$  IU/l; progesterone  $\geq 1$  ng/ml); and number of follicles on day of HCG injection.

#### RNA isolation and microarray hybridization

Total RNA was extracted using TRIzol<sup>®</sup> reagents according to the manufacturer's recommendation (Life Technologies, Inc., USA) from specimens taken on day HCG +7 from five patients who had received standard-dose ganirelix, four patients who had received high-dose ganirelix, and five patients who had received buserelin. Biotin-labelled cRNA probes were hybridized onto the GeneChip HG\_U133A (Affymetrix, High Wycombe, UK) at the Organon Gene Chip Platform in Newhouse (UK), as described by Horcajadas *et al.* (2005). Gene expression profiles of the study samples were compared with profiles from samples that were taken at day LH +2 and LH +7 of the natural cycle. These samples were processed as described in Riesewijk *et al.* (2003). All data were normalized within Rosetta Resolver.

As described by Horcajadas *et al.* (2005), Spotfire DecisionSite 7.2 (Spotfire, Göteborg, Sweden) was used to perform principal component analysis to make a general comparison of the expression profiles of the different samples. To identify significant changes in expression levels between sample sets, a one-way ANOVA with build ratio was

calculated using the values from day LH +7 in natural cycles as a baseline. To select regulated genes, criteria as described by Horcajadas *et al.* (2005) were used. To confirm the differential expression, real-time quantitative polymerase chain reaction (Q-PCR) was performed for a subset of regulated genes according to the method described by Horcajadas *et al.* (2005).

#### Statistical methods

This study evaluated the endometrial effects of two ganirelix treatments versus the traditional long protocol of buserelin during rFSH stimulation treatment in oocyte donors. The endometrial development during the natural cycle was used as a reference. The main aim of this trial was not to show superiority of either treatment but only to perform a descriptive evaluation of endometrial maturation after different controlled ovarian stimulation regimens using several available techniques: ultrasound, hormonal measurements, histological dating, steroid receptors, scanning electron microscopy and gene arrays. This implies that it is not the statistical power which is of interest, but rather the precision of the estimates. With 10 evaluable subjects in each treatment group, the endometrial date can be estimated with a precision (SE) of  $-0.5$  days (assuming that the SD is 3 days).

Only the subjects who were treated according to the protocol and who underwent biopsy on day HCG +2 and day HCG +7 were considered for evaluation. Evaluation of normal endometrial development during the natural cycle was based on data from a subgroup of subjects who participated in a natural cycle prior to the treatment cycle and had biopsies on day LH +2 and LH +7. For all parameters, summary statistics were presented and no formal statistical testing was performed.

## Results

### Subject characteristics

Demographic characteristics for the evaluable subjects were similar in all treatment groups. The mean age ranged from 24.6 to 26.0 years, mean weight from 55.2 to 60.4 kg and mean body mass index from 21.1 to 23.1 kg/m<sup>2</sup>. On average, the median cycle length was comparable, ranging from 28.5 to 30.1 days.

### Treatment and stimulation characteristics

Table I presents an overview of the treatment and stimulation characteristics and present median values and ranges. The mean number of GnRH analogue treatment days was 5.7 (standard-dose ganirelix), 5.1 (high-dose ganirelix), and 22.7 (buserelin). None of the subjects needed an increased buserelin dosage. The mean number of rFSH treatment days and the total rFSH dose were 9.8 and 1713 IU (standard-dose ganirelix), 9.4 and

**Table 1.** Treatment and stimulation characteristics (31 evaluable subjects)

	Ganirelix 0.25 mg/day	Ganirelix 2 mg/day	Buserelin long protocol
GnRH analogue duration (days) <sup>a</sup>	6 (3–9)	5 (2–7)	23 (15–31)
rFSH duration (days) <sup>a</sup>	10 (7–13)	10 (7–11)	9 (7–12)
Total rFSH dose (IU) <sup>a</sup>	1425 (1050–3150)	1500 (950–1750)	1350 (1050–1800)
Follicles on the day of HCG <sup>b</sup>			
≥11 mm	13	15	14
≥15 mm	9	11	10
≥17 mm	7	7	7

<sup>a</sup>Values are median (range).

<sup>b</sup>Values are median.

1467 IU (high-dose ganirelix), and 8.9 and 1335 IU (buserelin) respectively. On the day of HCG, there was a mean of 14.3, 16.3 and 13.4 follicles  $\geq 11$  mm; 9.7, 9.6 and 9.6 follicles  $\geq 15$  mm; and 7.3, 6.4 and 6.5 follicles  $\geq 17$  mm in the standard-dose ganirelix, high-dose ganirelix and buserelin groups respectively.

### Serum hormone profiles

Figure 1 shows the median serum LH, FSH,  $E_2$  and progesterone values during ovarian stimulation and in the luteal phase. Serum LH values decreased during stimulation in both ganirelix groups. In the buserelin group, LH values were already low at the start of rFSH stimulation and remained low during ovarian stimulation. On the day of HCG, LH values were highest in the buserelin group. In the luteal phase, LH decreased to very low levels in all three treatment groups. In the high-dose ganirelix group, one LH rise ( $\geq 10$  IU/l) occurred prior to the first ganirelix injection. During ganirelix treatment, no LH rises occurred. During buserelin treatment, four subjects had an LH rise, of which three occurred on the day of HCG with values of between 10.0 and 11.0 IU/l. All five LH rises observed were associated with a concomitant progesterone rise ( $\geq 3.2$  nmol/l).

Similarly, serum FSH values started at a lower median level in the buserelin group. Despite the increase from day 1 onwards, as a result of the rFSH administration, serum FSH levels remained lower in the buserelin group than in either ganirelix group. During the luteal phase, serum FSH levels decreased comparably in all three treatment groups.

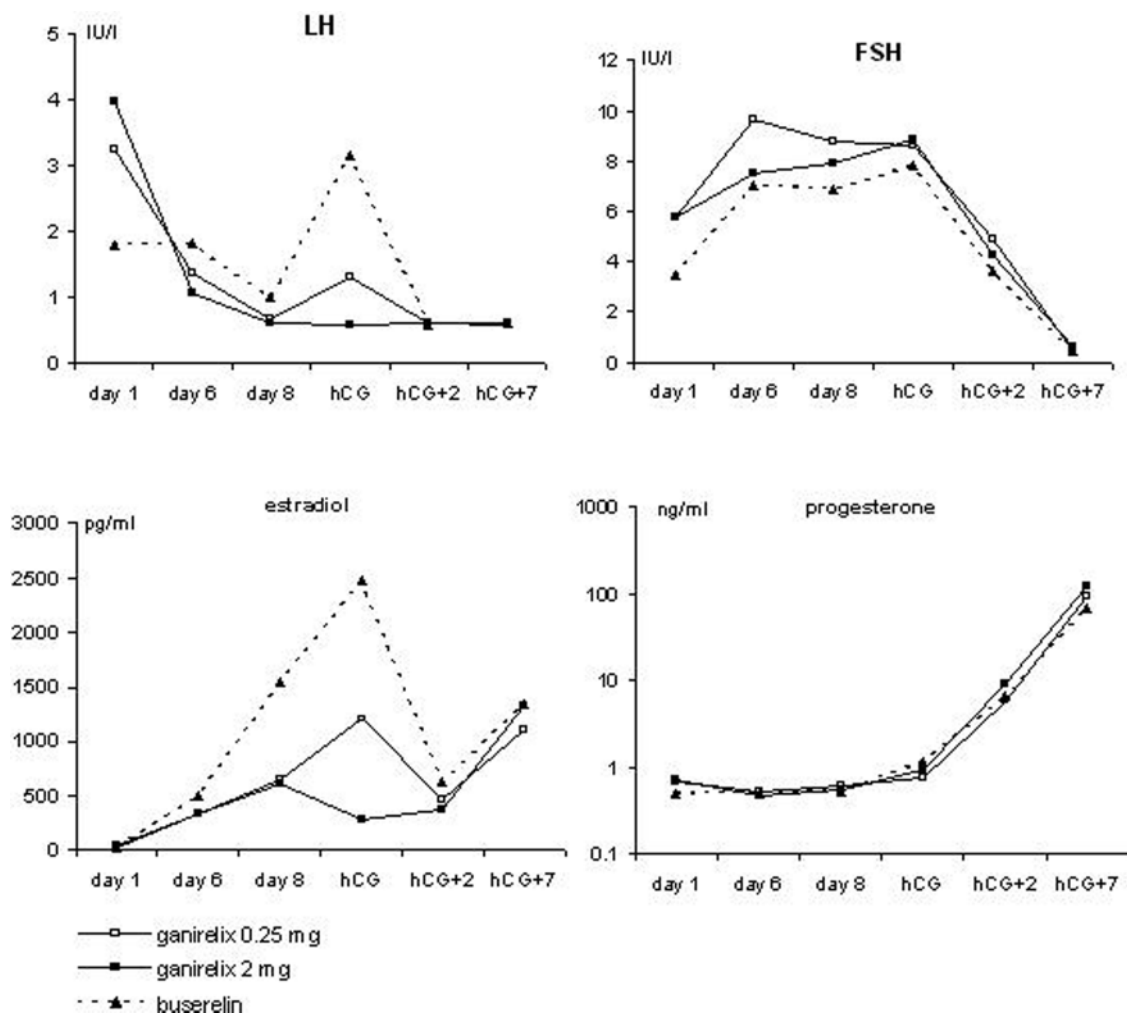
Serum  $E_2$  levels were equally low in all treatment groups at the start of stimulation. From day 8 until the day of HCG injection,  $E_2$  levels were highest in the buserelin group. The  $E_2$  levels were equally low in all three treatment groups on day HCG +2 and with a similar increase afterwards.

In all treatment groups, serum progesterone values remained low from the start of stimulation up to and including the day of HCG. After HCG injection, serum progesterone values showed a comparable increase in all three treatment groups as a result of the progesterone treatment given for luteal support.

### Endometrial development

#### Endometrial thickness and pattern (ultrasound)

No relevant difference in endometrial thickness was observed between the three treatment groups: on day HCG +2, median values were 9 mm in the standard-dose ganirelix group and



**Figure 1.** Median serum hormone values on days 1, 6 and 8 of stimulation; on (or just before) the day of HCG; and during the luteal phase [i.e. 2 and 7 days after HCG injection (restricted to evaluable subjects with  $\geq 8$  days of rFSH treatment)].

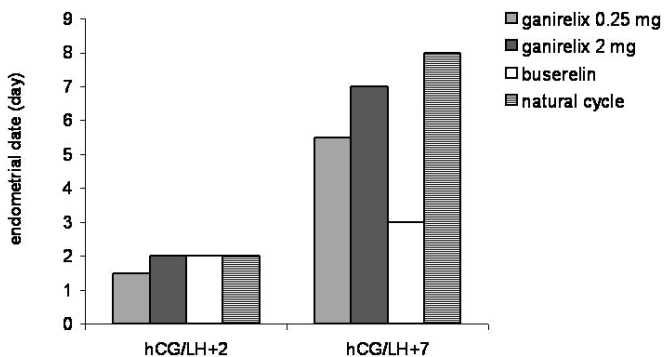
buserelin group and 11 mm in the high-dose ganirelix group; mean values ranged from 9.4 to 10.3 mm. On day HCG +7, the median value was 11 mm in all three treatment groups; the range was 7–13 mm for standard-dose ganirelix, 9–17 mm for high-dose ganirelix, and 6–14 mm for buserelin. On both days, the highest mean values were measured in the high-dose ganirelix group.

On day HCG +2, ultrasonography of the endometrium in most subjects showed a multilayered pattern: 58% for the subjects in the standard-dosage ganirelix group, 56% of those in the high-dosage ganirelix group, and 80% of those in the buserelin group. In two subjects (one in each ganirelix group: 8.3 and 11.1% respectively) a non-multilayered solid pattern was seen. On day HCG +7, the endometrium showed a non-multilayered (solid) pattern in all subjects. In the ganirelix groups, most of these samples showed a solid pattern (92 and 89% in the standard- and high-dose groups respectively), compared to 60% in the buserelin group.

### Endometrial dating (histology)

Figure 2A shows the median endometrial dates scored on day HCG +2 and HCG +7 (treatment cycles) and day LH +2 and LH +7 (natural cycles). On day HCG +2 (or day LH +2 in natural cycles), the median (mean) endometrial dates (i.e. post-ovulation day number) were 1.5 (0.1) for standard-dose ganirelix, 2 (1.8) for high-dose ganirelix, 2 (1.4) for buserelin, and 2 (0.9) in the natural cycles. On day HCG +7, the median (mean) endometrial dates were 5.5 (5.8) for standard-dose ganirelix and 7 (6.9) for high-dose ganirelix; these values approach those observed on day LH +7 in the natural cycles: 8 (7.3). In the buserelin group, a lower median (mean) endometrial date of 3 (3.0) was scored on that day.

Figure 2B shows the individual scores of endometrial dates. Although there is considerable inter-individual variation, the shifts from day HCG +2 to day HCG +7 observed in both ganirelix groups are comparable to the shifts seen in the natural cycle. The shifts observed in the buserelin group appear to be smaller. Figure 2B also shows that the very low score (−6) in two subjects from the standard-dose ganirelix group on day 2 after HCG accounts for the lower mean endometrial date on that day.



**Figure 2.** (A) Median endometrial dating (days) according to Noyes' criteria evaluated on day 2 and 7 after HCG injection in treatment cycles (31 subjects) or after LH peak in natural cycles (12 subjects).

### Estrogen and progesterone receptor expression (histology)

Figure 3 summarizes the H-scores, which express the staining percentage and staining intensity for estrogen and progesterone receptor proteins.

On day HCG +2, estrogen receptor expression was comparable in all three treatment groups, being highest in the glandular tissue and lowest in the stromal tissue. The mean H-scores for estrogen receptor expression ranged from 1.3 (standard-dose ganirelix) to 1.7 (buserelin) in glandular tissue and from 1.1 (both ganirelix groups) to 1.4 (buserelin) in luminal tissue. Almost no estrogen receptor expression was observed in the stromal tissue of treatment groups (mean H-score was 0.2 in all treatment groups). In the natural cycle group, the mean H-scores for estrogen receptor expression were also highest in glandular and luminal tissue (1.9 and 1.2 respectively) and lowest in the stromal tissue (0.9), though the latter was higher than in the treatment groups.

On day 7 (HCG +7 or LH +7), all groups showed a decreased estrogen receptor expression in luminal and glandular tissue compared to day 2 values. Like day 2 values, the estrogen receptor expression on day 7 in the stromal tissue was extremely low in the treatment groups (mean H-score of 0.2 to 0.3), and again lower than in the natural cycle (mean H-score of 0.6).

On day HCG +2, progesterone receptor expression was highest in the glandular tissue in all treatment groups (mean H-scores ranged from 1.3 to 1.9) and in the natural cycle group (mean H-score of 1.7). For the luminal and stromal tissue, similar H-scores were observed in all treatment groups and in the natural cycle group (mean values ranging from 0.7 to 1.3).

On day HCG +7, little or no progesterone receptor expression (mean H-score  $\leq 0.2$ ) was observed in any of the tissue types for all treatment groups. In the natural group, the progesterone receptor expression reached a mean H-score of 0.5 in the stromal tissue only.

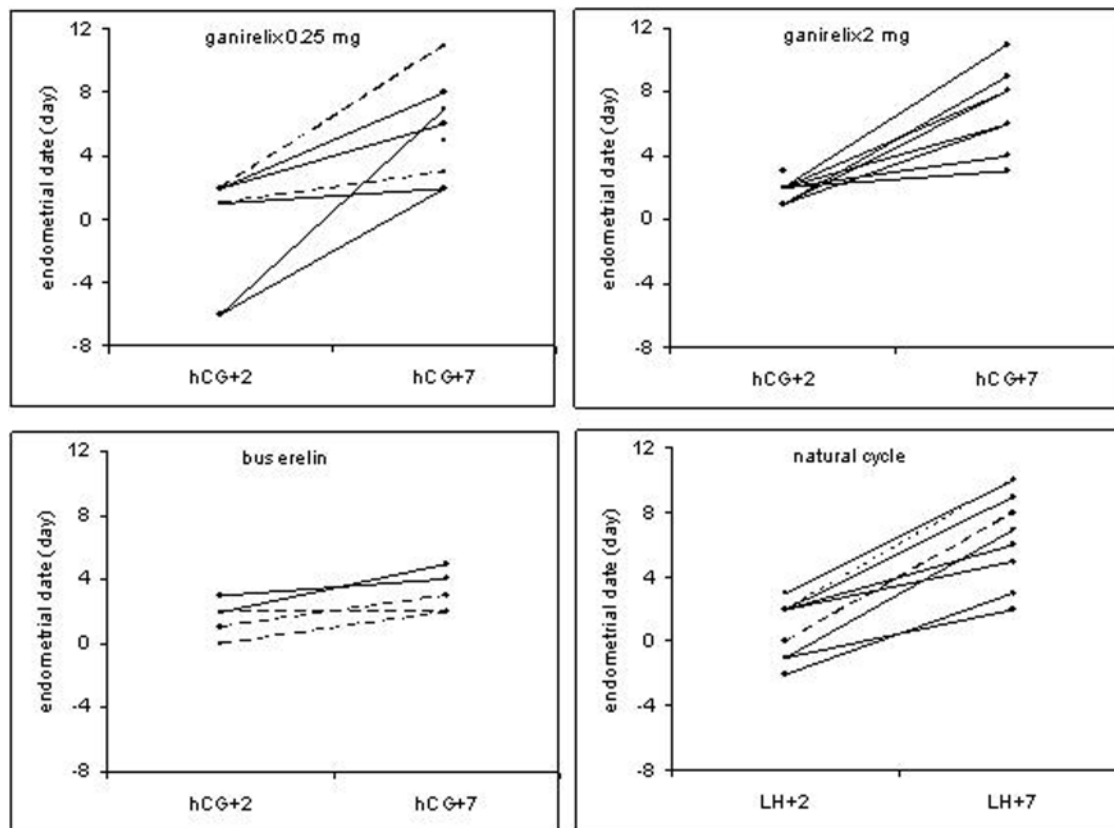
### Pinopode appearance (scanning electron microscopy)

On day HCG +2, no pinopodes at any stage of development were observed in any specimens from any of the treatment groups; however, a mean of 4.4% of cells from the natural cycle showed pinopodes (developing, developed, or regressing) at day LH +2. On day 7 after HCG/LH, the mean percentage of cells with any type of pinopodes was 6.0% for standard-dose ganirelix, 6.6% for high-dose ganirelix, and 5.9% for the natural cycles but only 2.6% for buserelin. Pinopodes are visible in Figure 4, which shows a representative specimen from the ganirelix high-dose group.

### Gene expression

Principal component analysis revealed limited variation between the natural cycle and the ganirelix regimens or buserelin regimen for day LH +7 or HCG +7 respectively (see Figure 5), whereas the natural cycle samples for day LH +2 can be clearly distinguished as a separate group.

For each of the treatment regimens, a similar number of genes was differentially expressed (defined as a  $\geq 100\%$  increase or  $\geq 50\%$  decrease in expression) between treated and natural cycles: 91 genes for the standard-dose ganirelix regimen,



**Figure 2. (B)** Individual endometrial dating (days) according to Noyes' criteria evaluated on day 2 and 7 after HCG injection in treatment cycles (31 subjects) or after LH peak in natural cycles (12 subjects; straight lines represent a single case, interrupted lines represent two cases, dotted lines represent three cases).

112 genes for the high-dose ganirelix regimen, and 122 genes for the buserelin regimen. Previous research had identified 1398 genes that are differentially expressed within the window of implantation (between day LH +2 and LH +7) and are therefore potentially important for the implantation process and endometrial receptivity (Horcajadas *et al.*, 2005). Of the genes with differential expression between treated and natural cycles, 50 (55%) of the 91 genes in the standard-dose ganirelix specimens, 23 (21%) of the 112 genes in the high-dose ganirelix specimens, and 85 (70%) of the 122 genes in the buserelin specimens belong to the window of implantation group (see Table II).

Regulation of a subset of genes was confirmed by Q-PCR (see Figure 6). The regulation observed in the microarray and the Q-PCR is consistent, although the level of regulation varied between methods. For four genes in the subset that were investigated by Q-PCR, samples from the ganirelix group resembled samples from the natural cycle more closely than did samples from the buserelin group.

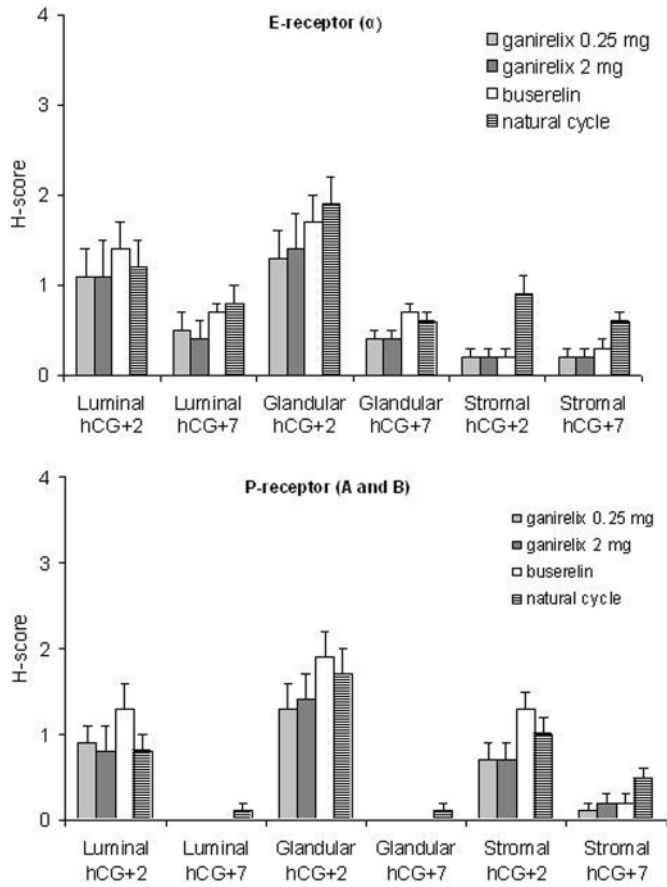
#### Outcome of oocyte donation

In total, 12 subjects in the standard-dose ganirelix group, nine subjects in the high-dose ganirelix group, and 10 subjects in the buserelin group donated oocytes to 18, 17 and 16 recipients respectively. Embryo transfer could not be performed in five subjects (two in the standard-dose ganirelix group and three in

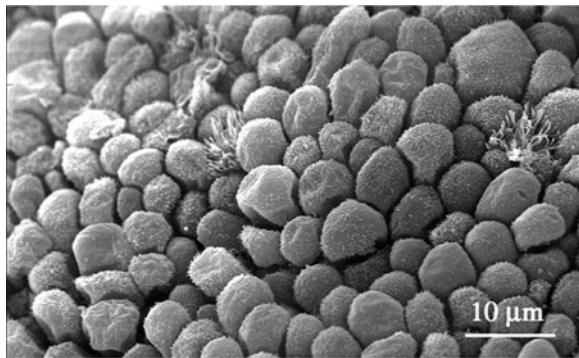
the high-dose ganirelix) because of embryo–endometrial asynchrony, whereas poor embryo quality prohibited embryo transfer in another three subjects (two in the high-dose ganirelix group and one in the buserelin group). Eventually, pregnancy was achieved in nine, six and seven women who received oocytes from the standard-dose ganirelix, high-dose ganirelix, and buserelin group respectively (pregnancy rate per cycle of 50, 35 and 44% and a pregnancy rate per transfer of 56, 50 and 47% in the oocyte recipients).

#### Discussion

A precondition for successful establishment of pregnancy is that the endometrium must be receptive for implantation of the blastocyst. The transient period of endometrial receptivity, called the 'implantation window', is thought to span ~6 days, i.e. from day 19 to 24 of the menstrual cycle or from 5 to 10 days after ovulation (Navot *et al.*, 1991; Wilcox *et al.*, 1999). This implantation window might be shifted after controlled ovarian stimulation, as advanced endometrial development was observed after ovarian stimulation with either a GnRH agonist (Ubaldi *et al.*, 1997) or a GnRH antagonist (Kolibanakis *et al.*, 2002). Specifically, these trials show that when the endometrium is advanced by >2 days as compared to the chronological date, the chance of achieving a pregnancy is substantially reduced.

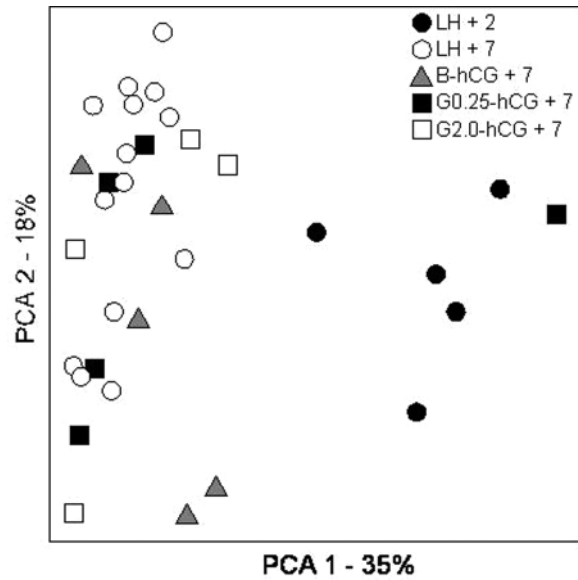


**Figure 3.** Estrogen receptor ( $\alpha$ ) and progesterone receptor (A and B) protein expression; mean H-scores ( $\pm$  SEM) for luminal epithelium, glandular, and stromal tissue on day 2 and 7 after HCG injection in treatment cycles (31 subjects) or after LH peak in natural cycles (12 subjects).



**Figure 4.** This scanning electron micrograph of a sample taken on day 7 after HCG administration from an oocyte donor in the ganirelix 2 mg group shows that the high-dose ganirelix treatment can produce the required epithelial response.

In the current descriptive trial, the impact of standard (0.25 mg/day) and high-dose (2 mg/day) GnRH antagonist treatment on the endometrial development in the early and mid-luteal phase of oocyte donors is further investigated. As there is no unequivocal marker of receptivity, several common markers of endometrial development were evaluated at day LH +2 and LH +7 (natural cycles) or HCG +2 and HCG +7 (treatment cycles),



**Figure 5.** Outcome of Principal Component Analysis (PCA) analyses of 33 endometrial samples using 500 randomly selected genes. Percentage of variation for the PCA1 and PCA2 axis are 35 and 18% respectively. Closed circles: LH +2 samples; open circles: LH +7 samples; triangles: buserelin HCG +7 samples; closed squares: ganirelix standard dose HCG +7 samples; open squares ganirelix high dose HCG +7 samples.

representing pre-receptive and receptive endometrium, respectively. The endometrial development in natural cycles and in GnRH agonist (long protocol) treatment cycles was used as a reference. To reflect clinical practice, progesterone supplementation was given in the luteal phase of the treatment cycles until the last biopsy was performed. No clear differences in endometrial characteristics were found between the standard and the high-dose GnRH antagonist regimen groups. In comparison with the GnRH agonist regimen, both GnRH antagonist regimens were associated with endometrial development that more closely resembled that found in the natural (unstimulated) cycle.

Treatment and stimulation characteristics observed in this study correspond to most of the results reported in other studies that compared ganirelix treatment with GnRH agonist treatment in COS (Out and Mannaerts, 2002): the use of either ganirelix treatment (as compared with buserelin treatment) resulted in a considerably reduced duration of GnRH analogue treatment (by ~2.5 weeks), higher serum LH and FSH values at the start of ovarian stimulation (no down-regulation), and lower serum  $E_2$  values at the end of stimulation. The duration of ovarian stimulation and total amount of rFSH administered were comparable among the three treatment groups investigated, and no distinct differences were observed in the number of follicles on the day of HCG administration. The relatively high LH serum values observed in the buserelin group on the day of HCG administration are due to the LH rises on this day. To check the impact of these (relatively low) LH rises on endometrial development, an additional evaluation was carried out excluding these subjects. This evaluation revealed no differences as compared to the evaluation which included all evaluable subjects (data not shown).

**Table 11.** Number of genes with differential regulation between the natural cycle (day LH +7) and the treatment regimens (day HCG +7) and within the window of implantation for the three treatment regimens<sup>a</sup>

Regimen/direction of regulation <sup>b</sup>	No. of genes	Window of implantation genes	
		Typically up-regulated <sup>c</sup> (n = 894)	Typically down-regulated <sup>c</sup> (n = 504)
Gonirelix 0.25 mg/day			
Up	22	0	4
Down	69	46	0
Gonirelix 2 mg/day			
Up	88	0	7
Down	24	15	1
Buserelin long protocol			
Up	22	3	4
Down	100	76	2

<sup>a</sup>Differential regulation was defined as a  $\geq 100\%$  increase or  $\geq 50\%$  decrease in expression.

<sup>b</sup>Compared to values on day LH +7 of natural cycle.

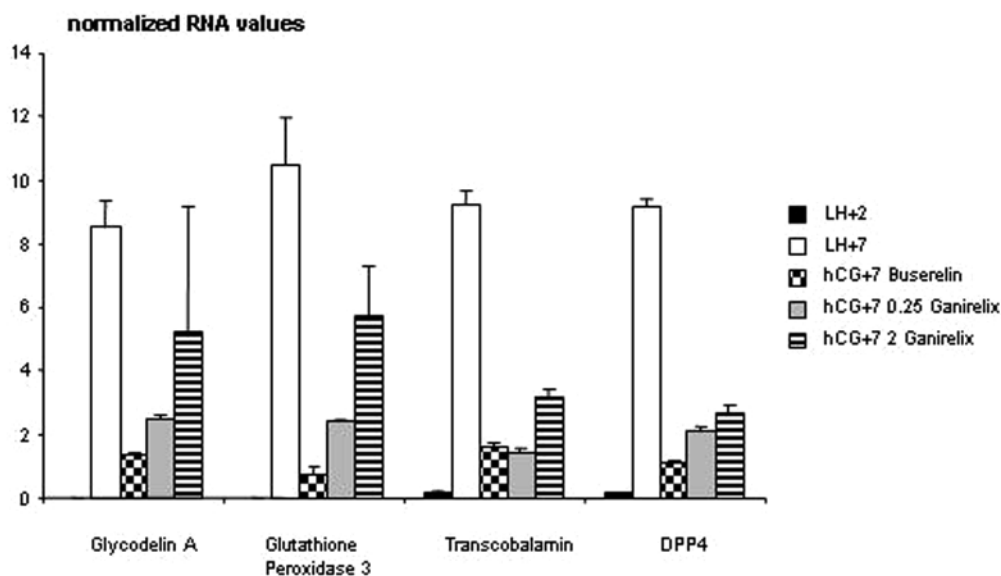
<sup>c</sup>Genes whose expression is typically up-regulated or typically down-regulated during the window of implantation (day 2–7 after the LH surge), according to Horcajadas *et al.* (2005).

Panel A

	Average Expression Values				
	Natural cycle		Buserelin	Gonirelix 0.25 mg/day	Gonirelix 2.0 mg/day
	LH+2	LH+7	hCG+7	hCG+7	hCG+7
Day					
Glycodelin A	64	5002	2733	3199	4694
Glutathione peroxidase 3	134	3945	1959	2387	3574
Transcobalamin	41	1085	148	138	297
DPP4	-34*	409	5	-9*	41

\* Negative values indicate absence of expression of this gene as determined by microarray

Panel B

**Figure 6.** Comparison of the microarray (A) and the quantitative PCR (B) data for four selected genes on day 2 and day 7 after the LH surge in natural cycles and day 7 after HCG administration in stimulated cycles.



Ultrasound evaluations of the endometrium in the luteal phase did not find any relevant differences between the three treatment groups. In all subjects, the endometrial thickness was  $\geq 7$  mm (ganirelix groups) or  $\geq 6$  mm (buserelin group) on day HCG +7. Thus, all of the subjects exceeded the threshold (5 mm endometrial thickness) considered necessary to permit implantation (Friedler *et al.*, 1996). On day HCG +2, a multi-layered endometrial pattern was observed in nearly 60% (ganirelix groups) and 80% (buserelin) of the subjects, whereas the endometrial biopsies of all subjects showed a non-multilayered (solid) pattern on day HCG +7. Although a multilayered pattern on the day of HCG or oocyte retrieval has been associated more frequently with a conception cycle (Gonen and Casper, 1990; Sharara *et al.*, 1999), the predictive value of endometrial assessments by ultrasound is considered to be very low (Friedler *et al.*, 1996; Leibovitz *et al.*, 1999).

In the natural cycles, timing of the endometrial biopsy was related to day of detection of LH in urine using a commercial ovulation prediction kit, whereas this was related to the day of injection of HCG in the stimulated cycles. This difference might result in a maximum deviation of  $\sim 1$  day in timing of the biopsy of the natural cycles versus the stimulated cycles (Ghazzeeri *et al.*, 2000), and may have caused additional variation in the natural cycle group.

Results of endometrial dating according to histological criteria indicate a normal endometrial development in the early luteal phase (day HCG +2) in all treatment groups as compared to the natural cycle data. However, biopsies from the mid-luteal phase (day HCG +7) showed a slightly delayed or normal endometrium (as compared with that in the natural cycle) in both ganirelix groups and a delayed endometrium in the buserelin group. Comparison of these results with other published studies is complicated because different stimulation protocols are used, luteal phase support is missing, or biopsies are performed at different time points. Nevertheless, the general trend observed in the literature—an advanced endometrium is observed just before or at oocyte retrieval, but ‘in phase’ endometrium is observed in the early luteal phase (Bourgain and Devroey, 2003)—is somewhat different from the results in the current trial.

The expression of estrogen and progesterone receptors in all treatment groups roughly followed the pattern of the natural cycle studied in this trial (i.e. a decline in steroid receptor staining in the course of the luteal phase regardless of whether luminal, glandular or stromal tissue was inspected). This is in line with other published data that showed that in natural cycles, endometrial estrogen and progesterone receptors were found to be maximally expressed in the peri-ovulatory and early luteal phase and to be suppressed toward the mid-luteal phase (Tamaya *et al.*, 1986; Garcia *et al.*, 1988).

Pinopodes, which are flower-like extrusions of the apical epithelial cell membrane, can be observed with scanning electron microscopy in the middle of the implantation window, i.e. around day 6–8 after ovulation (Nikas, 1999). The expression of fully developed pinopodes, which is limited to  $<48$  h, is strongly correlated with implantation following embryo transfer (Nikas, 1999). In the present study, endometrial biopsies of both ganirelix groups and the natural cycle group showed a similar, albeit low, expression of pinopodes on day HCG +7. In the buserelin

group, a less pronounced presence of pinopodes was observed on that day. In other studies of endometrial maturation in COS cycles, an accelerated appearance of pinopodes was observed, which is indicative of an advanced endometrial maturation (Kolb and Paulson, 1997; Develioglu *et al.*, 1999; Nikas *et al.*, 1999). Because high pre-ovulatory progesterone serum values ( $>6$  ng/ml) strongly correlated with advanced endometrial maturation, the absence of endometrial advancement in the treatment groups of the present study may be explained by the relatively low progesterone values on the day of HCG injection (Figure 2).

Gene expression profiles of the three different treatment groups were largely comparable to that of the natural cycle. In each of the treatment groups, expression of  $\sim 100$  genes was different from that in the natural cycle. When specifically investigating for genes whose expression is regulated during the window of implantation (WOI genes), more genes were differentially expressed compared to the natural cycle in the buserelin group than in either the low- or the high-dose ganirelix groups. This suggests that the expression profile of WOI genes is closer to the natural cycle profile in the ganirelix groups than in the buserelin group. The microarray data are therefore in good agreement with the results of the morphological and histological parameters tested.

Besides the evaluation of the endometrium in oocyte donors, the clinical outcome in the oocyte recipients was evaluated. Reported pregnancy rates were good and comparable between the treatment groups, further supporting Kol *et al.*'s findings that the high ganirelix doses in the dose-finding study had no detrimental effect on oocyte or embryo quality. In addition, the current trial does not reveal any differences between the standard- and high-dose ganirelix for the most common endometrial markers, suggesting that relatively high exposure to GnRH antagonist during stimulation does not significantly affect endometrial development. Thus, the low implantation and low pregnancy rates in the higher dose groups in the ganirelix dose-finding study seems to remain unexplained by this study. Thus, the low implantation and low pregnancy rates in the higher dose groups of the ganirelix dose-finding study are unlikely to be related to direct or indirect effects of the GnRH antagonist on the endometrium. One may speculate on other possible factors that could affect the implantation potential of transferred embryos, such as embryo exposure to remaining levels of GnRH antagonist in the high dose groups (Casan *et al.*, 1999; Raga *et al.*, 1999), or the impact of too low endogenous LH at the end of the follicular phase in a GnRH antagonist protocol. Interestingly, retrospective analysis of endogenous LH during GnRH antagonist treatment seems to favour low LH levels (Kolibianakis *et al.*, 2004) or shows no effect (Merviel *et al.*, 2004). Clearly, the current study did not address those questions and additional prospective controlled trials will be essential to substantiate the most likely explanation for the outcome of the GnRH antagonist dose-finding trial. A possible difference between the GnRH antagonist protocol used in the current study and that used in previous Phase III trials was the application of strict and early criteria for HCG, which has been shown to affect the probability of pregnancy (Kolibianakis *et al.*, 2004). By applying these strict criteria, estradiol levels, and probably also progesterone values, might not rise as much as compared to when HCG is given at a later stage. Applying less strict criteria for

HCG might consequently result in a further advanced endometrium. Nevertheless, for the current trial, the criteria used in the three treatment arms were comparable, meaning that this will not have an impact on the comparison performed.

In conclusion, the endometrial development in the early and mid-luteal phase revealed no relevant difference after daily treatment with standard-dose (0.25 mg/day) or high-dose (2 mg/day) GnRH antagonist in women undergoing COS for oocyte donation. In comparison with the buserelin regimen, the ganirelix regimens were associated with endometrial development that more closely resembled that in natural (unstimulated) cycles.

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