Inhibition of ovulation by administration of estetrol in combination with drospirenone or levonorgestrel: Results of a phase II dose-finding pilot study

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ABSTRACT

Objectives The aim of the study was to evaluate the efficacy of different dosages of estetrol (E_4) combined with one of two progestins in suppressing the pituitary–ovarian axis and ovulation in healthy premenopausal women.

Methods This was an open, parallel, phase II, dose-finding, pilot study performed in healthy women aged 18 to 35 years with a documented ovulatory cycle before treatment. For three consecutive cycles in a 24/4-day regimen, participants received 5 mg or 10 mg E_4/3 mg drospirenone (DRSP); 5 mg, 10 mg or 20 mg E_4/150 μg levonorgestrel; or 20 μg ethinylestradiol (EE)/3 mg DRSP as comparator. Pituitary–ovarian axis activity and the occurrence of ovulation were evaluated by monitoring follicular size, serum levels of follicle-stimulating hormone, luteinising hormone, estradiol and progesterone during treatment cycles 1 and 3. Endometrial thickness was evaluated throughout the trial, and the return of ovulation was evaluated after the last intake of medication.

Results A total of 109 women were included in the trial. No ovulation occurred in any treatment group. Ovarian activity inhibition seemed proportional to the E_4 dosage: the highest suppression was observed in the 20 mg E_4 group and was very similar to that observed with EE/DRSP. Endometrial thickness was suppressed to the same extent in all groups. Post-treatment ovulation occurred in all participants between 17 and 21 days after the last active treatment. The study combinations were well tolerated and safe.

Conclusions Combined with a progestin, E_4 adequately suppresses ovarian activity, particularly when given at a dosage above 10 mg/day.

KEYWORDS Estetrol; Estrogen; Oral contraception; Ovulation inhibition; Progestin

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INTRODUCTION

Estetrol (E_4) is a naturally occurring estrogen discovered in 1965[1]. E_4 is produced exclusively and in large amounts by the human fetal liver. E_4 has a relatively low affinity for the estrogen receptor (ER), but this is largely compensated by its high oral bioavailability (80% in contrast to 1% for estradiol [E_2]) and a long half-life of approximately 28 h (in contrast to 3.6 h for E_2). It binds to both ERα and ERβ, with a four- to fivefold preference for ERα. After its initial discovery, research on E_4 was performed for approximately 20 years in unsuccessful attempts to discover its function or to correlate its maternal plasma levels with fetal well-being. Thereafter, scientific interest in the hormone declined. In recent years, preclinical and clinical therapeutic studies have shown that E_4 might be an effective drug for several indications, including contraception, as it was notably shown to inhibit ovulation in cycling rats in a dose-dependent manner[2,3].

Some evidence suggests that E_4 may be suitable as a daily oral contraceptive and has several benefits in comparison with the currently available estrogen. Most marketed combined oral contraceptives (COCs) contain the potent synthetic estrogen ethinylestradiol (EE). EE has been shown to be safe but causes subjective side effects and increases the hepatic production of several coagulation factors, resulting in a prothrombotic status[4]. The most serious adverse effects of EE are cardiovascular complications, both arterial and venous, and in particular an increased risk of venous thromboembolism (VTE)[5,6]. These cardiovascular complications are rare but serious, especially when they occur in young, healthy women. The risk of VTE has been reduced by decreasing the EE dosage in COCs and it could also be lowered by replacing EE with the natural estrogen E_2. There are currently two COCs on the market that contain E_2 instead of EE: a sequential COC containing estradiol valerate (E_2V) and dienogest (DNG) and a monophasic COC containing E_2 and nomegestrol acetate. Recent epidemiological data suggest that the risk of VTE for users of COCs containing E_2V and DNG is similar to that for users of COCs containing second-generation progestins[7]. Because E_4 has minimal impact on the hepatic production of coagulation factors, it is hypothesised that the VTE risk will also be reduced by using the natural estrogen E_4 instead of EE [Kluft C, *et al.*, submitted].

In addition, in contrast to EE or E_2, E_4 does not inhibit the cytochrome P450 enzymes and should consequently not interfere with the metabolism of other drugs[8]. It is excreted in the urine as inactive sulfo- and glucurono-conjugates that do not interfere with the biliary system and therefore would not increase the incidence of gallbladder diseases as do classical COCs[9]. E_4 metabolism has not been shown to produce active metabolites, in contrast to E_2, whose metabolism leads to the production of carcinogenic catechol estrogen metabolites[10]. Finally, recent clinical and experimental *in vitro* and animal studies demonstrate a minimal impact of E_4 on normal and malignant breast cells[11–13].

The present study was performed to investigate the effects of different doses of E_4 in combination with two different progestins, drospirenone (DRSP) and levonorgestrel (LNG), on ovarian follicular activity and ovulation, in comparison to the registered COC EE/DRSP (Yaz; Bayer HealthCare Pharmaceuticals, Berlin, Germany). In addition, pituitary–ovarian function, the effect on endometrial thickness and the return of ovulation were investigated.

METHODS

This single centre, open, parallel, phase II, dose-finding pilot study was performed on a limited number of healthy female volunteers. The study was conducted in a clinical research centre (Dinox BV) in Groningen, the Netherlands. The trial was registered in the Netherlands Trial Register (www.trialregister.nl) under the registration number NTR2102. Compliance with Good Clinical Practice and the statistical and clinical study report were verified by an independent auditor.

Participants

All trial participants gave their written informed consent, and the study was approved by the independent ethics committee Stichting Therapeutische Evaluatie Geneesmiddelen (Duivendrecht, the Netherlands). The main inclusion criteria were as follows: age 18 to 35 years; ovulation in the pretreatment cycle between cycle day 9 ($\pm$ 1) and day 24 ($\pm$ 1), with a subsequent progesterone concentration $\geq$ 16 nmol/l and a luteal phase duration of at least 6 ($\pm$ 1) days; body mass index (BMI) of 18 to 30 kg/m^2; and good physical and mental health. Exclusion criteria were as follows: contraindication for contraceptive steroids; clinically relevant abnormal laboratory results; a long duration of the washout cycle after stopping hormonal contraception for more than 42 days; pregnancy; lactation; pregnancy during accurate hormonal contraceptive use in the past; history of breast cancer; abnormalities of the uterus or ovaries; abnormal cervical smear in the last 3 years or at screening; renal insufficiency; hepatic dysfunction; adrenal insufficiency; status postpartum or postabortion in the last 2 months; and a history (within 12 months) of alcohol or drug abuse. Use of the following drugs within two cycles prior to the start of study medication were also exclusion criteria: hepatic enzyme-inducing medicinal products; sex steroids; herbal remedies containing St John's Wort; antihypertensive drugs; phytoestrogens; investigational drugs in the last 2 months; and an injectable hormonal method of contraception in the last 6 months.

Before inclusion in the study, all participants underwent a general physical and gynaecological examination, including electrocardiogram, transvaginal ultrasonography (TVUS), breast examination and cervical smear (if no smear result had been obtained within the last 3 years). Haematological and clinical chemical blood parameters were determined, and urinalysis was performed.

The participants received financial compensation for their participation in the trial.

Study design

Participants who were using hormonal contraception at the start of the study discontinued its use after completion of the current cycle and then had a washout cycle. All participants had to use barrier contraception methods throughout the study. The pretreatment cycle started on the first day of spontaneous menstrual blood loss after the washout cycle (if any). Participation in the study was accepted only if ovulation occurred on or before day 24 (± 1) of the pretreatment cycle, if the progesterone concentration was ≥ 16 nmol/l and if the next menstruation did not start within 6 (± 1) days after ovulation. Eligibility was evaluated by monitoring follicular growth in the pretreatment cycle by TVUS, which was performed every 3 (± 1) days. After ovulation was documented by TVUS, a blood sample was taken 2 (± 1) days later to determine the progesterone concentration. If the progesterone concentration was in the postovulatory range but below 16 nmol/l another blood sample was taken 4 (± 1) days after ovulation.

During the first and the third treatment cycles, TVUS and blood sampling were performed every third (± 1) day from day 3 (± 1) to day 24 (± 1). TVUS and blood sampling were also performed on day 3 (± 1) of the second cycle. If a follicle with a diameter ≥ 13 mm was observed at day 24 (± 1) of the first or third cycle or at day 3 (± 1) of the second cycle, TVUS and blood sampling were continued every 3 (± 1) days until the follicle disappeared.

During the spontaneous cycle following the three treatment cycles, TVUS was performed every third (± 1) day from day 3 onwards until ovulation was observed. A blood sample was taken 2 (± 1) days after ovulation to determine the progesterone concentration. If the progesterone concentration was in the postovulatory range but below 16 nmol/l, another blood sample was taken 4 (± 1) days after ovulation. A follow-up visit was performed on day 3 (± 1) of the cycle after the post-treatment cycle. At the follow-up visit, physical and gynaecological examinations were performed.

Urine pregnancy tests were performed before the first intake of study medication and several times during the course of the study. Haematological and clinical chemical blood determinations and urinalysis were performed at screening and during the post-treatment cycle. At all visits during the study, participants were questioned for adverse events and use of concomitant medication.

Treatment

There were six treatment groups: (i) 5 mg E_4 combined with 3 mg DRSP (5 mg E_4/DRSP); (ii) 10 mg E_4 combined with 3 mg DRSP (10 mg E_4/DRSP); (iii) 20 μg EE combined with 3 mg DRSP (EE/DRSP); (iv) 5 mg E_4 combined with 150 μg LNG (5 mg E_4/LNG); (v) 10 mg E_4 combined with 150 μg LNG (10 mg E_4/LNG); and (vi) 20 mg E_4 combined with 150 μg LNG (20 mg E_4/LNG). All participants were stratified according to the day of ovulation in the pretreatment cycle and then assigned to one of the treatment groups. E_4 was supplied as tablets of 5 or 10 mg, in blister packs. DRSP was supplied as tablets of 3 mg, and LNG as tablets of 150 μg, both in blister packs. EE/DRSP was supplied as tablets in the original blister pack. Blinding was therefore not possible.

Production, packaging and labelling of the study medication were performed according to Good Manufacturing Practice guidelines (Haupt Pharma, Münster, Germany). The chemical synthesis of E_4 was performed by Cambridge Major Laboratories Europe (Weert, the Netherlands). A quality control of the tablets was performed at their release, and studies were conducted to evaluate the stability of the products for periods of time beyond the duration of the study. Oral treatment was started on the first day of menstruation following the pretreatment cycle and was administered for three cycles once daily in the morning at approximately the same time, which was recorded in a diary. In each cycle, participants treated with E_4 used the study medication for 24 days, followed by 4 days without medication. Participants treated with EE/DRSP used 24 active tablets followed by four placebo tablets.

Measurements

TVUS was performed using a Voluson E8 device (GE Healthcare, Kretztechnik GmbH & Co OHG, Zipf, Austria). The mean diameter of the bidirectional measurement of the largest follicle in each ovary and the double-layer endometrial thickness were assessed. Serum levels of follicle-stimulating hormone (FSH), luteinising hormone (LH), E2 and progesterone were determined in each blood sample. Blood samples were processed to serum and stored at −20°C until assays were performed. FSH, LH and progesterone levels in serum were determined by the Immulite 2000 immunoassay system (Siemens Healthcare GmbH, Erlangen, Germany). Because of significant crossreactivity between E_4 and E_2 using the commercially available ligand-binding assay, the E_2 concentrations were determined using the API 4000 LC/MS/MS system (Applied biosystems/MSD Sciex, Waltham, MA, USA).

At several time points in the study, extra blood samples were taken to measure various liver parameters. In addition, bone turnover markers and growth endocrine parameters were determined, and pharmacokinetic parameters were measured in the blood and urine. The methods and results of these assessments will be reported separately.

Sample size

The study was explorative. Its aim was to gather information that would help to decide which dose regimen should be selected for future studies. Therefore, the sample size was not calculated but arbitrarily assigned to 18 women per group. Based on this sample size, the upper limit of the unidirectional confidence interval of the ovulation rate in the absence of ovulation would be 5% when considering no intra-subject correlation (i.e., no ovulation in any of the three treatment cycles for the same participant) or 14% when considering perfect intra-subject correlation (i.e., one ovulation in each treatment cycle for the same participant). This sample size was considered acceptable for a dose-finding pilot study.

Analysis

The primary efficacy variable was the ovulation rate, i.e., the number of ovulations per number of cycles per treatment group. Ovulation was defined using the Hoogland score, which is based on the combination of maximum follicular diameter and concentrations of E_2 and progesterone during a treatment cycle[14] (Figure 1A). Hoogland scores were determined for treatment cycles 1 and 3. In addition, summary statistics of the largest follicle size per time point and the maximum follicle size per participant over the entire treatment period were calculated.

Secondary study objectives were to investigate pituitary–ovarian function, effect on endometrial thickness and return of ovulation. The mean and maximum serum concentrations of E_2, progesterone, FSH and LH per cycle were calculated. Summary statistics were calculated for the maximum endometrial thickness per woman per cycle. The return of ovulation was evaluated by assessing the day of ovulation in the post-treatment cycle.

Differences in the maximum follicle diameter and endometrial thickness for treatment cycles 1 and 3, comparing the E_4 groups versus EE/DRSP, the different E_4 dose groups and DRSP versus LNG, were analysed using a random effects repeated measures model. Hormone concentrations on day 3 of cycle 1 and pooled results on day 24 in cycles 1 and 3 were analysed statistically using a random effects repeated measures analysis model with pre-treatment day 3 values as covariate after logarithmic

(A)

Score	Activity	Follicle size (nm)	E2		Progesterone	
			nmol/L	pg/mL	nmol/L	pg/mL
1	No activity	≤ 10	-		-	
2	Potential activity	> 10	-		-	
3	Non active FLS	> 13	≤ 0.1	≤ 27.2	-	
4	Active FLS	> 13	> 0.1	> 27.2	≤ 5	≤ 1.57
5	LUF	> 13, persisting	> 0.1	> 27.2	> 5	> 1.57
6	Ovulation	> 13, ruptured	> 0.1	> 27.2	> 5	> 1.57

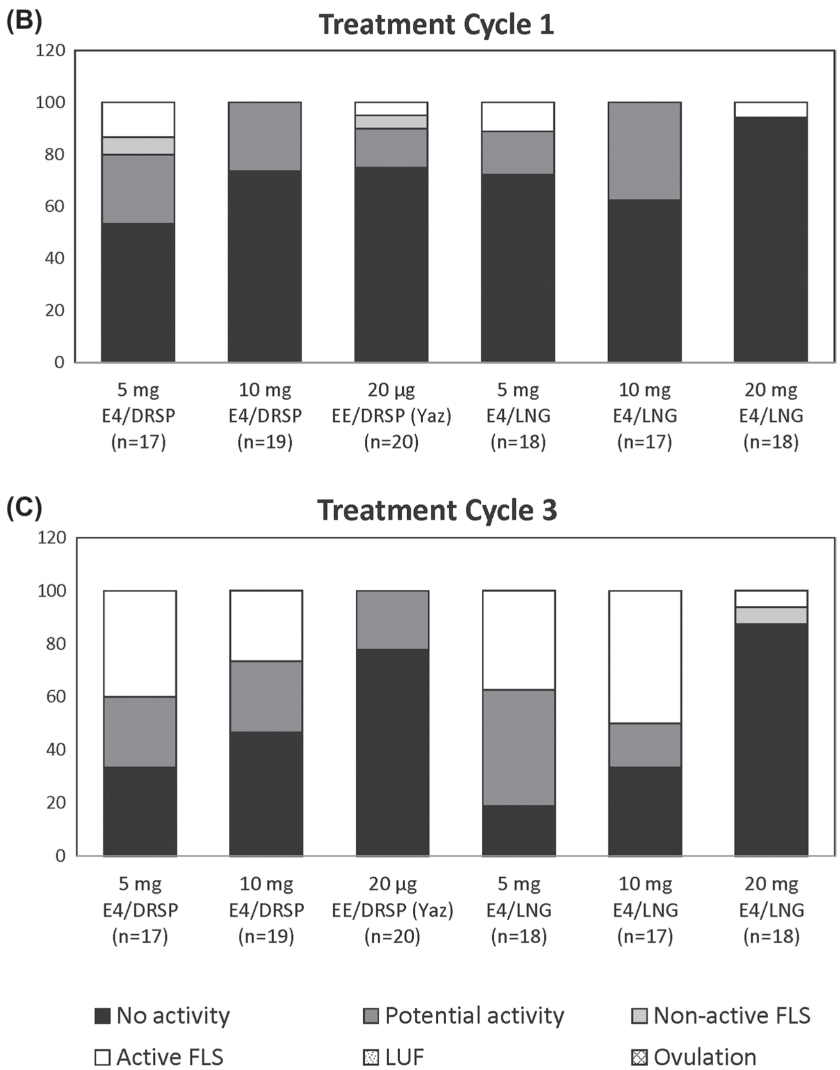

Figure 1 Ovulation inhibition according to the Hoogland score (A). Hoogland scores obtained during cycle 1 (B) and cycle 3 (C) with the different combinations tested during the trial. Results are expressed in percentage of participants.

transformation. Differences in the return of ovulation day comparing the E_4 groups with the EE/DRSP group, comparing the different E_4 doses, and comparing DRSP with LNG were analysed by analysis of covariance with day of ovulation in the pretreatment cycle as the covariate. In the statistical analyses, $p < 0.01$ was used as the criterion for statistical significance.

RESULTS

Study population

The study was performed between November 2009 and November 2010. In total, 210 women were screened: 99 were screening failures and 111 were included and assigned to a treatment group. The most common reasons for screening failure were menstrual cycle deviations, in particular a washout cycle of more than 42 days after stopping COC, no ovulation until cycle day 24 in the pretreatment cycle, or a low progesterone concentration after ovulation in the pretreatment cycle. The participant disposition is shown in Figure 2.

The demographic and pretreatment cycle characteristics were generally similar across the treatment groups (Table 1). However, compared with the other groups, the mean BMI was lower in the 5 mg E_4/LNG and 10 mg E_4/LNG groups, and the percentage of smokers was lower in the 10 mg E_4/LNG and 20 mg E_4/LNG groups.

Ovulation rate

The distribution of the Hoogland scores in treatment cycles 1 and 3 in the different treatment groups is depicted in Figure 1B & 1C. In none of the treatment cycles was the Hoogland score higher than 4, so there were no luteinised unruptured follicles (LUFs) or ovulations. During treatment cycle 1, in all treatment groups, the majority (80% or more) of participants had no ovarian activity (Hoogland score 1) or potential activity (Hoogland score 2). The remaining participants had a non-active follicle-like structure (FLS) (Hoogland score 3) or active FLS (Hoogland score 4). For the E_4 treatment groups, the number of participants with non-active FLS or active FLS was higher in treatment cycle 3 compared with treatment cycle 1. During treatment cycle 3, the majority (50% or more) of the participants had no activity or potential activity. Despite the low number of participants in each group, it appears that increasing the dose of E_4 was associated with an increased suppression of ovarian activity, particularly in treatment cycle 3, during which the percentage of participants with non-active FLS or active FLS was lowest in the 20 mg E_4/LNG group (12.6%) and comparable to the EE/DRSP group (0%).

Ovarian and pituitary function

The maximum values of the largest follicular diameter during the entire treatment period are shown in Figure 3A. The mean values of the largest follicular diameter at each time point during treatment cycles 1 and 3 are depicted in Figure 3B.

The mean maximum follicular diameter in treatment cycle 1 and 3 decreased significantly with increasing E_4 dose ($p < 0.0001$) and did not differ between the DRSP and the LNG groups. The mean maximum follicular diameter in the 5 mg E_4 groups was higher than in the EE/DRSP group ($p < 0.0001$). The difference between the 10 mg E_4 groups and the EE/DRSP group almost reached significance ($p = 0.0133$).

Table 2 shows the mean and maximum FSH, LH, E_2 and progesterone concentrations in treatment cycles 1, 2 and 3. Pooled FSH and LH concentrations on day 24 of cycle 1 and cycle 3 were significantly lower with increasing E_4 dose ($p < 0.0001$

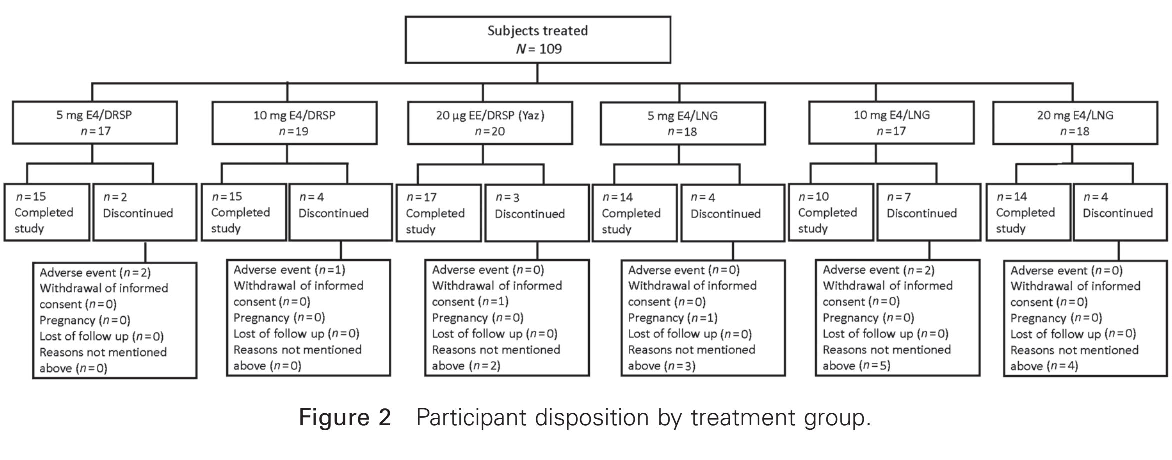

Figure 2 Participant disposition by treatment group.

Table 1 Demographic and baseline characteristics.

Parameter	5 mg E_4/DRSP ($n=19$)	10 mg E_4/DRSP ($n=19$)	20 µg EE/DRSP ($n=20$)	5 mg E_4/LNG ($n=18$)	10 mg E_4/LNG ($n=17$)	20 mg E_4/LNG ($n=18$)	Overall ($n=111$)
Mean age, years (SD)	24.3 (3.11)	23.7 (3.67)	23.4 (3.87)	22.3 (2.65)	22.4 (2.42)	21.1 (2.30)	22.9 (3.20)
BMI, kg/m^2							
Mean (SD)	22.54 (2.33)	23.20 (3.21)	23.03 (2.93)	21.51 (1.70)	21.78 (2.52)	24.28 (3.37)	22.74 (2.83)
Range	18.3–26.1	18.8–30.0	19.2–28.3	18.2–24.5	18.7–27.4	19.1–29.8	18.2–30.0
Race, *n* (%)							
White or Caucasian	16 (84.2)	18 (94.7)	19 (95.0)	16 (88.9)	17 (100)	16 (88.9)	102 (91.9)
Black or African American	1 (5.3)	0	0	0	0	2 (11.1)	3 (2.7)
Asian	2 (10.5)	0	1 (5.0)	0	0	0	3 (2.7)
Other	0	1 (5.3)	0	2 (11.1)	0	0	3 (2.7)
Mean duration of menstrual cycle, days (SD)	28.8 (1.81)	28.3 (0.75)	28.7 (1.29)	27.8 (2.13)	28.0 (0.38)	28.5 (3.22)	28.4 (1.86)
Gravidity, *n* (%)							
0	14 (73.7)	18 (94.7)	19 (95.0)	18 (100.0)	16 (94.1)	16 (88.9)	101 (91.0)
≥1	5 (26.3)	1 (5.3)	1 (5.0)	0 (0.0)	1 (5.9)	2 (11.1)	10 (9.0)
Parity, *n* (%)							
0	16 (84.2)	19 (100)	19 (95.0)	18 (100.0)	17 (100.0)	17 (94.4)	106 (95.5)
≥1	3 (15.8)	0 (0.0)	1 (5.0)	0 (0.0)	0 (0.0)	1 (5.6)	5 (4.5)
Smoking habits, *n* (%)							
Non-smoker	12 (63.2)	13 (68.4)	12 (60.0)	11 (61.1)	14 (82.4)	15 (83.3)	77 (69.4)
Smoker	5 (26.3)	5 (26.3)	7 (35.0)	6 (33.3)	3 (17.6)	3 (16.7)	29 (26.1)
Former smoker	2 (10.5)	1 (5.3)	1 (5.0)	1 (5.6)	0 (0.0)	0 (0.0)	5 (4.5)

SD, standard deviation

and $p = 0.0078$, respectively). There were no statistically significant differences in FSH and LH concentrations when the DRSP and the LNG groups were compared. Pooled FSH and LH concentrations on day 24 of cycle 1 and cycle 3 were significantly higher in the 5 mg E_4 and 10 mg E_4 groups than in the EE/DRSP group ($p < 0.0001$).

Mean and maximum E_2 concentrations decreased with increasing E_4 concentration in the study medication combinations, and the lowest mean E_2 concentration was observed in the 20 mg E_4/LNG group (50 ± 10 pmol/l at cycle 3) and was comparable to that of the EE/DRSP combination (40 ± 10 pmol/l at cycle 3). The pooled E_2 concentrations on day 24 of cycle 1 and cycle 3 were significantly lower with increasing E_4 dose ($p < 0.0001$). There was no statistically significant difference in E_2 concentrations between the DRSP and LNG groups. The pooled E_2 levels on day 24 of cycles 1 and 3 were significantly higher in the 5 mg E_4 groups than in the EE/DRSP group ($p = 0.0066$ and $p = 0.0001$, respectively); no significant difference was observed when comparing the 10 mg E_4 groups and the EE/DRSP group ($p = 0.0708$).

There were no discernible differences in mean or maximum progesterone concentrations among the treatment groups. All measured progesterone concentrations during treatment cycles 1, 2 and 3 were below 5 nmol/l, indicating absence of a LUF or ovulation, except for one measurement (a participant included in the 10 mg E_4/DRSP group had a progesterone concentration of 5.69 nmol/l on day 3 of treatment cycle 1, probably due to incomplete regression of a corpus luteum from the pretreatment cycle). Progesterone concentrations did not statistically differ between the E_4 groups and the EE/DRSP group, nor between the different E_4 dose groups, nor between the DRSP and the LNG groups.

(A) Maximum follicle size (mm) for the entire treatment period

	Mean (SD)	Minimum	Maximum		Mean (SD)	Minimum	Maximum
5 mg E4/DRSP	16.03 (6.64)	8.9	30.5	5 mg E4/LNG	14.28 (6.67)	11.7	28.1
10 mg E4/DRSP	13.33 (6.21)	8.1	29.7	10 mg E4/LNG	13.48 (5.79)	11.2	29.6
20 µg EE/DRSP	11.05 (5.08)	6.8	27.6	20 mg E4/LNG	9.72 (4.42)	8.4	23.7

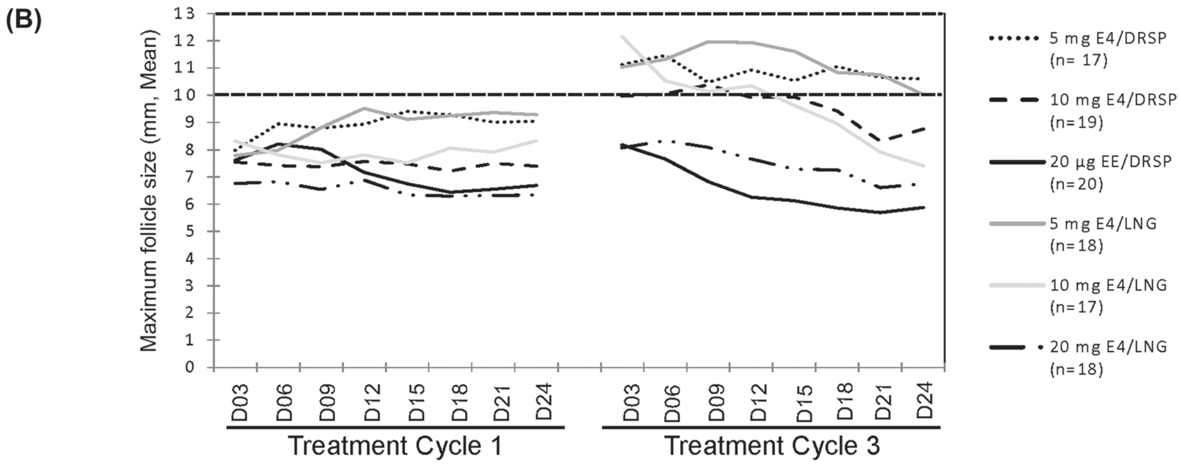

Figure 3 Mean (± SD), minimum and maximum value of the largest follicular diameter per participant in each group over the entire treatment period (A). Mean diameter of the largest follicle (mm) measured in each treatment group every 3 days during cycles 1 and 3 (B).

Endometrium

The mean endometrial thickness decreased in the treatment cycles compared with the pre- and post-treatment cycles, with no dose-related trends or significant differences between participants treated with increasing doses of E_4 combined with DRSP or LNG (Figure 4A, B).

Return of ovulation

Return of ovulation was measured by monitoring follicular growth in the posttreatment cycle until ovulation occurred. During the post-treatment cycle, all participants ovulated within 21 days after stopping treatment. Those treated with 5 or 10 mg E_4/DRSP had their first day of ovulation approximately 17 days after the last treatment (mean 17.6 and 17.1 days, respectively). The mean number of days to first ovulation was longer for participants treated with an E_4/LNG combination (20.5, 20.8 and 21.0 days for the 5, 10 and 20 mg E_4 groups, respectively). No difference was observed with increasing dose of E_4. The mean number of days to first ovulation after the last active treatment with EE/DRSP was 20.6 days. The statistical analysis did not show any significant differences between the E_4 groups and the EE/DRSP group, nor between the different E_4 dose groups, nor between the DRSP and the LNG groups.

Safety and tolerability

The study combinations were well tolerated. No serious adverse events occurred. Table 3 shows the drug-related adverse events (i.e., considered possibly, probably or definitely related to treatment) reported by at least two participants in one of the treatment groups. Drug-related adverse events were reported by 50–82.4% of participants across the treatment groups. The most common drug-related adverse events were lower abdominal pain, nausea, headache, dysmenorrhoea, breast enlargement and acne. No overall trends were noted for the frequency of drug-related adverse events, when treatment groups were compared by dose of E_4, between those treated with E_4 and those treated with EE or between those treated with E_4/DRSP or E_4/LNG, except the incidence of headache, which was higher in participants treated with E_4 compared with EE (not significantly

Table 2 Effects of treatment on pituitary–ovarian axis parameters*

	5 mg E_4/DRSP (n = 17)		10 mg E_4/DRSP (n = 19)		20 µg EE/DRSP (n = 20)		5 mg E_4/LNG (n = 18)		10 mg E_4/LNG (n = 17)		20 mg E_4/LNG (n = 18)	
Parameter	*Mean (SD)*	*Maximum*	*Mean (SD)*	*Maximum*	*Mean (SD)*	*Maximum*	*Mean (SD)*	*Maximum*	*Mean (SD)*	*Maximum*	*Mean (SD)*	*Maximum*
LH, IU/l												
Baseline	4.84 (3.67)		5.83 (3.24)		4.31 (1.30)		4.47 (1.39)		4.79 (1.72)		4.08 (1.58)	
Treatment cycle 1	5.50 (2.85)	7.28 (3.18)	6.26 (2.87)	8.55 (3.41)	3.38 (1.75)	5.43 (2.13)	5.77 (2.62)	8.05 (3.40)	5.52 (1.83)	8.01 (2.24)	3.15 (1.56)	4.87 (2.18)
Treatment cycle 2	6.38 (3.95)	7.01 (3.65)	7.14 (3.89)	7.57 (3.68)	4.46 (2.40)	4.89 (2.73)	7.69 (4.78)	7.82 (4.73)	7.01 (2.71)	7.10 (2.81)	3.94 (1.81)	4.11 (1.98)
Treatment cycle 3	5.69 (3.25)	7.92 (3.88)	6.77 (3.77)	8.92 (4.55)	2.77 (2.31)	5.01 (3.27)	5.71 (2.27)	8.49 (3.06)	5.89 (2.06)	8.51 (3.12)	4.13 (1.76)	6.44 (2.52)
FSH, IU/l												
Baseline	6.23 (1.57)		6.71 (1.70)		5.82 (1.48)		5.50 (1.32)		6.10 (2.08)		5.12 (1.36)	
Treatment cycle 1	6.66 (1.78)	7.91 (2.08)	6.39 (1.76)	7.64 (2.40)	4.19 (1.83)	5.94 (1.85)	5.82 (1.27)	7.22 (1.37)	6.17 (1.75)	7.56 (2.16)	3.67 (1.70)	4.58 (1.87)
Treatment cycle 2	6.34 (1.65)	6.87 (1.67)	6.99 (1.66)	7.18 (1.42)	5.49 (1.95)	5.89 (2.16)	6.59 (1.73)	6.77 (1.69)	6.72 (1.75)	6.76 (1.78)	4.82 (1.75)	4.98 (1.76)
Treatment cycle 3	6.59 (1.59)	8.35 (1.37)	6.63 (1.52)	7.89 (1.65)	3.14 (2.01)	5.72 (2.00)	5.75 (1.77)	7.06 (1.79)	5.97 (1.71)	7.50 (2.11)	4.60 (1.63)	6.18 (2.10)
E_2, pmol/l												
Baseline	170 (210)		130 (60)		130 (170)		100 (60)		120 (50)		100 (50)	
Treatment cycle 1	80 (30)	120 (50)	60 (20)	110 (70)	50 (20)	140 (120)	80 (60)	80 (40)	50 (20)	80 (40)	50 (10)	80 (60)
Treatment cycle 2	200 (150)	320 (350)	140 (120)	190 (220)	100 (150)	170 (160)	150 (90)	180 (170)	180 (170)	180 (170)	60 (50)	70 (110)
Treatment cycle 3	120 (120)	270 (270)	110 (150)	200 (260)	40 (10)	280 (290)	120 (110)	170 (110)	70 (40)	170 (110)	50 (10)	90 (90)
Progesterone, nmol/l												
Baseline	1.50 (0.54)		1.66 (0.87)		1.50 (0.42)		1.57 (0.48)		1.69 (1.02)		1.53 (0.69)	
Treatment cycle 1	1.34 (0.34)	1.83 (0.49)	1.39 (0.46)	2.36 (1.14)	1.35 (0.41)	2.04 (0.64)	1.39 (0.39)	1.91 (0.53)	1.16 (0.35)	1.74 (0.57)	1.39 (0.55)	1.87 (0.79)
Treatment cycle 2	1.30 (0.34)	1.47 (0.50)	1.16 (0.54)	1.20 (0.57)	1.25 (0.57)	1.34 (0.62)	1.34 (0.56)	1.36 (0.54)	1.10 (0.53)	1.11 (0.53)	1.50 (0.69)	1.51 (0.69)
Treatment cycle 3	1.33 (0.30)	1.91 (0.54)	1.15 (0.39)	1.69 (0.73)	1.23 (0.48)	1.73 (0.72)	1.28 (0.34)	1.89 (0.61)	1.04 (0.35)	1.41 (0.50)	1.41 (0.54)	1.95 (0.79)

SD, standard deviation.
*Mean (± SD) and maximum (mean ± SD) values exposed for all parameters measured during treatment cycles 1, 2 and 3.

(A) Maximum endometrial thickness (mm, Mean [SD])

	Pre T cycle	Cycle 1	Cycle 3	Post T cycle		Pre T cycle	Cycle 1	Cycle 3	Post T cycle
5 mg E4/DRSP	9.91 (2.03)	5.87 (1.42)	5.81 (1.27)	9.11 (1.93)	5 mg E4/LNG	9.47 (2.27)	5.58 (1.15)	6.16 (1.29)	8.96 (1.97)
10 mg E4/DRSP	9.61 (1.59)	7.70 (3.04)	6.17 (1.44)	9.37 (2.08)	10 mg E4/LNG	9.51 (2.06)	6.19 (1.40)	6.04 (1.14)	9.41 (1.13)
20 µg EE/DRSP	8.74 (1.86)	5.92 (1.09)	5.90 (1.20)	8.85 (1.86)	20 mg E4/LNG	10.17 (2.36)	6.08 (1.38)	6.34 (1.27)	9.69 (2.15)

(B)

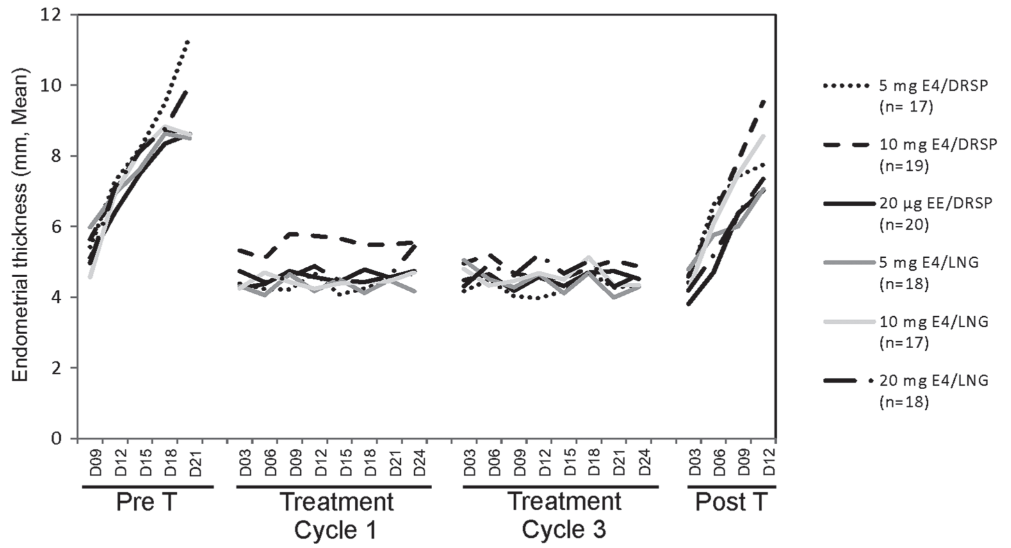

Figure 4 Maximum (mean ± SD) endometrial thickness value observed for each group during the trial (A). Mean endometrial thickness assessed in each treatment group every 3 days across the pretreatment cycle, treatment cycles 1 and 3, and post-treatment cycle (B). D, day; Pre T, pretreatment cycle; Post T, post-treatment cycle.

different), and the incidence of acne, which was significantly higher in participants treated with E_4/LNG compared with E_4/DRSP.

DISCUSSION

Findings and interpretation

The results of this study demonstrate that E_4 in combination with DRSP or LNG effectively inhibits ovulation. There were no ovulations or LUFs during the treatment cycles in all treatment groups, showing the efficacy of 5, 10 and 20 mg E_4 combined with DRSP or LNG in a regimen of 24-treatment days followed by a 4-day treatment-free period.

Ovarian suppression, as determined by maximum follicular diameter, mean and maximum E_2 concentration and Hoogland score, was adequate in all E_4 treatment groups. There were no discernible differences in the degree of ovarian suppression between the two progestins. However, a difference could be observed between the different E_4 doses. Ovarian suppression was most pronounced at the highest E_4 dose (20 mg E_4). In addition, suppression of gonadotropins was most pronounced in the highest dosage group. The stronger ovarian and pituitary suppression in this group could already be observed in the first treatment cycle and was more apparent in the third treatment cycle. Ovarian suppression in the 20 mg E_4/LNG group was comparable to that in the EE/DRSP group, and also with ovarian suppression reported with other registered COCs containing EE or E_2[15–22].

Endometrial thickness was reduced in the treatment cycles compared with the pre- and post-treatment cycles. The results were comparable in all treatment groups, including the EE/DRSP group. Apparently, E_4 in combination with a progestin has a similar effect on endometrial growth to that of EE/DRSP.

Table 3 Drug-related adverse events reported by at least two participants in any treatment group. Data expressed in number (%) of participants.

Parameter	*5 mg E_4/DRSP (n = 17)*	*10 mg E_4/DRSP (n = 19)*	*20 μg EE/DRSP (n = 20)*	*5 mg E_4/LNG (n = 18)*	*10 mg E_4/LNG (n = 17)*	*20 mg E_4/LNG (n = 18)*
Any adverse effect	13 (76.5)	11 (57.9)	12 (60.0)	14 (77.8)	14 (82.4)	9 (50.0)
Lower abdominal pain	4 (23.5)	1 (5.3)	2 (10.0)	1 (5.6)	1 (5.9)	0
Nausea	2 (11.8)	2 (10.5)	3 (15.0)	1 (5.6)	1 (5.9)	2 (11.1)
Irritability	0	0	2 (10.0)	0	0	1 (5.6)
Headache	4 (23.5)	6 (31.6)	2 (10.0)	4 (22.2)	4 (23.5)	4 (22.2)
Dizziness	0	0	2 (10.0)	0	0	1 (5.6)
Affect lability	1 (5.9)	0	2 (10.0)	1 (5.6)	0	0
Decreased libido	0	0	1 (5.0)	2 (11.1)	1 (5.9)	0
Dysmenorrhoea	6 (35.3)	3 (15.8)	2 (10.0)	1 (5.6)	2 (11.8)	2 (11.1)
Breast enlargement	3 (17.6)	2 (10.5)	0	0	0	1 (5.6)
Breast tenderness/pain	2 (11.8)	3 (15.8)	3 (15.0)	0	1 (5.9)	2 (11.1)
Acne	0	1 (5.3)	0	3 (16.7)	4 (23.5)	3 (16.7)
Seborrhoea	0	0	0	1 (5.6)	2 (11.8)	0
Hot flush	2 (11.8)	0	0	0	0	0

The first post-treatment ovulation occurred approximately 17 days after the last treatment day in the E_4/DRSP groups, and 21 days after the last active treatment in the E_4/LNG and EE/DRSP groups. An explanation for the difference between the two progestins cannot be given. For all treatment groups, the time period until the first ovulation was comparable to the duration of a normal follicular phase, confirming adequate ovarian suppression during treatment.

The different combinations were safe and well tolerated. The reported adverse events were the same as those previously described with other marketed COCs. When comparing both progestins, the incidence of headache was higher in the E_4/DRSP groups, whereas the incidence of acne was higher in the E_4/LNG groups. No E_4 dose-related trends could be observed in the frequency or severity of the reported adverse events.

Strengths and weaknesses of the study

This study represents the first attempt to combine E_4 and LNG or DRSP to achieve blockade of ovulation for three cycles. The study was conducted using state-of-the-art methodologies and by an experienced scientific team. Even if the number of participants included in each treatment arm was limited, the primary objective of the study was achieved, as no ovulation occurred in any patient.

Because the primary objective of this exploratory study was to evaluate ovulation inhibition in the different groups, the sample size was not powered to perform a safety comparison between the tested combinations. Therefore, larger studies will be needed to confirm the safety profile and tolerability of an E_4-containing COC.

Differences in the results and conclusions

Animal studies performed in female rats, and human studies performed in postmenopausal women, have already demonstrated the significant dose-dependent inhibitory effect of E_4 on central gonadotropin secretion[3,23–25]. The results of the present study confirm these previous data, as with a fixed dose of progestin higher doses of E_4 were associated with a more profound inhibition of ovarian activity.

A previous study showed that the 24/4-day regimen is associated with greater inhibition of ovarian function than the conventional 21/7-day regimen[26]. Administering the E_4 combinations following that regimen might also have contributed to the total absence of Hoogland scores 5 and 6 in our study.

Finally, recent physiological studies reveal critical requirements for membrane ERα in ovarian function and thereby in fertility[27]. Transgenic mice lacking the membrane ERα do not ovulate, demonstrating that this receptor is essential for ovulation.

E_4 selectively activates the nuclear ERα but antagonises the membrane ERα[28]. This selective blockade of the membrane ERα could contribute to the blockade of ovulation.

Relevance of the findings: Implications for clinicians

Women with intermenstrual bleeding have significantly larger FLS and significantly higher E_2 levels than those without intermenstrual bleeding. High ovarian suppression is classically positively correlated with improved cycle control characterised by less frequent intermenstrual bleeding[29]. When administered at a dosage above 10 mg/day, E_4 appears to be a promising alternative estrogen for use in contraception. Because doses of 20 mg E_4 combined with a progestin suppress ovarian activity as efficiently as 20 μg EE/DRSP or other registered COCs containing EE or E_2, an additional phase II study should be able to more precisely delineate the best E_4 dose regimen between 10 and 20 mg that provides an acceptable pattern of intermenstrual spotting and bleeding.

E_4 exhibits several unique features that could make it suitable as an alternative estrogen for use in a COC. These advantages were evaluated in previous trials and have been reported elsewhere. First, E_4 has a high oral bioavailability associated with a long half-life of approximately 30 h, allowing daily administration. Furthermore, E_4 is an end-product of estrogen metabolism in the human foetus. Metabolism through oxidation does not occur. In non-pregnant women, E_4 is rapidly and almost completely excreted in the urine as a conjugate (glucuronide and sulphate). In contrast to EE and E_2, E_4 is less subject to biliary excretion and enterohepatic recirculation[30]. Therefore, it is tempting to speculate that COCs containing E_4 would not result in an increased risk of hepatobiliary diseases as observed among users of EE-containing medications[9]. Furthermore, because E_4 has a minimal impact on production of coagulation factors in the liver, the VTE risk might also be reduced compared with EE-containing COCs [Kluft C. *et al.*, submitted].

Unanswered questions and future research

In addition to ovulation inhibition, it is necessary to assess tolerability and bleeding pattern when defining the adequate dosage of a new estrogen to be incorporated in a COC. The present study, with its relatively small sample size and short treatment duration, was not designed to evaluate properly the bleeding pattern and safety aspects of the different combinations tested. A larger dose-finding study aiming at assessing the tolerability, acceptability and bleeding characteristics of different E_4-containing combinations is therefore necessary.

As mentioned above, larger trials will also be needed to fully characterise the contraceptive efficacy and safety profile of an E_4-containing COC among women of different ethnicities, with different health-related characteristics (e.g., BMI, smoking habits) and of different ages. Only large and sufficiently long-term studies will be able to answer these questions.

CONCLUSION

The results of this study show that E_4 in combination with DRSP or LNG adequately suppresses ovarian activity and inhibits ovulation, particularly when given at a dosage above 10 mg/day. E_4 appears to be a promising alternative estrogen for use in contraception.

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REFERENCES

1. Hagen AA, Barr M, Diczfalusy E. Metabolism of 17-beta-estradiol-4-14-C in Early Infancy. *Acta Endocrinol* 1965;49:207–20.
2. Visser M, Coelingh Bennink HJ. Clinical applications for estetrol. J *Steroid Biochem Mol Biol* 2009;114(1–2): 85–9.
3. Coelingh Bennink HJ, Skouby S, Bouchard P*, et al.* Ovulation inhibition by estetrol in an in vivo model. *Contraception* 2008;77:186–90.
4. Cleuren AC, Van der Linden IK, De Visser YP*, et al.* 17alpha-ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor alpha. *J Thromb Haemost* 2010;8:1838–46.
5. Lidegaard O, Nielsen LH, Skovlund CW, *et al.* Risk of venous thromboembolism from use of oral contraceptives containing different progestogens and estrogen doses: Danish cohort study, 2001–9. *BMJ* 2011; 343:d6423.
6. Lidegaard O, Lokkegaard E, Jensen A, *et al*. Thrombotic stroke and myocardial infarction with hormonal contraception. *N Engl J Med* 2012;366(24):2257–66.
7. Lidegaard O. Thromboembolic complications in users of estradiolvalerate /dienogest oral contraceptives 2013, 24 May 2013. Copenhagen. Accessed 22 December 2014. Available from: http://www.lidegaard.dk/Slides/OC%20epidem/PP-VTE%2013-05-23%20Qlaira.pdf
8. Visser M, Foidart JM, Coelingh Bennink HJ. In vitro effects of estetrol on receptor binding, drug targets and human liver cell metabolism. *Climacteric* 2008; 11(Suppl. 1):64–8.
9. Thijs C, Knipschild P. Oral contraceptives and the risk of gallbladder disease: a meta-analysis. *Am J Public Health* 1993;83:1113–20.
10. Yagi E, Barrett JC, Tsutsui T. The ability of four catechol estrogens of 17beta-estradiol and estrone to induce DNA adducts in Syrian hamster embryo fibroblasts. *Carcinogenesis* 2001;22:1505–10.
11. Singer C, Coelingh BH, Natter C, *et al.* Anti-estrogenic effects of the fetal estrogen estetrol in women with estrogen-receptor positive early breast cancer. *Carcinogenesis* 2014;35:2447–51.
12. Gérard C, Blacher S, Communal L, *et al.* Estetrol is a weak estrogen antagonizing estradiol-dependent mammary gland proliferation. *J Endocrinol* 2015;224:85–95.
13. Visser M, Kloosterboer HJ, Coelingh Bennink HJT. Estetrol prevents and suppresses mammary tumors induced by DMBA in a rat model. *Horm Mol Biol Clin Invest* 2012;9:95–103.
14. Hoogland HJ, Skouby SO. Ultrasound evaluation of ovarian activity under oral contraceptives. *Contraception* 1993;47:583–90.
15. Coney P, DelConte A. The effects on ovarian activity of a monophasic oral contraceptive with 100 microg levonorgestrel and 20 microg ethinyl estradiol. *Am J Obstet Gynecol* 1999;181(5 Pt 2):53–8.
16. Duijkers IJ, Klipping C, Grob P, *et al.* Effects of a monophasic combined oral contraceptive containing nomegestrol acetate and 17 beta-estradiol on ovarian function in comparison to a monophasic combined oral contraceptive containing drospirenone and ethinylestradiol. *Eur J Contracept Reprod Health Care* 2010; 15:314–25.
17. Duijkers IJ, Klipping C, Verhoeven CH, *et al.* Ovarian function with the contraceptive vaginal ring or an oral contraceptive: A randomized study. *Hum Reprod* 2004;19:2668–73.
18. Endrikat J, Parke S, Trummer D, *et al.* Ovulation inhibition with four variations of a four-phasic estradiol valerate/dienogest combined oral contraceptive: results of two prospective, randomized, open-label studies. *Contraception* 2008;78:218–25.
19. Fitzgerald C, Feichtinger W, Spona J, *et al.* A comparison of the effects of two monophasic low dose oral contraceptives on the inhibition of ovulation. *Adv Contracept* 1994;10:5–18.
20. Rabe T, Nitsche DC, Runnebaum B. The effects of monophasic and triphasic oral contraceptives on ovarian function and endometrial thickness. *Eur J Contracept Reprod Health Care* 1997;2:39–51.
21. Rossmanith WG, Steffens D, Schramm G. A comparative randomized trial on the impact of two low-dose oral contraceptives on ovarian activity, cervical permeability, and endometrial receptivity. *Contraception* 1997;56:23–30.
22. Thomas K, Vankrieken L. Inhibition of ovulation by low-dose monophasic contraceptive containing gestodene. *Am J Obstet Gynecol* 1990;163(4 Pt 2):1404–10.
23. de Visser J, Coert A, Feenstra H, *et al.* Endocrinological studies with (7 alpha, 17 alpha)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one (Org OD 14). *Arzneimittelforschung* 1984;34:1010–7.
24. Visser M, Holinka CF, Coelingh Bennink HJ. First human exposure to exogenous single-dose oral estetrol in early postmenopausal women. *Climacteric* 2008; 11(Suppl 1.):31–40.
25. Visser M, Coelingh Bennink HJ. Estetrol, the new natural estrogen for clinical use in women. *Références Gynécologie Obstétrique* 2011;14:427–32.
26. Klipping C, Duijkers I, Trummer D*, et al.* Suppression of ovarian activity with a drospirenone-containing oral contraceptive in a 24/4 regimen. *Contraception* 2008;78:16–25.
27. Abot A, Fontaine C, Raymond-Letron I, *et al.* The AF-1 activation function of estrogen receptor alpha is

necessary and sufficient for uterine epithelial cell proliferation in vivo. *Endocrinology* 2013;154:2222–33.
28. Adlanmerini M, Solinhac R, Abot A, *et al.* Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. *Proc Natl Acad Sci USA* 2014;111: E283–E90.
29. Endrikat J, Gerlinger C, Plettig K, *et al.* A meta-analysis on the correlation between ovarian activity and the incidence of intermenstrual bleeding during low-dose oral contraceptive use. *Gynecol Endocrinol* 2003;17:107–14.
30. Coelingh Bennink HJ, Holinka CF, Diczfalusy E. Estetrol review: profile and potential clinical applications. *Climacteric* 2008;11(Suppl 1.):47–58.