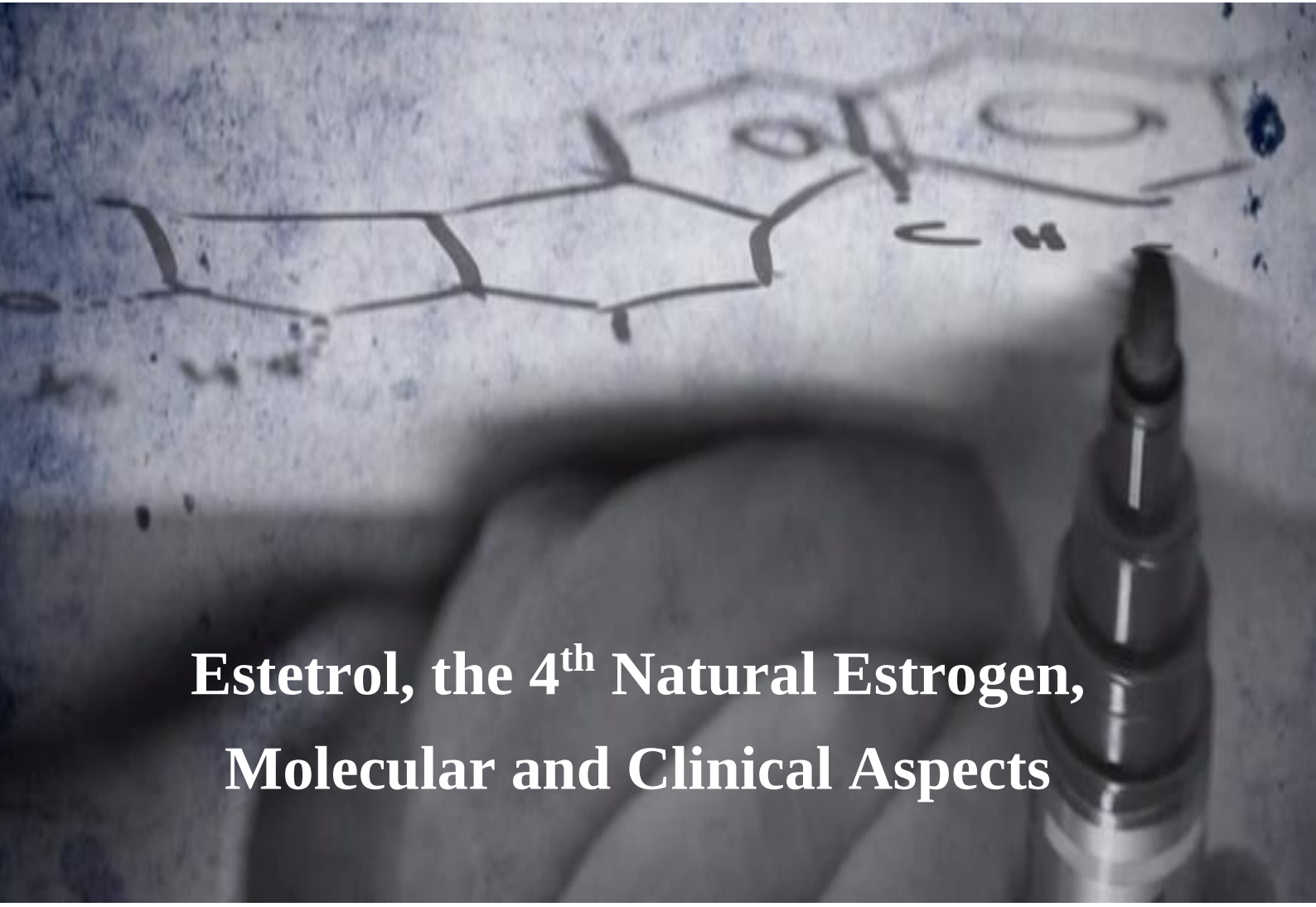

Estetrol, the 4th Natural Estrogen, Molecular and Clinical Aspects

A Dissertation submitted to the
University of Liège
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by

Marie Mawet, MD

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Thesis Director: Professor Jean-Michel Foidart

LIÈGE université

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Thesis Student:

Marie Mawet, MD

President of the Jury :

Professor Michèle Nisolle (Université de Liège)

Members of the Jury:

Professor Tommaso Simoncini (Universita di Pisa, Italy)

Professor Jean-Michel Dogné (Université de Namur)

Professor Albert Beckers (Université de Liège)

Professor Régis Radermecker (Université de Liège)

Thesis Director :

Professor Jean-Michel Foidart (Université de Liège)

DEDICATION

I dedicate this thesis to my family.

And to the Backstreet Boys.

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LIST OF ABBREVIATIONS

ACE	Angiotensinogen converting enzyme
ADH	Antidiuretic hormone
AF-1	Activation function 1
AF-2	Activation function 2
APC	Activated protein C
APCr	Activated protein C resistance
aPTT	Activated partial thromboplastin time
ARC	Arcuate nucleus
ATE	Arterial thromboembolism
$AUC_{0\text{-last}}$	Area under the concentration time curve up to the last measurable concentration
AVPV	Anteroventral periventricular nucleus
BMD	Bone mineral density
BMI	Body mass index
CBG	Corticosteroid binding globulin
CHC	Combined hormonal contraceptive
C_{max}	The maximum (or peak) serum concentration that a drug achieves
CMC	Chemistry, manufacturing and control
COC	Combined oral contraceptive
CON	Control
CPA	Cyproterone acetate
Ctr	Control
Ctrl	Control
CTX1	C-terminal telopeptide
CV	Coefficient of variation
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulfate
DHT	Dihydrotestosterone
dl	Deciliter
DMBA	7,12-Dimethylbenz(a)anthracene
DNA	Deoxyribonucleic acid
DNG	Dienogest
DRSP	Drospirenone
DSG	Desogestrel
DVT	Deep vein thrombosis
E1	Estrone
E2	Estradiol
E2V	Estradiol valerate
E3	Estriol
E4	Estetrol

EE	Ethinylestradiol
e.g.	For example
EMA	European medicines agency
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
et al.	And others
FDA	Food and drug administration
FSH	Follicle-stimulating hormone
GH	Growth hormone
GM	Geometric mean
GnRH	Gonadotropin-releasing hormone
GSD	Gestodene
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HIE	Hypoxic-ischemic encephalopathy
HPO	Hypothalamic-pituitary-ovarian
HRT	Hormone replacement therapy
IC_{50}	Half maximal inhibitory concentration
ICH	International council for harmonisation
i.e.	That is
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IGFBP-1	Insulin-like growth factor-binding protein 1
IGFBP-3	Insulin-like growth factor-binding protein 3
IL-6	Interleukine-6
kg	Kilogram
l	Liter
LARC	Long lasting reversible contraceptive
LBD	Ligand-binding domain
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LNG	Levonorgestrel
mcg	Microgram
mg	Milligram
MISS	Membrane-initiated steroid signaling
ml	Milliliter
mmol	Millimole
mRNA	Messenger ribonucleic acid
N	Newton
NC	Not calculated
ND	Not done

nmol	Nanomole
NO	Nitric oxide
NOMAC	Nomegestrol acetate
NRG	Norgestimate
OH	Hydroxyl group
ORO	Oil red-O
OVX	Ovariectomized
PAI	Plasminogen activator inhibitor
pg	Picogram
PK	Pharmacokinetic
PMDD	Premenstrual dysphoric disorder
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SHBG	Sex hormone binding globulin
TFPI	Tissue factor pathway inhibitor
T_{max}	The time taken to reach the maximum concentration
tPA	Tissue plasminogen activator
TST	Tail skin temperature
USA	United States of America
VTE	Venous thromboembolism

ACKNOWLEDGEMENTS

Life begins at the end of your comfort zone (Neale Donald Walsh).

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Marie Mawet

2020, Liège

CHAPTER 1: INTRODUCTION

1. Combined oral contraceptive: mechanism of action, history and prevalence of use

Combined hormonal contraceptive (CHC) is a class of birth control methods composed of two hormonal compounds: a progestin and an estrogen. These drugs can be administered by different routes including vaginal (using a vaginal ring) and transdermal (using a patch), but the oral route remains currently the most popular route of administration. The oral form of CHC is called "combined oral contraceptive" (COC), commonly referred as the "pill".
The primary mechanism of action of a CHC is the inhibition of the ovulation by a central feedback mechanism exerted by the combination on the hypothalamic-pituitary-ovarian (HPO) axis.

1.1. Menstrual cycle

A spontaneous menstrual cycle is classically divided in two phases (Figure 1). First the *follicular phase*, starting on the first day of the menstruation and ending on the day of ovulation. The follicular phase lasts classically 14 days but its length varies from a woman to another and also varies across a woman's life. The second phase is the *luteal phase*, starting on the day of ovulation and ending on the first day of the next menstruation. In normal circumstances, this phase lasts 14 days and its length is less submitted to inter- and intra-subject variability (Monis and Tetrokalashvili 2020).
Menstrual cycle is controlled at central level by the hypothalamus which releases gonadotropin-releasing hormone (GnRH) in a pulsatile way (Gharib, et al. 1990). For now, the mechanisms regulating GnRH secretion by the hypothalamus are still unknown. Some key mechanisms involved will be detailed hereafter (see Section 1.2).
GnRH stimulates the pulsatile secretion of both gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by the anterior pituitary gland. The follicular phase is characterized by an increasing secretion of FSH. This allows for the development of follicles in the ovaries. Multiple FSH stimulated-Graafian follicles produce estradiol (E2), and consequently, in parallel with the follicular development, E2 serum level increases throughout the follicular phase. At the uterine level, E2 exerts a proliferative action on the endometrial cells which proliferate, and the endometrial glands increase in size. Consequently, the endometrium increases in size and thickness. The estrogen also stimulates crypts in the cervix to produce cervical mucus permeable to sperm cells. During the follicular phase, LH plasma level remains low until approximately 48 hours before the ovulation when LH begins to increase dramatically probably under the influence of the high circulating E2 level. The LH

surge triggers the rupture of one of the follicles (the so called *dominant follicle*) and, consequently, the release of the oocyte. This phenomenon referred to the *ovulation* marks the beginning of the luteal phase (Gharib, et al. 1990; Kanasaki, et al. 2017).

During the luteal phase, the ruptured follicle becomes a corpus luteum, an endocrine structure synthetizing both progesterone and E2. The luteal phase is thus characterized by a rise in serum progesterone level while E2 level plateaus. Both hormones act on the endometrium: the glands increase in size and become actively secretory while the stroma presents an edematous change (Gharib, et al. 1990; Kanasaki, et al. 2017).

If no fertilization occurs, the corpus luteum involutes and both progesterone and E2 production decreases. The hormone drop leads to a constriction of spiral arteries leading to necrosis of the upper layer of the endometrium which sheds. This constitutes the first day of the menstruation and the first day of the next menstrual cycle (Gharib, et al. 1990).

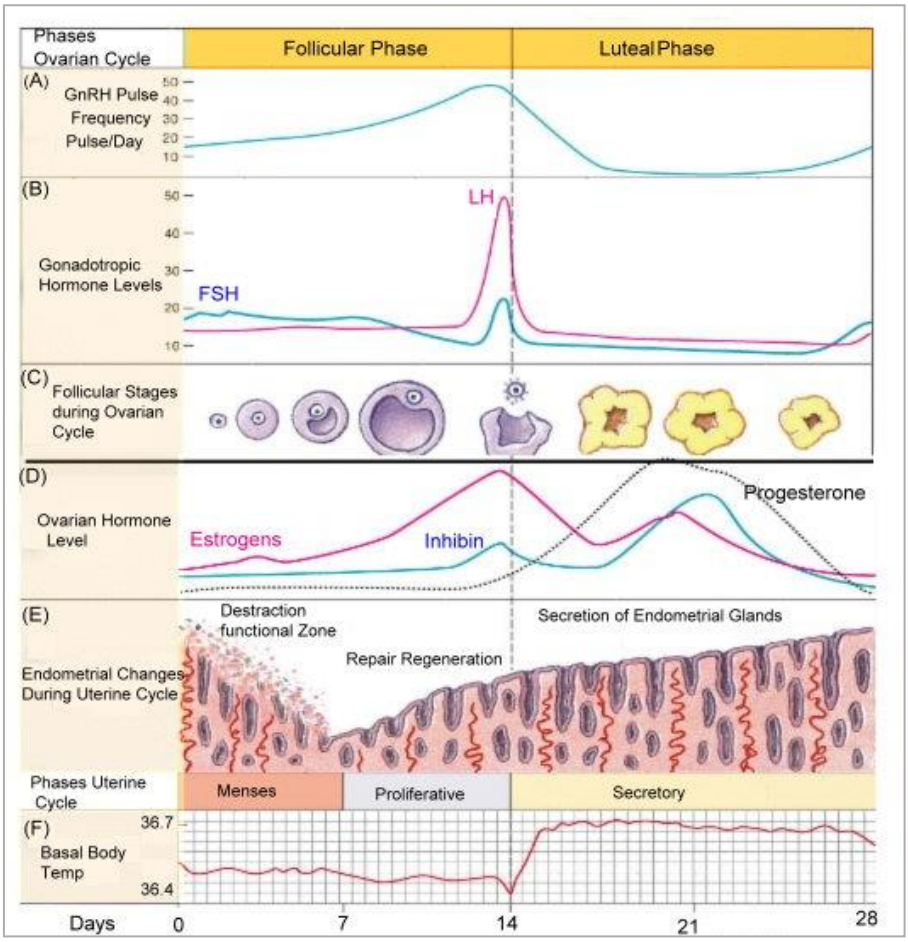

Figure 1. Normal menstrual cycle. GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

1.2. Intra-hypothalamic regulation of GnRH secretion

Estrogens, and particularly E2, play a major role in the feedback mechanisms which regulate GnRH pulsatile secretion by the hypothalamus. However, GnRH neurons do not express estrogen receptors (ERs). This paradox has been partly solved at the end of the 1990's with the discovery of kisspeptin and its key role in the regulation of GnRH secretion (Acevedo-Rodriguez, et al. 2018).

As shown in Figure 2, Kisspeptin, a neuropeptide encoded by Kiss1 gene, is secreted by kisspeptin neurons located in the hypothalamus, either in the arcuate nucleus (ARC) or in the anteroventral periventricular nucleus (AVPV). Kisspeptin binds to Kiss1 receptor. GnRH neurons do not express ERs but do express Kiss1 receptors, while kisspeptin neurons express ERs, mainly estrogen receptor alpha (ERα). Circulating E2 modulates the secretion of kisspeptin, which, subsequently, modulates the secretion of GnRH by GnRH neurons. The negative feedback is done by down-stimulating the kisspeptin secretion by the ARC-derived kisspeptin neurons while the positive feedback leading to the LH surge is driven by the AVPV-derived kisspeptin neurons (Mittelman-Smith, et al. 2012; Oakley, Clifton, and Steiner 2009; Wahab, et al. 2016).

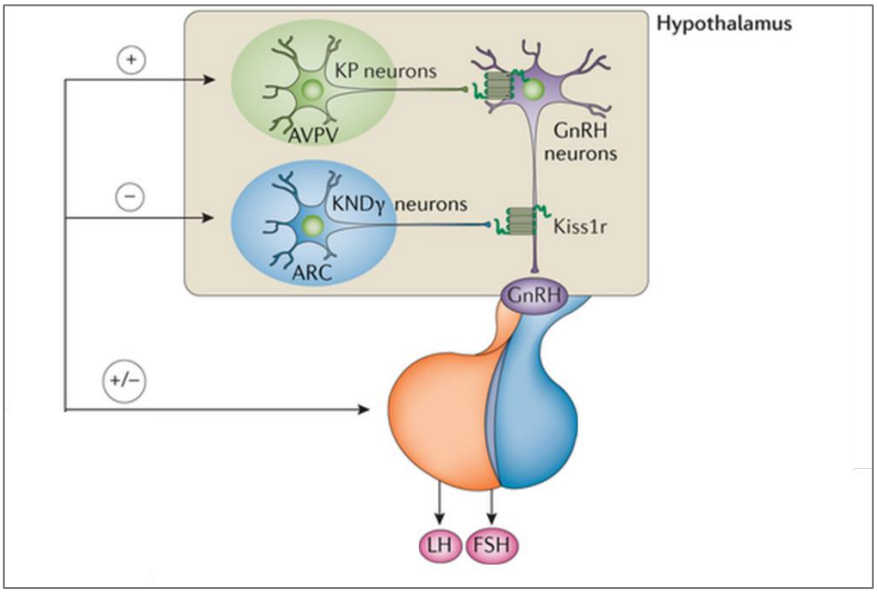

Figure 2. Hypothalamic kisspeptin has an action on the GnRH neurons. GnRH in turn modulates secretion of pituitary LH and FSH. LH and FSH act on ovaries to modulate steroidogenesis and oogenesis. ARC, arcuate nucleus of the hypothalamus; AVPV, anteroventral periventricular nucleus of the hypothalamus; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; KNDγ neurons, KP, kisspeptin; LH, luteinizing hormone (Wahab, et al. 2016).

Although it is now well established that kisspeptin regulates GnRH pulsatility, E2 remains the key element controlling the secretion of kisspeptin by kisspeptin neurons. As proposed by Micevych *et al*., E2 stimulates kisspeptin secretion at different levels (Micevych, Wong, and Mittelman-Smith 2015). First, during proestrus, E2 stimulates directly the transcription of kisspeptin by activating the nuclear and membrane ER present on kisspeptin neurons. Second, E2 also activates in these neurons the nuclear ERα to stimulate the synthesis of the membrane form of the Progesterone receptor (Figure 3, A and C). Third, rising levels of E2 induce the synthesis of progesterone by the neighboring astrocytes (Figure 3, B). This progesterone produced by the central nervous system is called *neuroprogesterone*. Neuroprogesterone, via membrane progesterone receptors present on the kisspeptin neurons, stimulates the secretion of kisspeptin.

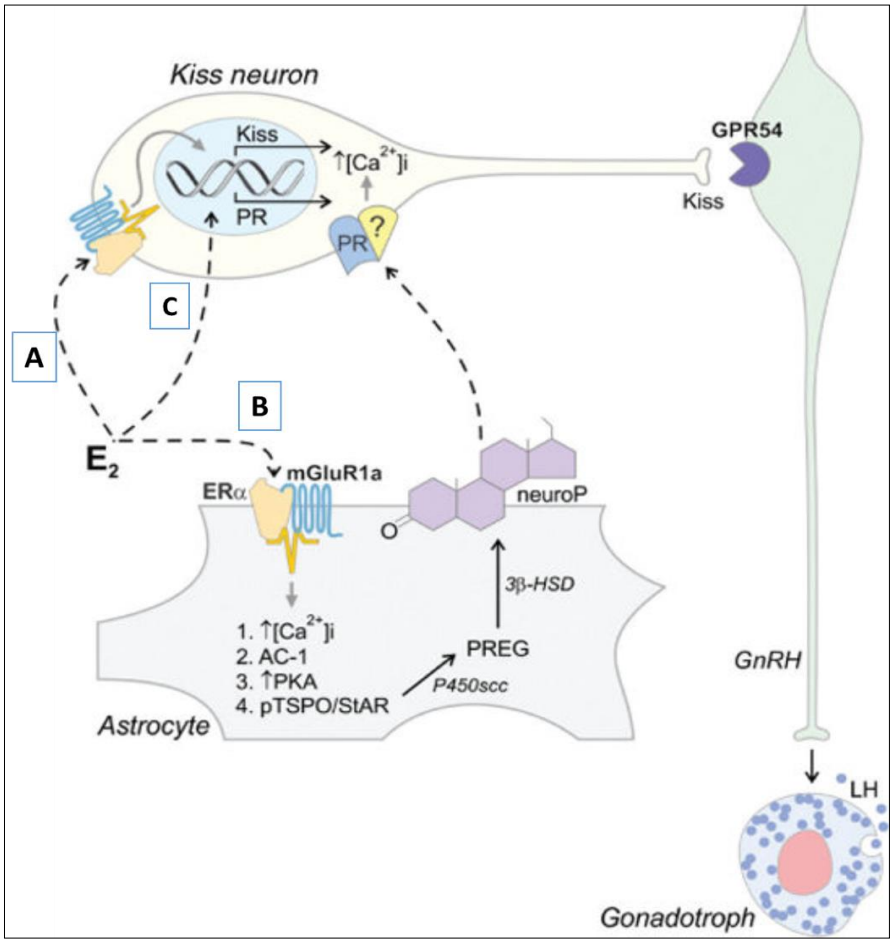

Figure 3. A model showing proposed estradiol (E2) actions on hypothalamic cells. In Kiss1 neurons, E2 acts at both membrane and nuclear estrogen receptors. During diestrus, classical nuclear E2 signaling induces PR expression in kisspeptin (Kiss1) neurons in the RP3V. On proestrus, rising E2 leads to transactivation of mGluR1a in astrocytes, which increases [Ca2+]i leading to stimulation of a kinase cascade resulting in the activation of translocator protein (TSPO) and steroid acute regulatory protein (StAR), which results in an increase of cholesterol import into the mitochondrion—the rate limiting step in steroidogenesis. The resulting pregnenolone (PREG) is converted to progesterone (neuroP) by 3β-HSD. Simultaneously, E2 activates an ERα-mGluR1a complex in neurons leading to the expression of Kiss1. Newly synthesized neuroP diffuses out of the astrocytes and activates E2-induced PR, which have been trafficked to the Kiss1 neuronal membrane. This leads to a series of events culminating in Kiss1 secretion onto GPR54 expressing GnRH neurons. We hypothesize that signaling through the membrane PR involves transactivation of another receptor (indicated by [?]) to stimulate [Ca2+]i, and induce Kiss1 release, activating GnRH neurons and triggering the E2-induced LH surge from anterior pituitary gonadotrophs (Micevych, Wong, and Mittelman-Smith 2015).

1.3. Ovulation inhibition by progesterone, the first step in the development of COC

The first step paving the route of hormonal contraception was the understanding that pregnancy is a period during which no ovulation occurs. It was hypothesized that the high amount of progesterone produced during pregnancy was responsible for the blockade of ovulation. This theory was confirmed in the 1930s when Makepeace *et al*. demonstrated that exogenous progesterone administration was able to block the ovulation in rabbit (Makepeace, Weinstein, and Friedman 1937). In 1957, a first human study was conducted in Harvard by the gynecologist John Rock who administered to 27 women 300 mg progesterone/day for 3 months. Ovulation was inhibited in 85% of the women (Pincus 1958). Subsequently, synthetic compounds with progestogenic activity were developed and tested in women. The goal was to identify a compound with the anti-ovulatory activity of progesterone but at lower dosage. *Noretynodrel* appeared to be the best candidate as it was associated with less androgenic activity than the other compounds in animal studies. It was therefore selected for human evaluation (Garcia, Pincus, and Rock 1956).

1.4. Why combining an estrogen compound to the progestin?

The human studies with the progestin noretynodrel were successful: ovulation inhibition was adequate, and incidence of breakthrough bleeding was considered acceptable. Each month, the subjects were asked to observe a few days pill-free period to allow withdrawal bleeding.
Further analysis of the progestin used in the study revealed that the tested noretynodrel was contaminated by 4-7% of *mestranol*, an estrogenic compound intermediate in the chemical synthesis of Noretynodrel. The product was further purified in order to keep mestranol level at 1%. However, the subsequent study revealed that the bleeding pattern deteriorated. In order to restore a satisfactory bleeding profile, it was decided to intentionally associate mestranol to noretynodrel. The first *combined* oral contraceptive was born (Dhont 2010). In 1969, mestranol was replaced by *ethinylestradiol* (EE) in most COCs. Mestranol is in fact rapidly transformed into its active metabolite EE after absorption. Consequently, a lower amount of EE was needed to achieve the efficacy of mestranol.
Theoretically, both progestin and estrogen components included in COC are able to block the ovulation by exerting a central feedback mechanism at the hypothalamic and pituitary levels leading to a decrease in GnRH pulsatility and, consequently in LH and FSH synthesis and secretion. By abolishing the LH surge, no ovulation occurs. In practice, the progestin component is by far the principal actor in this central feedback activity. It also enhances the contraceptive action by thickening the cervical mucus, avoiding the ascent of the sperm into the upper genital tract. The estrogen component contributes to the central feedback mechanism by reinforcing the activity of the progestin but, as shown in the first studies on COC, its principal role is to prevent unwanted spotting and bleeding due to endometrial atrophy (Nelson, Cwiak, and Cates 2011).

1.5. Prevalence of use

The first COC, Enovid® 5 mg (5 mg Noretynodrel and 75 mcg Mestranol), was approved for contraceptive use by the Food and Drug Administration (FDA) in the United States of America (USA) on February 15th, 1960 (Figure 4). It was initially only approved for married women. Enovid® became rapidly popular demonstrating, if still necessary, the need for reliable and convenient birth control methods: after two years, 1.2 million American women were taking the pill; after three years, the number almost doubled, to 2.3 million (Nikolchev 2010). The first approval for a COC in Belgium occurred in 1968.

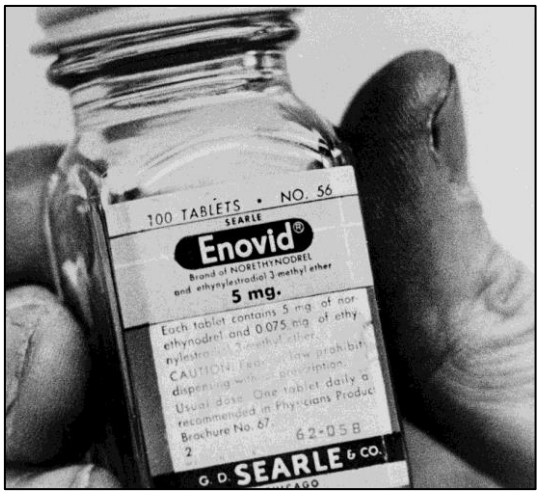

Figure 4. Enovid®, the first commercialized combined oral contraceptive containing 5 mg noretynodrel and 75 mcg mestranol.

Today, it is estimated that worldwide, 8.8% of the married or in-union women aged 15 to 49 years old are using a COC as contraceptive method. Prevalence of COC use is maximal in Europe (23.1% of the women in Belgium, 39.5% in France and almost 50% in Portugal) and in North America (16.0% in the USA). The prevalence of use is increasing in Africa even if it remains relatively low in Middle and Western Africa (2.1 and 3.2% of the women in average, respectively). In Japan and China, the use of COC is historically low (1.1% and 1.2% of the women in average, respectively) (UnitedNations 2015). In the USA, it is the most popular contraceptive method: 4 out of 5 sexually active women have ever used COC. It represented 9 720 000 COC users in the USA in 2012 (GuttmacherInstitute 2020).

2. How to Measure the Efficacy of a Contraceptive Method?

The efficacy of a COC is measured during clinical studies using the *Pearl Index* which is an estimation of the number of unintended pregnancies in 100 woman-years. The Pearl Index is traditionally calculated by dividing the number of observed pregnancies during a clinical trial by the number of months or cycles in the study, and then multiplying by 1200 (when months are the denominator) or by 1300 (when the number of 28-day cycles is the denominator). Thus, a lower Pearl Index is representative of a lower pregnancy risk. The Pearl Index is a practical tool to compare the efficacy of different contraceptive methods, different COCs and, for a same method, different populations (*e.g.* efficacy of COC in obese versus non-obese women) (Pearl 1933).

The *overall Pearl Index* (which takes into account all the pregnancies that have occurred during a clinical trial) is the sum of two Pearl Indexes: the *method failure Pearl Index*, which takes into account only the pregnancies due to an inefficacy of the contraceptive method tested, and the *user failure Pearl Index*, which takes into account only the pregnancies due to incorrect use of the contraceptive method by the subject. In other words, the overall Pearl Index is indicative of the risk of getting pregnant with a given contraceptive method in the "real-life" (also called *typical use*) while the method failure Pearl Index is indicative of the risk of getting pregnant when a given contraceptive method is used correctly (also called *perfect use*).

Table 1 displays the efficacy of different contraceptive methods when used perfectly and in real-life settings. From these data, it appears that the risk of pregnancy with a COC is very low when it is used perfectly (0.3%) while, in practice, the risk dramatically increases to 9% (Trussell 1995).

Table 1. Percentage of women experiencing an unintended pregnancy during the first year of typical use and the first year of perfect use of contraception. Data from the United States of America (Trussell 1995).

	Percentage of women experiencing an unintended pregnancy within the first year of use.	
Methods	***Typical use***	***Perfect use***
No method	85	85
Spermicides	28	18
Fertility awareness – based methods	24	
- **Standard days method**		5
- **TwoDay method**		4
- **Ovulation method**		3
- **Symptothermal method**		0.4
Withdrawal	22	4
Sponge		
- **Parous women**	24	20
- **Nulliparous women**	12	9
Condom		
- **Female (FC)**	21	5
- **Male**	18	2
Diaphragm	12	6
Combined pill and progestin-only pill	9	0.3
Evra® patch	9	0.3
Nuvaring®	9	0.3
Depo-Provera®	6	0.2
Intrauterine contraceptives		
- **Copper T**	0.8	0.6
- **Mirena®**	0.2	0.2
Implanon®	0.005	0.005
Female sterilization	0.5	0.5
Male sterilization	0.15	0.10

The huge difference between the efficacy of COC in case of perfect use and typical use shows that user's compliance is the main driving factor influencing a COC efficacy. To improve this, different parameters may be used: first, by choosing progestin and estrogen compounds with high anti-gonadotropic activity and long half-life (above 24 hours), the risk of pregnancy is

lower because a good ovarian function inhibition is still achieved even in case of missed pill. Secondly, the choice of the therapeutic regimen will influence the efficacy of the method. Finally, improving the safety and tolerability profile of the COC will in turns positively affect subject's compliance.

All these parameters have been taken into account in the development of the different COCs since 1960s, and are further discussed in the sections below.

The main disadvantage of the Pearl Index is that failure rates of birth control methods usually decrease throughout the cycles of use, as the women who are the most likely to rapidly become pregnant drop early from the study for pregnancy reason (this covers mainly non-compliant users but also women for whom the contraceptive method does not work). This decrement in pregnancy rate is not visible with a global score such as the Pearl Index. To overcome this, in addition to the Pearl Index, the *life table method* should also be used to study the contraceptive efficacy of a method. This method is a decrement table displaying a separate efficacy rate for each cycle (or month) of the study (Trussell, et al. 1990). Those data are often displayed in a figure (an example of such a figure is given in Figure 5).

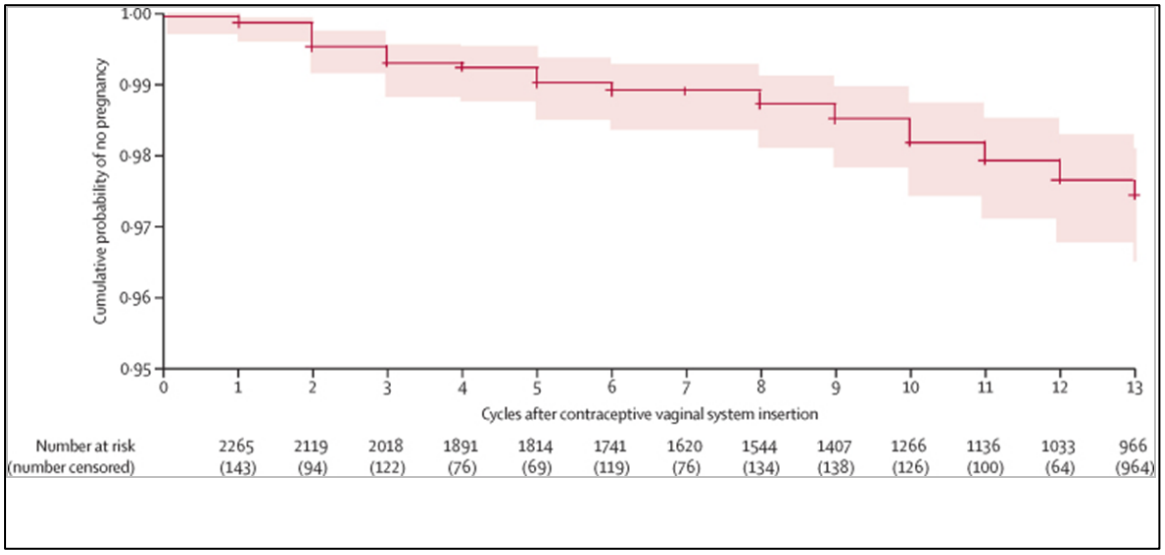

Figure 5. Example of life table analysis: Efficacy of the 1-year (13-cycle) segesterone acetate and ethinylestradiol contraceptive vaginal system (Archer, et al. 2019).

3. Evolution of progestins and their classification

If the estrogen compound has not changed for about 40 years, extensive research was carried out to develop new progestins. The goal was here to decrease the androgenic activity of the first available progestin responsible for unwanted side effects such as acne and hirsutism, likely to decrease subject tolerance and compliance (Burkman, Bell, and Serfaty 2011).

A second important reason to develop new progestins was to solve the deleterious metabolic impact of the first available progestins.

3.1. Progestin classification

The different types of progestins used in combined contraception may be classified in different ways.
A first classification is based on the steroids from which the progestin derivates: testosterone, progesterone or spironolactone (Figure 6).

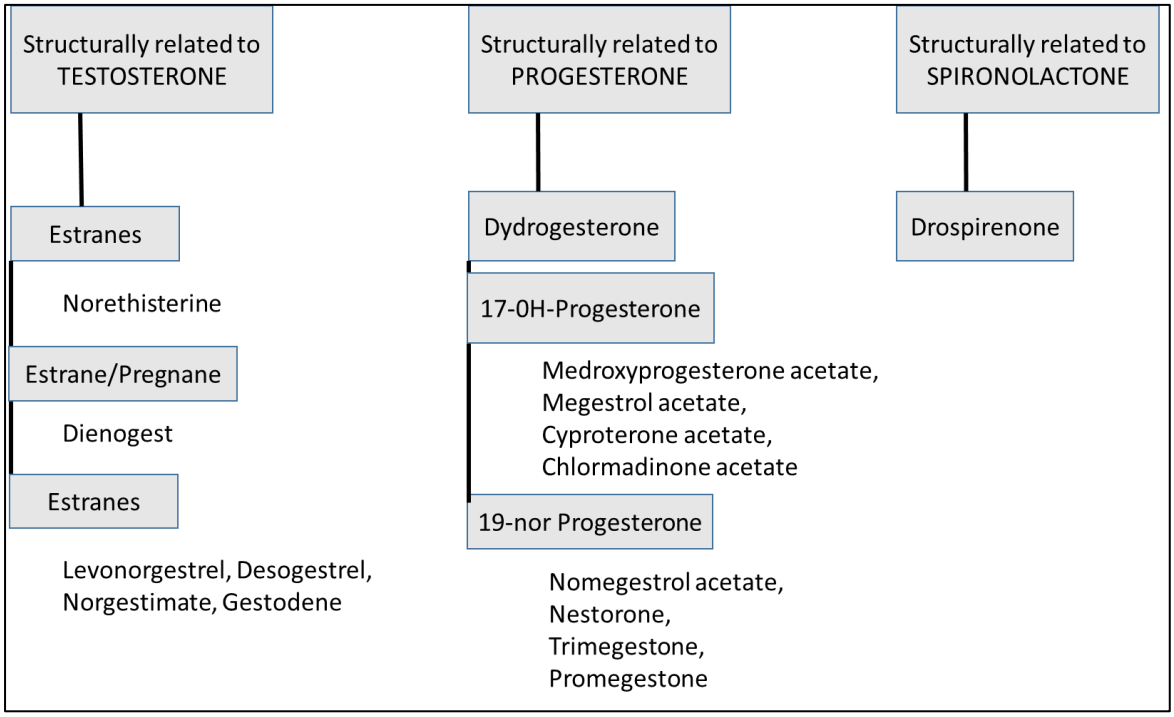

Figure 6. Classification of the available progestins based on the steroids from which they derivate (Sitruk-Ware 2004).

Another way to classify progestins is based on their activity on the steroid receptors (Table **2**). Indeed, progestins do not only interact with the progesterone receptor but also binds to the androgen receptor, ER, glucocorticoid receptor, and mineralocorticoid receptor. Some progestins exert agonist activity on these receptors while other are neutral or antagonist. The affinity for the steroid receptors varies also from a compound to another (Nath and Sitruk-Ware 2009). As a result, each progestin displays a different pattern of steroid activity: for example, levonorgestrel (LNG) is more androgenic than dienogest (DNG) and displays a glucocorticoid activity. In clinical settings, this is translated by a higher incidence of acne, hirsutism, water retention and metabolic impairment in LNG-containing COCs users. *Drospirenone* (DRSP) is a derivative from spironolactone, an anti-mineralocorticoid agent used in the treatment of arterial hypertension. Drospirenone has 8-fold the anti-mineralocorticoid activity of spironolactone and, therefore, less water retention effect is observed with DRSP-containing COCs. As a consequence, blood pressure and body weight are more likely to remain stable with this type of COCs. In addition, DRSP has an antiandrogenic profile leading to a positive impact on acne and hirsutism (see Section 6.2 for more details) (Sitruk-Ware 2004).

Table 2. Biological activities of progestins and interaction with the steroid receptors other than progesterone receptor (Nath and Sitruk-Ware 2009).

Progestogens	*Androgenic*	*Antiandrogenic*	*Glucocorticoid*	*Antimineralocorticoid*
Progesterone	-	+/-	+/-	+
Medroxyprogesterone acetate	+/-	-	+/-	-
Norethisterone	+	-	-	-
Levonorgestrel	+	-	+/-	-
Dienogest	-	+	-	-
Drospirenone	-	+	-	+++
Nomegestrol acetate	-	+/-	-	-
Trimegestone	-	+/-	-	+/-
Nestorone	-	-	-	-

+, effective; +/-weakly effective; -, not effective

In everyday clinical practice, an often-used classification to categorize the different COCs is based on their "generations". This classification is more related to the chronological appearance of the different combinations than on a scientific basis. It is however the most often used classification in clinical settings. *First-generation COCs* includes norethisterone- and norethindrone acetate-containing pills; *second-generation COCs* include LNG-containing pills; *third-generation COCs* include desogestrel (DSG)-, gestodene (GSD)- and norgestimate (NRG)-containing pills; and *fourth generation COCs* include DRSP- or any other new progestin-containing pills (International Planned Parenthood Federation 2013). Although this generation-based classification of COCs is nowadays questioned by experts, it has been in use for almost three decades in the scientific community, including in the most important epidemiological studies, and therefore will be used in this work for the sake of clarity (Creinin and Jensen 2020).

3.2. Impact of COC on lipid and carbohydrate metabolism

Estrogens administrated alone induce an increase in high-density lipoprotein (HDL) cholesterol and a decrease in low-density lipoprotein (LDL) cholesterol, a pattern usually seen as beneficial on a cardiovascular point of view. Estrogens alone however also lead to an increase in triglycerides level. The changes seen in lipids parameters appear to be different when the estrogen compound is combined to a progestin, and they also vary in function of the progestin androgenicity (Beasley, et al. 2012).

The combination of androgenic progestins (*e.g.* LNG) to EE negatively impacts the HDL/LDL cholesterol ratio (LDL-cholesterol increases; HDL-cholesterol decreases). On the opposite, less androgenic progestins, and particularly anti-androgenic progestins such as DRSP, rather maintain the positive changes in cholesterol levels seen with estrogens alone (Gaspard, et al. 2004).

For note, the increase in triglycerides level is higher with anti-androgenic combinations (EE/DRSP or EE/cyproterone acetate) than with more androgenic combinations (Gaspard, et al. 2004; Amiri, et al. 2020).

The impact of COC on carbohydrate metabolism is complex and can be simplified this way:

- Early formulations containing a high dose of EE (≥75 mcg/day) negatively impair glucose tolerance assessed using oral glucose tolerance test. Therefore, reducing the estrogen content in COC has improved glucose tolerance, without completely normalizing it (Wynn, et al. 1979).
- For current low dose-COCs (*i.e.* EE ≤ 35 mcg/day), the degree of glucose tolerance impairment appears to vary in function of the progestin used in the combination: administration of androgenic progestins may cause a state of insulin resistance, as suggested by a slight reduction of oral glucose tolerance accompanied with slight hyperinsulinism. In the opposite, the combination of less androgenic progestins improves the glucose tolerance (e.g. EE/DSG). Gaspard and co-workers even demonstrated that a 1- year treatment with a combination of the anti-androgenic progestin DRSP with 30 mcg EE is neutral on the glucose parameters (Gaspard, et al. 2003).

It was thought that the negative metabolic changes seen with androgenic progestins may worsen the global cardiovascular risk of COC users (Lete, et al. 2015). This was supported by epidemiological data showing that COC use increases the relative risk of arterial thromboembolism (ATE). However, it appeared that it is not the progestin androgenicity that determines this enhanced ATE risk in COC users, but rather the dose of estrogen in the preparation: the relative risk of ATE increases by a factor of 0.9 to 1.7 with COCs containing EE at a dose of 20 mcg, and by a factor of 1.3 to 2.3 with those containing EE at a dose of 30 to 40 mcg (Lidegaard, et al. 2012). In conclusion, the cardiovascular negative effect linked to the metabolic changes of androgenic progestins is currently more theoretical than clinically proven.

To note, smoking and particularly, heavy smoking in women above 35 years old, is one important risk factor of ATE in COC users, and is therefore a contraindication to COC use (CDC 2016).

4. Increased venous thromboembolic risk with combined hormonal contraception

Shortly after the launch of the first COC, reports of an increased incidence of venous thromboembolism (VTE) in COC users were published (Jordan and Anand 1961; Inman, et al. 1970). This remains today the main concern with COC use. Extensive *in vitro*, *in vivo* and epidemiological research has been undertaken in order to understand the mechanism of this complication and to better characterize the risk.
In this thesis, we will first review the normal hemostasis and the common etiology of VTE. We will then focus on the changes in hemostasis associated with COC. A specific attention will be given to the role of the type of progestin on the VTE risk. Finally, we will discuss how this VTE risk is evaluated in clinical studies and the strategies developed to mitigate this risk.

4.1. Overview of Hemostasis

Hemostasis is defined as the physiological response to vascular injury that provides control of blood loss (Castellanos 2011). Hemostasis is a balanced and tightly controlled process divided in three consecutive steps (Morayma Reyes 2019):

- *Primary hemostasis*: formation of a platelet plug at the site of vascular injury. This step necessitates the interaction of the damaged endothelium with von Willebrand factor and platelets.
- *Secondary hemostasis*: stabilization of the platelet plug with fibrin. This second step necessitates the activation of the coagulation factors.
- *Tertiary hemostasis*: dissolution of the stabilized platelet plug via a process called fibrinolysis. This allows to return to a normal endothelium with a normal vessel lumen size.

The complex process leading to the formation of fibrin is called the *coagulation cascade*. For decades, it was thought to be initiated by two distinct pathways (extrinsic and intrinsic pathways), both pathways converging to activate the prothrombinase complex, composed of activated factor X, activated factor V, phospholipids, and calcium. This complex transforms inactive prothrombin into active thrombin that converts fibrinogen into fibrin. The role of platelets in coagulation was considered independent. Nowadays, this theory is abandoned in favor of the *cell-based model of coagulation cascade* which starts with the exposure of tissue factor from tissue factor-bearing cells once blood vessels are damaged. The complete process requires 3 phases as described in Figure 7 (Perez-Gomez and Bover 2007; De Caterina, et al. 2013).

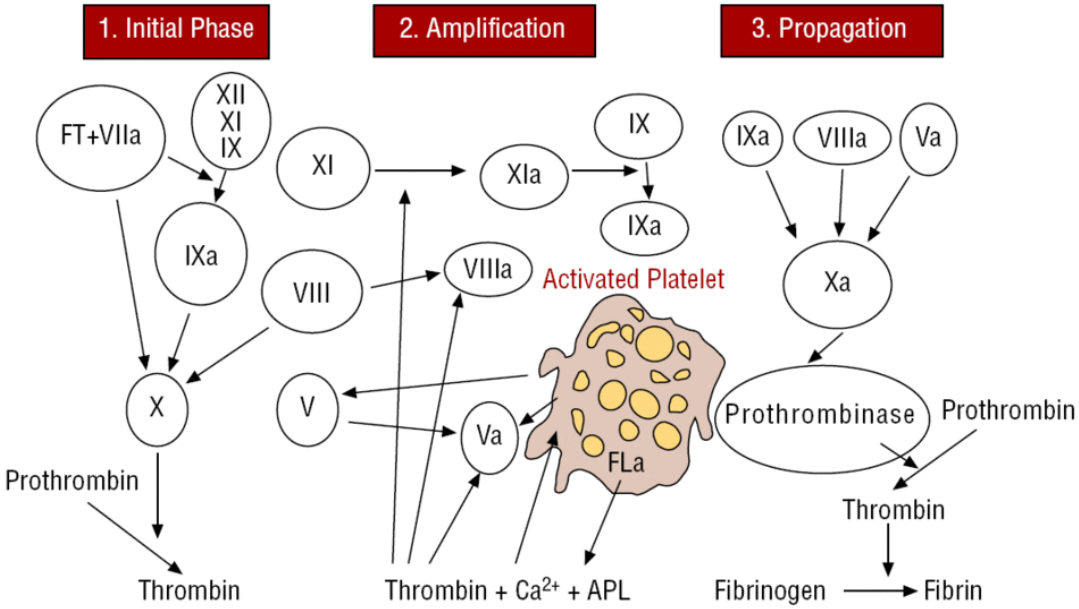

Figure 7. The new coagulation cascade (Perez-Gomez and Bover 2007):
<u>Initial Phase:</u> The tissue factor-factor VII complex activates factor X, either directly or indirectly via factor IX, and transforms prothrombin into thrombin in small amounts that are insufficient to complete the process of fibrin formation.
<u>Amplification Phase:</u> The thrombin that has been formed, along with calcium from the blood and negatively charged phospholipids derived from platelets, actively participates in a positive feedback process for the activation of factors XI, IX, VIII, and V, and, especially, to accelerate platelet activation. Simultaneously, the factors mentioned are attracted to the surface of the platelets, where very rapid and extensive activation and amplification occurs.
<u>Propagation Phase:</u> The amplification of the process through feedback mechanisms involving thrombin and platelets and the activation of all these factors allow large quantities of factor X to be activated and form the prothrombinase complex to convert prothrombin into thrombin and, through the action of thrombin, fibrinogen into fibrin. The final process, always occurring on the surface of the platelets, accelerates and leads to the explosive generation of large quantities of thrombin and fibrin.

An exaggerated propagation of the thrombus is prevented by the natural anticoagulants (mainly, tissue factor pathway inhibitor [TFPI], protein C, protein S and, antithrombin), and by the fibrinolysis. Table 3 summarizes their respective activity (De Caterina, et al. 2013).

Table 3. Factors involved in the inhibition of blood clot formation or degradation of blood clot.

Factor	Activity
Tissue factor pathway inhibitor	Inhibits the Tissue Factor/VIIa complex
Antithrombin	Inhibits thrombin, IXa, Xa, XIIa. Its activity is largely enhanced in the presence of heparin (physiologically secreted by mast cells).
Activated Protein C	Inhibits VIIIa and Va.
Protein S	Cofactor of Protein C and possesses also its own anticoagulant activity.
Plasmin	Breaks down fibrin.

a, activated

Fibrinolysis starts with the release of tissue plasminogen activator (tPA) from damaged or activated endothelial cells. Tissue plasminogen activator and circulating urokinase stimulate the conversion of circulating plasminogen into plasmin, an enzyme able to degrade fibrin into fibrin degradation products (*e.g.* D-dimer). Fibrinolysis is inhibited and controlled by different proteins: plasminogen activator inhibitor-1 and 2 (PAI-1 & 2) inhibit tPA and urokinase. PAI-1 itself is inhibited by activated protein C (APC) and upregulated by interleukine-6 (IL-6) a proinflammatory cytokine which downregulates the expressions of APC. APC is therefore an enhancer of fibrinolysis while IL-6 generates an hypofibrinolytic environment. Fibrin itself can bind tPA, greatly enhancing its ability to convert plasminogen to plasmin. A simple overview of fibrinolysis is depicted in Figure 8 adapted from Harvey (Harvey 2012).

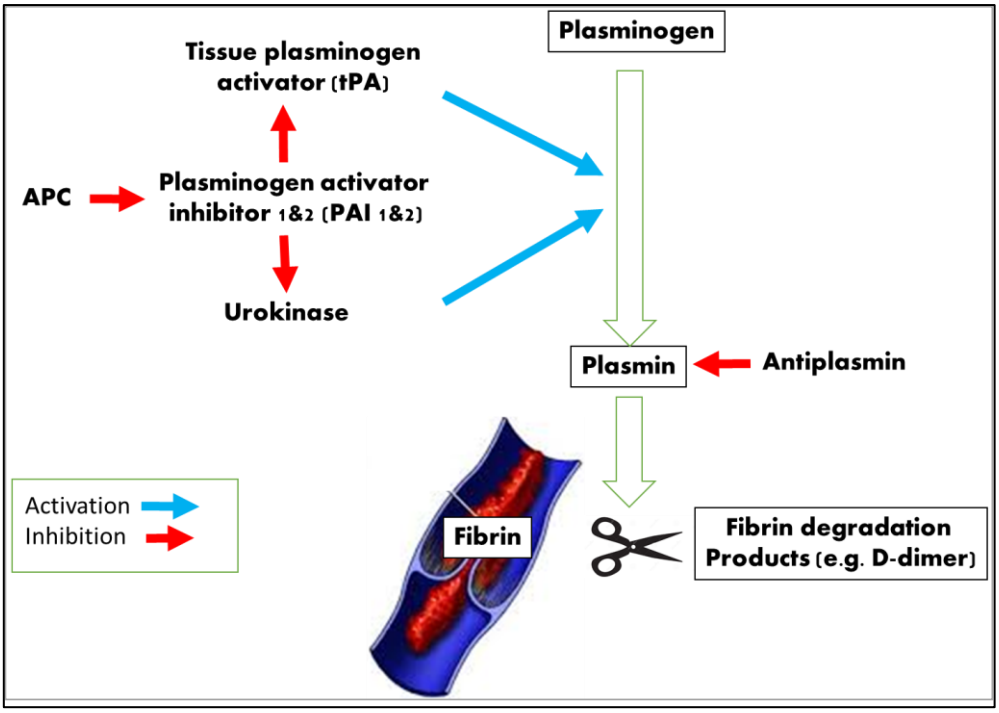

Figure 8. Simplified fibrinolysis. APC, activated Protein C; PAI 1&2, plasminogen activator inhibitor 1 and 2; tPA, tissue plasminogen activator (figure adapted from Harvey 2012).

4.2. Etiology of VTE under COC

4.2.1. Etiology and risk factors of VTE

Uncontrolled hemostasis may lead to the formation of a blood clot that obliterates a vein, a condition called *venous thromboembolism* (VTE). As initially suggested by Rudolf Virchow (1821-1902), three conditions predispose a subject to develop VTE (the Virchow's triad) (Kushner, West, and Pillarisetty 2020):

1. Endothelial damage
2. Blood stasis
3. Hypercoagulability (also referred as *thrombophilia*)

Nowadays, evidence suggests that endothelial damage plays an insignificant role in the development of VTE. Blood stasis and thrombophilia are thus the main pathogenic factors leading to thrombus formation in the veins (Mannucci and Franchini 2015). Blood stasis may be the consequence of different conditions (*e.g.* heart failure, immobilization, severe liver disease, etc); thrombophilia may be acquired (*e.g.* chronic inflammation, malignancy, obesity, etc) or inherited. The inherited thrombophilia can be grouped in three categories:

- Mutation of genes that encode for natural anticoagulant (antithrombin, protein C and protein S);
- Mutations of factor V Leiden (the most common one is called *Factor V Leiden*, less frequent mutations are Factor V Cambridge and Factor V Hong-Kong);
- Mutation of prothrombin (essentially the G20210A mutation).

Table 4 summarizes the risk factors for VTE development. Importantly, most of the patients with a VTE cumulate several risk factors at the time of VTE onset (*e.g.* inherited thrombophilia and COC use, or malignancy and immobilization) (Bauer 2020).

Table 4. Risk factors for the development of venous thromboembolism (Bauer 2020).

Inherited thrombophilia	Acquired risk factors
Factor V Leiden	Central venous catheter
Prothrombin G20210A mutation	Malignancy
Protein S deficiency	Surgery (especialy orthopedic)
Protein C deficiency	Trauma
Antithrombin deficiency	Immobilization
	Pregnancy
	Combined hormonal contraceptive
	Hormonal replacement therapy
	Chemotherapy
	Heart failure
	Antiphospholipid syndrome
	≥65 years
	Obesity
	Severe liver disease
	Inflammatory bowel disease
	Nephrotic syndrome

4.2.2. Effects of COC on hemostasis

The reason why COC use is associated with an increased VTE risk is still not fully understood, but all studies consider the estrogen as the principal responsible for this increased risk. The progestin modulates this risk as we will explain later (see section 4.6).

The central role of estrogen in the increased VTE risk relies on two observations: first, the VTE risk is correlated with the estrogen dose and, secondly, the progestin-only contraceptives are not associated with an increased risk of VTE (Piper and Kennedy 1987; Gerstman, et al. 1991; Lidegaard, et al. 2011).

Estrogens modify the liver synthesis of proteins, including the proteins of the coagulation. This effect varies with the type of estrogen. For example, estrone (E1) and EE stimulate the hepatic protein synthesis stronger than E2 (Lindberg, et al. 1989; Bagot, et al. 2010). It also varies with their route of administration: taken orally, E2 is converted to E1 in the intestine and liver. This conversion does not occur when E2 is administrated by the transdermal route. This absence of conversion in E1 explains the more neutral hepatic impact of dermally administrated E2 in comparison to oral administration (Bagot, et al. 2010).

In line with this, the administration of an EE-containing COC significantly modifies the levels of most of the hemostasis parameters. Table 5 below summarizes the changes observed with the administration of an EE-containing COC: it stimulates the production of several pro-coagulant factors while it inhibits the natural anticoagulant system (Private work from J. Douxfils, not published). As described in the above sections, a subtle balance between coagulation and anti-coagulation is required to avoid exaggerated thrombus formation. It appears that COC creates an imbalance favoring an hypercoagulable state, which is probably the key element to explain the higher VTE risk in COC users. This over activation of coagulation is also translated by the general increase in blood markers of coagulation activation (mainly D-dimers and prothrombin fragment 1+2), and by a reactional increase in the fibrinolysis process, biologically translated by an increase in tPA and a decrease in PAI-1 (Alhenc-Gelas, et al. 2004).

Importantly, these hemostasis changes are maximal during the first year of COC use, and appear to remain stable afterwards. Interestingly, the risk of VTE has also been demonstrated to be maximal during the first year of treatment (Bauer 2020; Alhenc-Gelas, et al. 2004).

Table 5. Changes in hemostasis parameters observed after the administration of ethinylestradiol-containing combined oral contraceptive.

Hemostasis parameter	EE-COC Effect
Coagulation factors	
Fibrinogen	↑
Prothrombin	↑
Factor V	= or ↓
Factor VII	↑
Factor VIII	= or ↑
Factor IX	↑
Factor X	↑
Factor XI	↑
Anticoagulant factors/processes	
Antithrombin	= or ↓
Protein C	= or ↑
Protein S	↓or = or ↑
Tissue factor pathway inhibitor	↓
APCsr (aPTT-based)	↓
APCsr (ETP-based)	↑
Fibrinolysis	
Tissue plasminogen activator	↑
Plasminogen	↑
A2-antplasmin	↑
Plasmin-α2antiplasmin complex	↑
Plasminogen activator inhibitor 1	↓
Coagulation activation markers	
D-dimer	↑
Prothrombin fragment 1+2	↑

ETP, endogenous thrombin potential; aPTT, activated partial thromboplastin time; EE, ethinylestradiol; COC, combined oral contraceptive.

4.2.3. COC use, a model of drug acquired thrombophilia

Mounting evidence suggests that the hemostasis changes seen in COC users are very close to those seen in subjects with the inherited thrombophilia *Factor V Leiden*. This is particularly true for third and fourth generation COCs.

Factor V Leiden thrombophilia results from an amino-acid substitution at nucleotide 1691 in the gene coding for the pro-coagulant factor V. This mutation confers a resistance to the natural anticoagulant activated Protein C (APC). This condition is called *APC resistance (APCr)*. As explained above, APC is a natural anticoagulant that cleaves and inactivates both activated factor V and VIII. In Factor V Leiden subjects, the inactivation of activated factor V is 10-fold slower than normal. This results in a prothrombotic state associated with elevated levels of D-dimer and Prothrombin F1+2.

Factor V Leiden is the most common inherited thrombophilia. Its prevalence is particularly high among Caucasians (in USA and European populations) and very low in Asian, African and indigenous Australian population. Prevalence in Europe is marked by a North-South gradient: the mutation is found in 10-15% of the population in Sweden, 7.1% in Northeastern France and 2-3% in Spain and Italy. Clinically, factor V Leiden is found in 20-25% of the subjects with VTE.

The diagnosis of Factor V Leiden is done with a coagulation screening test (APCr assay) and is confirmed genetically by the mutation on the Factor V gene (Kujovich 2011).

Combined oral contraceptive use is associated with an increase in APCr, even in the absence of Factor V Leiden. It is therefore suggested that the increased VTE risk associated with COC has the same physiopathologic determinism as the increased VTE risk seen in Factor V Leiden subjects; COC use being seen as a form of *acquired thrombophilia* (Alhenc-Gelas, et al. 2004). Estrogens lower the protein S which is an important co-factor of APC (Caine, et al. 1992). This is probably the mechanism by which COC confers resistance to APC (Hugon-Rodin, et al. 2017).

4.3. First attempt to reduce the VTE risk associated with the use of COC: lowering the estrogen content

It was thought that the use of EE instead of mestranol would improve the safety profile of COCs, particularly regarding the VTE risk (Aronson 2009). The initial EE dose used in COC was 150 mcg/day. There is no evidence that moving from mestranol to EE has significantly decreased the VTE risk.

To effectively reduce the VTE risk, a relevant approach consisted in a progressive reduction in EE content from >75 mcg down to 15 mcg EE. This was suggested to offer a better protection against VTE as shown in the epidemiologic study conducted by Böttiger and colleagues demonstrating that the incidence of VTE among COC users decreased in parallel to the progressive increased sales of COCs with a lower EE dose (Figure 9) (Bottiger, et al. 1980).

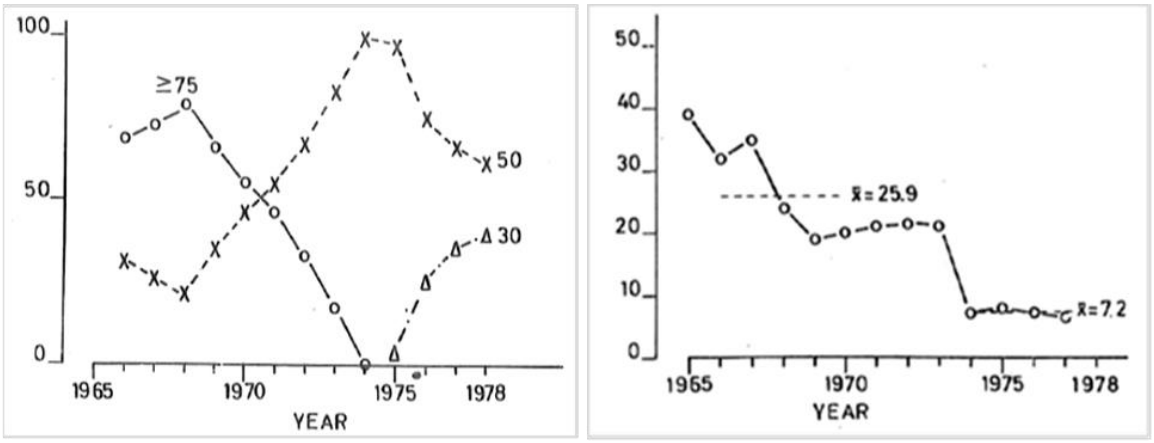

Figure 9. On the left, market share for COCs containing high (≥ 75 mcg), moderate (50 mcg) and low EE doses (30 mcg) in Sweden from 1966 to 1978. On the right, VTE episodes reported to the Swedish Adverse Drug Reaction Committee from 1965 to 1977 (per 100 000 COC users) (Bottiger, et al. 1980).

Beside the lower VTE risk, lowering the EE content in COC was also found to be beneficial for general tolerance to COC: lower complaints of estrogen-related adverse effects such as bloating, weight gain, breast tenderness and nausea were reported with the low estrogen combinations (Rosenberg, Meyers, and Roy 1999). Importantly, the contraceptive efficacy has not been affected by this reduction in estrogen content as shown in the efficacy studies performed with low dose COCs and summarized in a meta-analysis from the Cochrane group (Gallo, et al. 2013).

Nowadays COCs containing 20 to 35 mcg EE are most widely prescribed. The 30 mcg EE-containing COCs seem associated with a higher risk of VTE than contraceptives containing 20 mcg (Lidegaard, et al. 2009; van Hylckama Vlieg, et al. 2009). However, COCs containing 20 mcg EE or less are associated with an higher risk of bleeding disturbances than COCs containing 20 mcg EE (both amenorrhea or infrequent bleeding and irregular, prolonged, frequent bleeding, or breakthrough bleeding or spotting). This bleeding pattern issue precludes a further reduction of the EE content.

4.4. The role of Progestins on the VTE risk

4.4.1. Implication of progestin type on the VTE risk

Since the 1990s', several large studies have been conducted to assess the VTE risk with COC use. Studies were essentially conducted with second, third and fourth-generation COCs. The principal question that all these studies try to answer is the following: "In terms of VTE risk, is one COC generation safer than the other generations?". Three types of studies attempt to answer this question: 1) prospective randomized controlled studies, 2) observational cohort studies, and 3) reviews/meta-analyses integrating the two previous types of studies.

The principal studies (along with the study type, date and results) comparing the venous thromboembolic effects of EE/DRSP versus EE/LNG are listed in Table 6. This table illustrates the complexity of this problematic and explains why no clear answer to the above question is

currently possible. In general, prospective studies show an absence of increased risk or a slight increased risk of VTE with EE/DRSP in comparison to EE/LNG. It is however important to note that the treatment effects are associated with a 95% confidence interval which often incudes 1, suggesting a lack of robustness of the results. On the opposite, all retrospective studies conclude to an increased risk of VTE with EE/DRSP versus EE/LNG (ranging from 1.45 to 2.12). Based on the 95% confidence intervals (all above 1, except for one study), these studies seem more robust. These data have been extensively debated in the literature and there is no doubt that ink will continue to flow on this topic in the coming years.

Table 6. Studies evaluating venous thrombotic effects of ethinylestradiol/drospirenone versus ethinylestradiol/levonorgestrel

Study	Data origin	Study Period	Number of subjects treated with EE/DRSP	Number of subjects treated with EE/LNG	Treatment effect	95% CI
Prospective studies						
Dinger 2007	EURAS Study	2000-2005	16 534	15 428	HR: 1.0	0.6-1.8
Vlieg 2009	MEGA Study	1999-2004	33	858	OR: 1.7	0.7-3.9
Dinger 2010	German primary care sector	2002-2008	109	257	OR: 1.0	0.5-1.8
Jick 2011	PharMetrics database	2002-2008	434	433	OR: 2.4	1.7-3.4
LASS 2011	EURAS study + 5 year LASS extension	2000-2010	16 534	15 428	HR: 1.1	0.8-1.7
Parkin 2011	UK general practice reasearch database	2002-2010	43	233	OR: 3.3	1.4-7.6
Bergendal 2014	Thrombo embolism hormone study	2003-2009	66	173	OR: 2.0	0.9-4.3
Dinger 2014	INAS-OC study	2005-2013	15 542	10 254	HR: 1.3	0.63-2.5
Vinogradova 2015	QResearch & CPRD	2001-2013	611	3 923	OR: 1.75	1.43-2.12
Retrospective studies						
Lidegaard 2009	Four Danish registries	1995-2005	NR	NR	IRR: 1.64	1.27-2.10
FDA 2011	Kaiser Permanent (North & South Carolina) & the Medicaid Program (Washington & Tenessee)	2001-2007	142 166	198 839	HR: 1.45	1.15-1.83
Gronich 2011	Clalit clinical database	2002-2008	56 429	16 500	IRR: 1.65	1.02-2.65
Lidegaard 2011	Four Danish registries	2001-2009	NR	NR	IRR: 2.12	1.68-2.66
Sidney 2013	Kaiser Permanent (North & South Carolina) & the Medicaid Program (Washington & Tenessee)	2001-2007	109 070	137 311	HR: 1.57	1.13-2.18
Ziller 2014	IMS HEALTH Database	2005-2010	15 572	13 222	OR: 1.57	0.46-5.38

EE, ethinylestradiol; DRSP, drospirenone; LNG, levonorgestrel; CI, confidence interval; NR, not reported; HR, hazards ratio; OR, odds ratio; IRR, incidence rate ratio (Dinger, et al. 2010; Dinger, Bardenheuer, and Heinemann 2014; Dinger, Heinemann, and Kuhl-Habich 2007; Ziller, et al. 2014; Lidegaard, et al. 2009; Lidegaard, et al. 2011; Gronich, Lavi, and Rennert 2011; Sidney, et al. 2013; Jick and Hernandez 2011; van Hylckama Vlieg, et al. 2009; Bergendal, et al. 2014; Parkin, et al. 2011; Vinogradova, Coupland, and Hippisley-Cox 2015; FDA 2011)

The main advantage of observational cohort studies is undoubtedly their size: these studies encompass several hundred thousands of subjects, while prospective studies generally randomize several thousands of subjects. However, due to the retrospective nature of observational studies, they may be associated with biases (Larivee, et al. 2017):

1. The prevalent user bias: the different generations of COCs have arrived on the market at different times from 1970' (second generation) to 2000' (fourth generation). It is widely accepted that the risk of VTE is highest during the first months of COC use, and this is seen with all COC types. Due to their more recent appearance on the market, it is suspected that newer products may appear to have a higher risk, as a higher proportion of users of these products are short-term users compared with long-term users of older products.
2. The VTE misclassification bias: retrospective assessment of a VTE case may be difficult as the event generally occurred a few years before the study, the data may be not available to the authors or are very scares.
3. The confounding risk factors bias: as displayed in Table 4, a series of conditions exist that may impact a subject's risk of developing a VTE: intrinsic factors (*e.g.* age, body mass index [BMI], and pro-thrombotic genetic mutations) and extrinsic factors (*e.g.* immobility, surgery, and cast). Here again, it is thought that those confounding risk factors are better controlled in prospective studies than in retrospective studies.

In addition to the conflicting data reported in individual studies, the reviews and meta-analyses done on this topic have also generated inconsistent results, leading to new debates (Larivee, et al. 2017; Lidegaard 2018). In this thesis, we have decided to present the results obtained through a meta-analysis performed by the Cochrane group in 2014 because this review is considered strong and not much debated in the literature. It illustrates the relative risk with different COCs (Figure 10). In comparison to non-COC users, the risk of developing a VTE under COC treatment is in average doubled with second-generation COCs, multiplied by 3.5 with third generation COCs and multiplied by 4 with DRSP-COCs (fourth generation). To translate these data in actual values, if we assume that the risk of VTE in the general population of pre-menopausal women is 2.5/10 000 women-years (CI: 1.1-5.6), using second-generation COCs will lead to an additional 2.5 /10 000 women-years VTE case while the use of fourth generation will lead to an additional 7.5/10 000 women-years (de Bastos, et al. 2014).

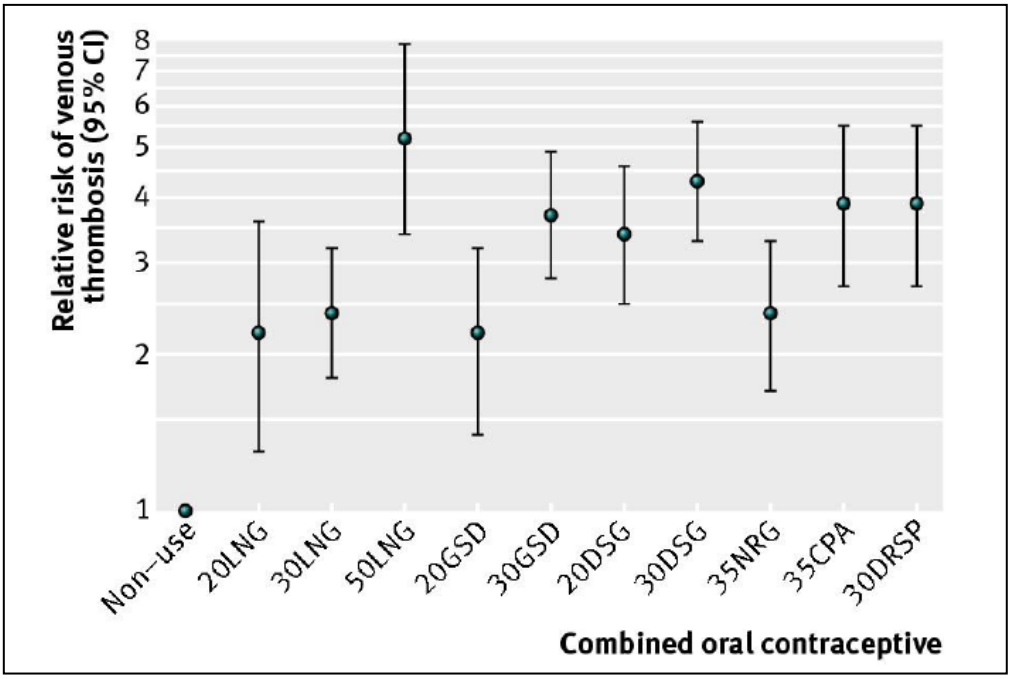

Figure 10. Overall relative risk (with 95% confidence interval) of developing VTE with different COCs in comparison to non-users. LNG, levonorgestrel; GSD, gestodene; DSG, desogestrel; NRG, norgestimate; CPA, ciproterone acetate; DRSP, drospirenone (de Bastos, et al. 2014).

Altogether, and although the risks differ between studies, from the large amount of results currently available, and based on the changes induced by the different COCs on the hemostasis parameters, it is reasonable to suggest that third and fourth generation COCs are associated with a higher VTE risk than second-generation COCs.

4.5. Benefit/risk balance

As described in the above section, developing a VTE under COC treatment remains a rare event (risk 2- to 4-fold higher than in non-users). In comparison, the risk is higher during pregnancy (4- to 5-fold higher than in non-pregnant women) and much higher during the 6 weeks that follows delivery (20-to 80-fold higher than in non-pregnant women) (James 2017). As COC use is very efficient in avoiding pregnancy, the benefit largely overweight the risk. However, COC users are in general young and healthy women, a population in which serious drug reaction is particularly undesired.

It is estimated that one third of deep vein thrombosis (DVT) cases will be complicated by a pulmonary embolism. Pulmonary embolism will lead to death in 9.7% to 12% of the cases. Beside this fatale outcome, 5% of the patients with pulmonary embolism will develop chronic pulmonary hypertension, a condition that requires long term management. It is also estimated that half of the patients with a DVT will develop a post-thrombotic syndrome characterized by

pain, heaviness and swelling of the damaged leg (particularly in the standing position) (de Bastos, et al. 2014).
A positive personal history of VTE will also necessitate the prescription of an antithrombotic prophylaxis during the subsequent pregnancies and post-partum times due to the higher risk of recurrence during this critical periods (van Vlijmen, et al. 2016).

4.6. The concept of estrogenicity

As progestin administered alone does not increase the VTE risk, it may be difficult to understand how the type of progestin used in a COC has the capacity to modify the resultant VTE risk of the combination. In that respect, the concept of *estrogenicity of a COC* may provide a good explanation (van Rooijen, et al. 2004).
The estrogenicity of a COC is the sum of the estrogenicity of both estrogen and progestin compounds: the estrogenicity depends on the potency and the dose of the estrogen compound and is modulated by the androgenic activity of the progestin. Consequently, an androgenic progestin, such as LNG, will display an anti-estrogenic profile which results in a lowering of the total estrogenicity of the preparation. In the opposite, a less androgenic progestin, such as DSG, or even, an anti-androgenic progestin, such as DRSP, will less (or even not in the case of DRSP) impact the estrogenicity associated with the estrogen compound and, consequently, the resultant estrogenicity remains high.
As described above, epidemiology shows that COC use increases the VTE risk and also that third and fourth generation COCs (*i.e.* higher estrogenic preparations) may be associated with a higher VTE risk than second generation COCs (*i.e.* lower estrogenic preparations). This is probably due to the direct impact of the estrogen environment on the synthesis of liver proteins, including synthesis of hemostasis parameters.

4.7. Biological surrogate markers of VTE risk

Given the very low incidence of VTE among COC users, only very large epidemiological studies encompassing several hundred thousand of subjects are able to assess the real risk of VTE associated with a specific COC. This type of study lasts several years, necessitates the participation of hundreds study centers, and therefore are not feasible before the market launch of the COC.
For the sake of rapidity, the ideal would be to define one or various biological markers that can predict the VTE risk with a specific COC in a smaller population (less than 100 subjects). Further, an easy and economically acceptable surrogate marker could be used to identify those women who are particularly at risk of developing VTE under COC, and consequently the physician could orient them to a safer product or an alternative contraceptive method. A series of markers have been tested so far and among all of them, two deserve attention: sex hormone binding globulin (SHBG) and ETP-based APCr.

Sex hormone binding globulin (SHBG)

Sex hormone binding globulin is a carrier protein transporting sex steroids in the blood. It is thus not a hemostasis parameter. Sex hormone binding globulin is produced by the liver and its production is proportional to the concentration in circulating sex hormones. Administration of a COC increases significantly the level of SHBG, and, interestingly, this increase seems to be proportional to the estrogenicity of the preparation: highly estrogenic COCs (third and fourth generations) are associated with a significantly higher increase in SHBG than the second generation COC. Some authors have also shown a correlation between the level of SHBG increase associated with a COC and the VTE risk seen in epidemiologic studies (Figure 11) (Odlind, et al. 2002).

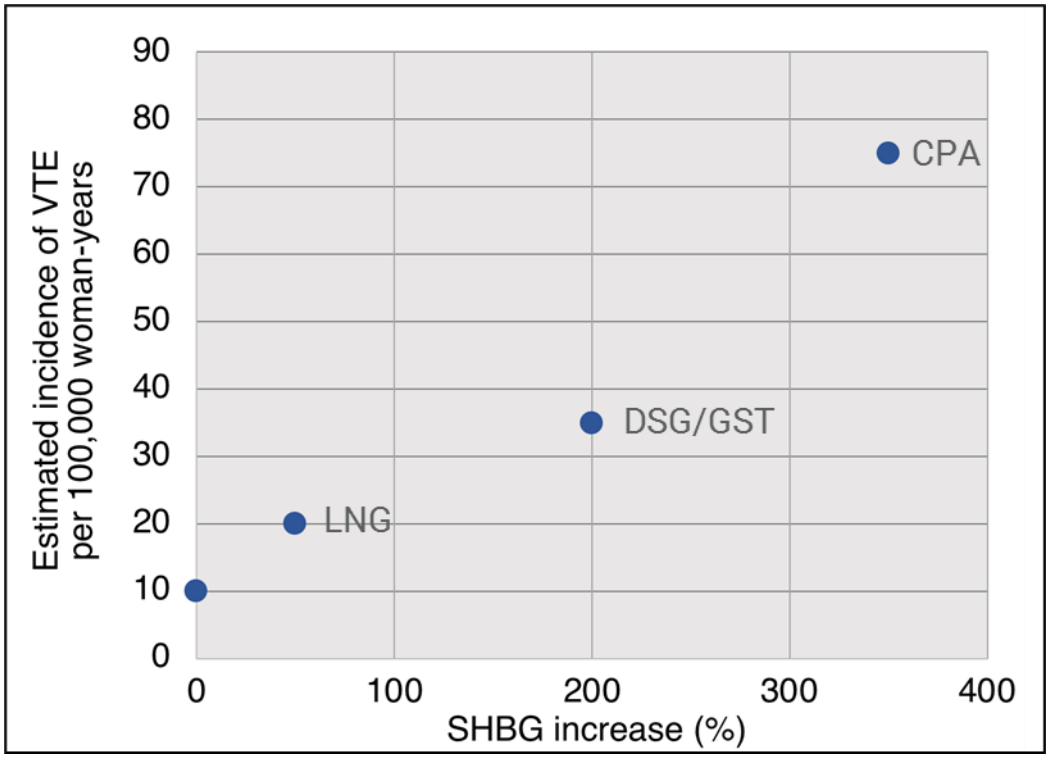

Figure 11. Estimated incidence of venous thromboembolism per 100,000 woman-years in relation to reported average increase in sex hormone binding globulin in untreated women and in women using combined preparations with 30–35 mg ethinyl estradiol and levonorgestrel, desogestrel/gestodene and cyproterone acetate (Odlind, et al. 2002). VTE, venous thromboembolism; LNG, levonorgestrel; DSG, desogestrel; GST, gestodene; CPA, cyproterone acetate; SHBG, sex hormone binding globulin.

ETP-based Activated Protein C resistance (APCr)

Different assays have been developed to assess the APCr of a subject. In the area of hemostasis changes induced by estrogens, two assays are particularly popular, namely:

1. activated partial thromboplastin time (APTT)-based APCr
2. endogenous thrombin potential (ETP)-based APCr.

APTT-based APCr consists in measuring the APTT in the presence of APC. The APTT is the time taken for a fibrin clot to form. This test is used to measure the activity of the intrinsic and

common pathways of the coagulation. In line with its anticoagulant activity, adding APC to the plasma will prolonge the APTT of the subject. In contrast, in a plasma with reduced APC sensitivity (*e.g.* Factor V mutations or COC use), the prolongation in the clotting time is less (Practical-Haemostasis 2020).

In practice, a normalized test is used to calculate the APTT-based APCr:

$\frac{APTT+APC}{APTT-APC}$ done with the plasma of the subject

divided by $\frac{APTT+APC}{APTT-APC}$ of a normal plasma pool

To be relevant, this test must be done in subjects who have a normal APTT which is not the case of subjects treated with COC. In addition, the test that was proposed initially was based on a simple APTT which is no longer the case for the test that are currently on the market. These tests aimed at assessing Factor V Leiden and therefore, strategies are used to make the tests insensitive to the presence of deficiencies or higher levels of coagulation factors, except for Factor V (*i.e.* by mixing the plasma of the subject with a factor V deficient plasma). Therefore, the usefulness of this test is limited (Practical-Haemostasis 2020) due to its lack of sensitivity towards the factor affected by COC and important in the setting of APCr, *i.e.* factor VIII, protein S and TFPI.

A more relevant assay in this context is the ETP-based APCr which quantifies the effect of APC on the endogenous thrombin potential (ETP) which is the area under the thrombin generation curve obtained during a thrombin generation assay from the patient (Figure 12) (de Visser, et al. 2005).

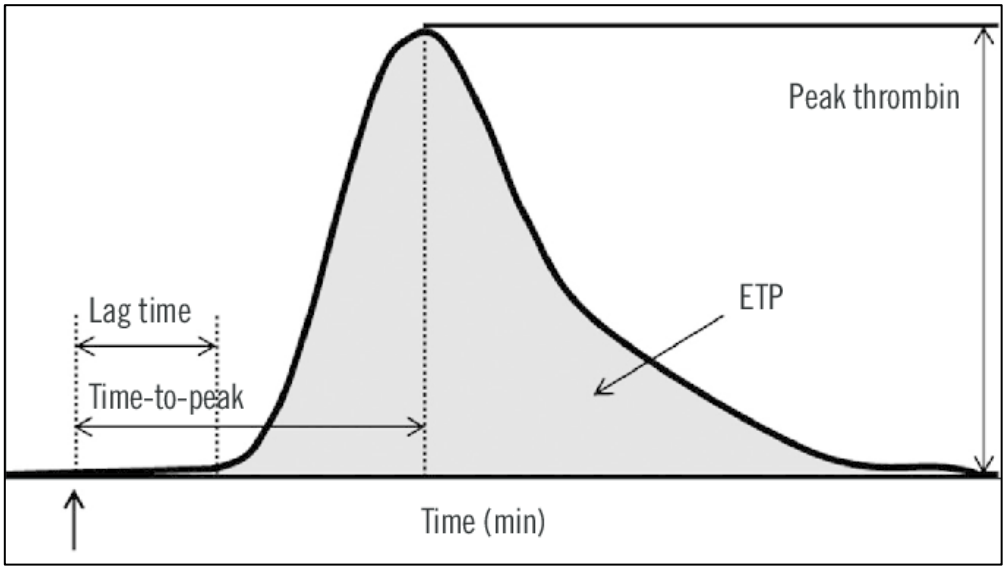

Figure 12. Thrombin generation curve obtained during a thrombin generation assay. ETP, endogenous thrombin potential.

In line with its anticoagulant activity, adding APC to the plasma will decrease the ETP of the subject. In contrast, in a plasma with reduced APC sensitivity (*e.g.* Factor V mutations or COC use), the decrease in ETP is less.
ETP-based APCr appears to be more sensitive to the effect of COC and is therefore considered as the reference test in this context (de Visser, et al. 2005).
In Section 4.2.3, we have seen how important APCr can be to explain the etiology of COC-induced VTE risk. It also appears that the level of APCr varies from a preparation to another: it is higher with third and fourth generation COCs than with second generation COC. Consequently, APCr has also been proposed as a reliable marker of VTE risk (Alhenc-Gelas, et al. 2004).

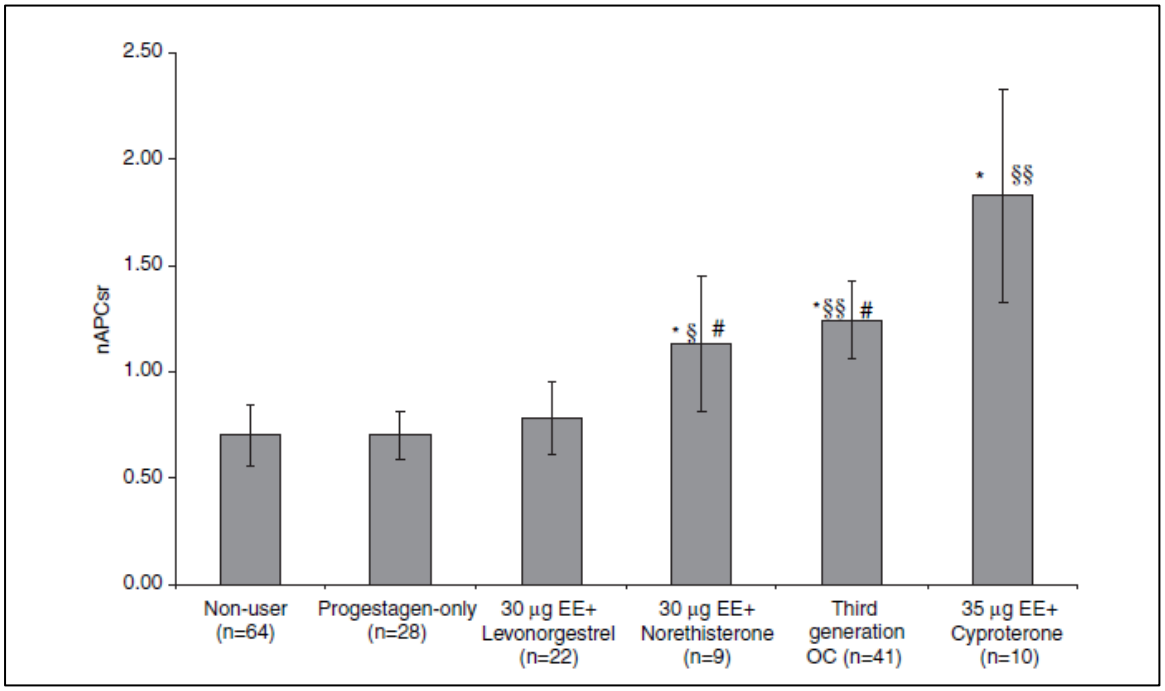

Figure 13. Mean values of ETP-based nAPCsr after adjustment for age and BMI by combined oral contraceptive use. Vertical lines are 95% confidence interval of the mean. Comparison vs. non-user: *:P < 0.01; comparison vs. 30 lg EE + levonorgestrel: §P < 0.05; §§P < 0.001; comparison vs. 35 lg EE + cyproterone:#P < 0.05 (Alhenc-Gelas, et al. 2004).

However, there was no standardized validated ETP-based APCr test at the time where most of the investigation on COC were done. Consequently, in the studies evaluating hemostasis with COCs, different methods were used precluding the possibility to compare those studies with each other. To confirm the relevance of using ETP-based APCr as a surrogate marker of VTE risk, standardization and harmonization of the method is mandatory and has recently been developed and presented in the works done by J. Douxfils and co-workers (Douxfils, et al. 2020; Morimont, et al. 2020).

4.8. New attempts to reduce the VTE risk

Besides decreasing EE content, another way to reduce the VTE and the metabolic risks associated with COC is to replace the potent EE by a more "liver friendly" estrogen. To achieve this, E2 was the first choice since it is the most abundant human adult estrogen and since it has been used for decades in therapeutic settings (notably, for the treatment of menopause related symptoms). However, the first attempts to introduce E2 in COC appeared complex (early 1980'). First, the pharmacokinetic (PK) properties of E2 make difficult to use this estrogen in a daily oral drug: E2 is subject to an extensive first-pass metabolism in the liver. As a result, only 5% of the oral E2 dose reaches the circulation. In addition, E2 binds to circulating proteins (SHBG and albumin) and consequently only 2% of the absorbed dose is active (Stanczyk, Archer, and Bhavnani 2013). This low bioavailability is largely responsible for the poor cycle control observed with the first E2-based COCs. Another explanation for the poor cycle control is the anti-estrogenic activity of progestin. Indeed, progestin stimulates the endometrial synthesis of the 17β-hydroxysteroid dehydrogenase which converts rapidly E2 to E1. Estrone, in the opposite of E2, cannot maintain stable endometrial proliferation (Stanczyk, Archer, and Bhavnani 2013). Due to this poor bleeding profile, initial attempts to develop E2-based COCs were unsuccessful (Jensen 2010; Mueck and Sitruk-Ware 2011). To avoid this problem, different approaches have recently been used in two new COCs.

The first E2-containing COC (called Qlaira® in Europe and Natazia® in the USA) was launched in 2010. This COC does not include E2 *per se* but E2 valerate (E2V), which acts as a prodrug of E2: after the liver has cleaved the molecule, E2 reaches the circulation and acts as active compound. Estradiol valerate is combined to the progestin DNG. In an attempt to provide a better control over the cycle, a quadriphasic regimen is used, meaning that the doses of E2V and DNG vary 4 times across the 28-day cycle: E2V varies from 1 mg to 3 mg per day and DNG varies from 0 mg to 3 mg per day.

The second marketed COC using E2 is called Zoely®. It is a monophasic combination of 1.5 mg E2 and 2.5 mg nomegestrol acetate (NOMAC). This progestin has been selected because it is thought to display less interference with E2 metabolism at the endometrial level and reduces accumulation of E1 in the endometrial cell which could contribute to the stabilization of the endometrium (Mueck and Sitruk-Ware 2011). Qlaira® but not Zoely® has been approved in the USA.

Clinical studies have shown that both E2-based COCs provide contraceptive efficacy similar to that of EE-COCs (Mansour, et al. 2011; Westhoff, et al. 2012; Palacios, et al. 2010; Bayer HealthCare Pharmaceuticals 2010). The published articles sponsored by the pharmaceutical companies developing these two COCs also report an acceptable bleeding pattern (Mansour, et al. 2011; Westhoff, et al. 2012; Palacios, et al. 2010; Bayer HealthCare Pharmaceuticals 2010). However, a recent publication from our group showed that the bleeding pattern of Qlaira® remains unsatisfactory (see Results section for more details) (Apter, et al. 2016).

Of course, the initial goal of replacing EE by E2 is to improve the hemostasis and metabolic profiles of the combination. Three and 6-cycles randomized controlled studies have shown that, indeed, in comparison to EE-containing COCs, the E2(V)-based COCs were associated with a

lower impact on metabolic and hemostasis parameters (Table 7). This suggests that E2-COCs use could be associated with a lower risk of developing cardiovascular events, including VTEs.

Table 7. Percentage change from baseline in the level of hemostasis and metabolic parameters with the use of Qlaira® (estradiol valerate/dienogest) and Zoely® (estradiol/nomegestrol acetate) in comparison to a second generation combined oral contraceptive (30 mcg ethinylestradiol/150 mcg levonorgestrel) (Junge, et al. 2011; Agren, et al. 2011).

	Mean (SD) % change from baseline to cycle 7		*Median (interquartile range) % change from baseline to cycle 6*	
	E2V/ DNG (n=30)	***30 mcg EE/ 150 mcg LNG (n=28)***	***E2/ NOMAC (n=60)***	***30 mcg EE/ 150 mcg LNG (n=58)***
Hemostasis Parameters				
Prothrombin fragment 1+2	-0.6 (30.3)	117.3 (358.0)	-1.7 (46.0)	13.5 (59.6)
D-dimer	-2.1 (43.5)	62.9 (99.5)	0.0 (0.0)	0.0 (68.0)
Prothrombin (Factor II)	ND	ND	-0.9 (18.5)	3.0 (22.7)
Fibrinogen	7.9 (13.7)	28.1 (29.8)	ND	ND
Factor VII activity	13.5 (14.9)	24.4 (20.9)	8.8 (63.3)	14.4 (42.0)
Coagulated activated Factor VII	ND	ND	1.0 (33.3)	-12.7 (24.1)
Factor VIII activity	6.9 (16.9)	7.5 (13.4)	4.8 (51.8)	6.8 (40.6)
Anti-thrombin III activity	0.8 (6.6)	-3.0 (8.4)	3.9 (13.4)	-3.6 (14.1)
Protein C activity	8.3 (11.9)	14.5 (17.0)	-3.1 (18.3)	8.2 (20.2)
Protein S activity	1.8 (7.5)	-11.7 (10.0)	4.7 (20.5)	-3.6 (17.5)
Free Protein S	ND	ND	13.3 (21.0)	11.9 (25.9)
ETP-based APC sensitivity ratio	ND	ND	60.0 (80.0)	146.4 (160.0)
aPTT-based APC sensitivity ratio	-5.3 (9.8)	-7.0 (5.6)	3.3 (16.4)	2.0 (14.9)
PAI-1 antigen	-10.6 (123.2)	-36.2 (48.6)	ND	ND
PAI-1 activity	-3.7 (13.3)	-5.1 (18.5)	ND	ND

SD, standard deviation; E2V, estradiol valerate; DNG, dienogest; EE, ethinylestradiol; LNG, levonorgestrel; E2, estradiol; NOMAC, nomegestrol acetate; ND, not done, APC, activated protein C; ETP, endogenous thrombin potential; aPTT, activated partial thromboplastin time; PAI, plasminogen activator inhibitor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1c, glycated hemoglobin.

Recently, a large international phase 4 study was completed which compared the incidence of serious cardiovascular events (including VTEs) in new users of the Qlaira (E2V/DNG) versus new users of other marketed COCs. In total, 50,203 women were included in this study for a mean follow up of 2.1 years: 20.3% were treated with E2V/DNG and 11.5% were treated with EE/LNG, the rest being treated with other marketed hormonal contraceptives. Number and incidence of serious cardiovascular events (VTE and ATE) recorded in the study are summarized in Table 8. Based on 9 and 6 cases of VTEs in the E2V/DNG and in the EE/LNG group, respectively and based on 1 ATE case in both groups, no statistical differences were found between the E2-based COC and the second generation.

Table 8. Number and incidence rate of venous and arterial thromboembolic events in a cohort study with Qlaira and other combined oral contraceptives.

	E2V/DNG		**EE/LNG**		**Other COCs**		**Non-users**	
	n	***Incidence* (95% CI)***	***n***	***Incidence (95% CI)***	***N***	***Incidence (95% CI)***	***n***	***Incidence (95% CI)***
VTE	9	7.4 (3.4-14.1)	6	8.2 (3.0-17.9)	33	8.3 (5.7-11.7)	4	2.1 (0.6-5.4)
ATE	1	0.8 (0.0-4.5)	1	1.0 (0.0-5.5)	15	2.4 (1.3-3.9)	2	0.8 (0.1-2.8)

* Incidence rate in events/10,000 woman-years

E2V, estradiol valerate; DNG, dienogest; EE, ethinylestradiol; LNG, levonorgestrel; COC, combined oral contraceptive; n, number; VTE, venous thromboembolism; ATE, arterial thromboembolism.

Two conclusions can be drawn from this study:

1. Replacing the potent EE by E2 in combination with a third generation progestin (DNG) maintains the VTE risk identical to that of a second generation COC;
2. The limited changes in hemostasis parameters generated in smaller studies (and summarized in Table 7) are generally in line with these epidemiological data, showing the validity of these parameters as surrogate markers of VTE risk.

5. New therapeutic regimens

COCs are generally administered in a 21/7 day regimen, *i.e.* 21 consecutive days of active COC intake followed by 7 days pause (also referred as the "hormone-free interval"). The goal is to mimic the classical 28-day menstrual cycle, comprising in average 7 days of menstrual bleeding in the majority of healthy women. In 2008, a large randomized-controlled trial conducted by Klipping *et al.* compared the administration of a same COC (20 mcg EE/3 mg DRSP) following the classical 21/7 day regimen to a new administration regimen consisting of 24 consecutive days of active COC intake followed by 4 days of placebo intake (24/4 day regimen) (Klipping, et al. 2008). The results demonstrated that moving to a shorter pill free interval and a longer active COC intake period had several advantages: with the 24/4 day regimen, the residual ovarian activity was lower, with a significantly lower incidence of escape ovulation (2% versus 8%). In addition to a better contraceptive efficacy, the higher ovarian activity suppression has also been associated with a better general tolerance to the COC, probably due to the more consistent suppression of endogenous E2 production: Endrikat *et al.* showed in a retrospective meta-analysis that the use of COCs leading to high ovarian activity suppression was positively correlated with improved cycle control (less frequent intermenstrual bleeding) and Sulak *et al.* demonstrated a decrease in headache severity when a same COC was administered in a 168/7 day regimen compared to the traditional 21/7 day regimen (Endrikat, et al. 2003; Sulak, et al. 2007). Similarly, a lower incidence of adverse reactions typically associated with hormone withdrawal, such as headache and breast tenderness, was observed in the study of Klipping *et al.* (Klipping, et al. 2008).

6. Non-contraceptive benefits of COC use

Besides the contraceptive efficacy, COC use is also associated with several non-contraceptive benefits. Some of them are simply explained by the ovulation inhibition or by the progestin impact on the endometrium while other benefits are still not clearly understood (Bahamondes, Valeria Bahamondes, and Shulman 2015). Importantly, these benefits may explain why COC use is associated with an overall absolute reduction in mortality in comparison to never users (reduction of 52 deaths per 100 000 woman years) (Hannaford, et al. 2010).
In addition to the common non-contraceptive benefits observed with all types of COCs, the EE/DRSP combinations are associated with specific additional benefits, generally related to DRSP anti-mineralocorticoid and anti-androgenic activities.

6.1. Non-contraceptive benefits common to all COCs

- Improvement of menstrual disorders: women suffering from heavy menstrual bleeding, dysmenorrhea and irregular menstruation can expect an improvement of these conditions by using a COC (Bahamondes, Valeria Bahamondes, and Shulman 2015).
- Lower incidence of endometrial cancer: different epidemiological studies conducted in the past 35 years have shown that the risk of endometrial cancer is decreased by about 50% in comparison to non-users (Dossus, et al. 2010; Hannaford, et al. 2010).
- Reduced mortality from ischemic heart disease: even if the use of COC is associated with a global increased risk of myocardial infarction, a large epidemiologic study has demonstrated that the risk of death from ischemic heart disease is lower among women who have ever used COC (Hannaford, et al. 2010; Roach, et al. 2015). However, in women above 35 years of age and who smoke, the risk of ATE is significantly increased, contraindicating the use of COC in this population.
- Improvement of endometriosis after surgical treatment: continuous use of COC after surgical treatment of endometriosis is associated with a lower recurrence rate of dysmenorrhea, pelvic pain and recurrence of endometrioma (Zorbas, Economopoulos, and Vlahos 2015).
- Lower incidence of ovarian cancer: the reduction of incidence of ovarian cancer appears to be proportional to the length of COC use. Women having used COC for 10 years or more can expect a 50% global reduction rate in comparison to never users (Havrilesky, et al. 2013).
- Lower incidence of colorectal cancer: Incidence of colorectal cancer is reduced by 20% in current COCs users. The mechanism underlying this reduction is currently not elucidated (Bosetti, et al. 2009).
- Lower incidence of ectopic pregnancy: by providing a good contraceptive efficacy but also by lowering the risk of pelvic inflammatory disease (Burkman, Schlesselman, and Zieman 2004).

6.2. The special case of EE/DRSP combinations

- Improvement of acne: Most probably due to the anti-androgenic activity of DRSP, YAZ® (20 mcg EE/3 mg DRSP) has been demonstrated to significantly improve acne in comparison to placebo (Koltun, et al. 2008; Maloney, et al. 2009). Based on these data, FDA approved YAZ® for the treatment of moderate acne in women at least 14 years old (only if the patient desires an oral contraceptive for birth control)(Bayer HealthCare Pharmaceuticals 2001). For note, this acne indication has not been approved in Europe because the VTE risk associated with YAZ® was considered to overweight the benefit in this case (EMA 2012).
- Improvement of premenstrual dysphoric disorder: Premenstrual dysphoric disorder (PMDD) is a severe form of premenstrual syndrome. It is characterized by moderate to severe mood deterioration, behavior and physical symptoms disrupting the life at work and at home. YAZ® was shown to decrease by about 50% the severity of the symptoms in women suffering from PMDD. The reason for this improvement is not fully elucidated. It is hypothesized that the low ovarian activity associated with this COC plays a central role by reducing the endogenous hormonal fluctuations (Pearlstein, et al. 2005; Yonkers, et al. 2005). Consequently, YAZ® has also been approved by the FDA for the treatment of PMDD for women who choose to use an oral contraceptive for contraception (Bayer HealthCare Pharmaceuticals 2001). For note, the European YAZ® label does not include this indication.
- Positive impact on body weight and blood pressure: Estrogen strongly stimulates the production of angiotensinogen by the liver. This protein is converted to a decapeptide called angiotensin I by ACE, the angiotensinogen converting enzyme. Angiotensin I is then converted into an octapeptide called angiotensin II. Angiotensin II activates the adrenal production and secretion of aldosterone and antidiuretic hormone (ADH) which both promote water/sodium retention. It also acts as vasoconstrictor. This explains both increased body weight and increased blood pressure in women treated by estrogens (in COC or in hormone replacement therapy [HRT]) (Figure 14).

  During a spontaneous menstrual cycle, with its strong antimineralocorticoid activity, progesterone secreted during the luteal phase counteracts the water-retention effect of the high estrogen levels seen at the end of the follicular phase. Progesterone is thus able to antagonize the aldosterone receptor. Biologically, this is translated by an increased natriuresis during the luteal phase. This increase in sodium excretion stimulates in turn the plasma renin activity and the production of aldosterone (Oelkers 2005; Oelkers 2002).

  Except DRSP, no other progestin used in COC or HRT display an anti-mineralocorticoid activity. In a majority of women treated by estrogen, the estrogen mediated increased Angiotensin II levels are balanced by a decreased production of renin. However, this negative feedback is absent in some women who will therefore develop arterial hypertension soon after starting an estrogen therapy (COC or HRT) (Oelkers 2005).

Drospirenone, with its potent anti-mineralocorticoid activity, counteracts the mineralocorticoid activity of estrogens mediated by angiotensin II and aldosterone. It has been shown that women treated with EE/DRSP are more likely to lose weight and to keep stable blood pressure values (Foidart, et al. 2000; Giribela, et al. 2015).

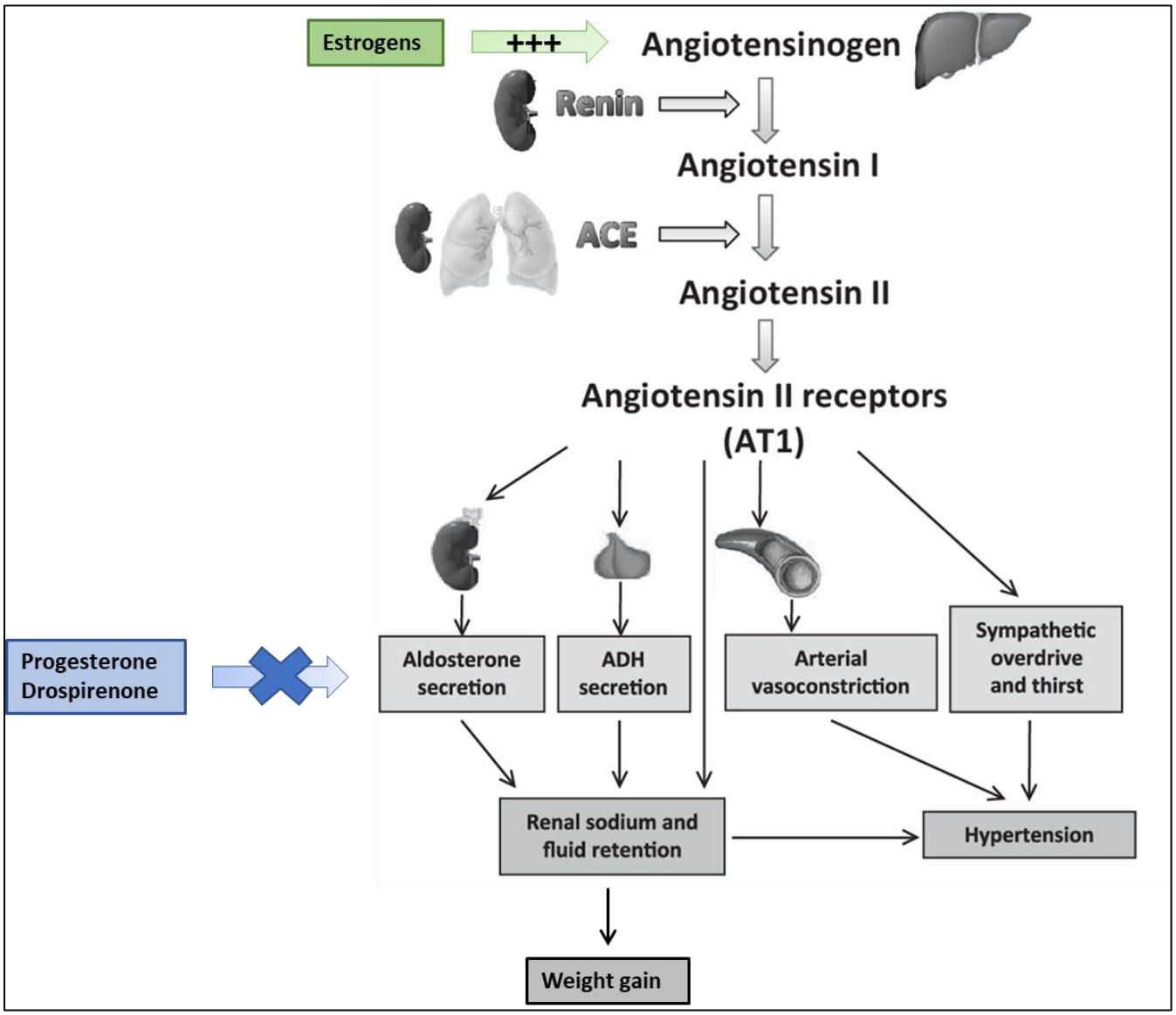

Figure 14. Renin–angiotensin system. Estrogens strongly increase angiotensinogen which in turn increases the level of aldosterone. Progesterone and drospirenone exert anti-aldosterone activity. ACE, angiotensinogen converting enzyme (Santos, Krieger, and Pereira 2012).

7. Estetrol

7.1. Estetrol

Estetrol (1,3,5(10)-estratrien-3,15α,16α,17β-tetrol), generally referred as *E4*, is a steroid hormone discovered in 1965 by Egon Diczfalusy and colleagues at the Karolinska Institute in Stockholm. To isolate E4, Diczfalusy and his team analyzed 200 liters of urines coming from late pregnant women (Zucconi, et al. 1967). Estetrol was not found in non-pregnant women nor in other mammalians tested, and therefore it was concluded that E4 is a human and pregnancy specific steroid.

Estetrol is the fourth estrogen of the naturally occurring estrogens family. "Naturally occurring" means that the compound is produced endogenously in mammalians, like it is the case of E1, E2 and estriol (E3). Structurally, E4 is characterized by the presence of four hydroxyl groups (OH). This explains the abbreviation "E4", using the same abbreviation system as for the other naturally occurring estrogens: estrone (E1, one OH group), estradiol (E2, two OH groups) and estriol (E3, three OH groups). Figure 15 displays the molecular structure of E4 in comparison with that of E1, E2 and E3.

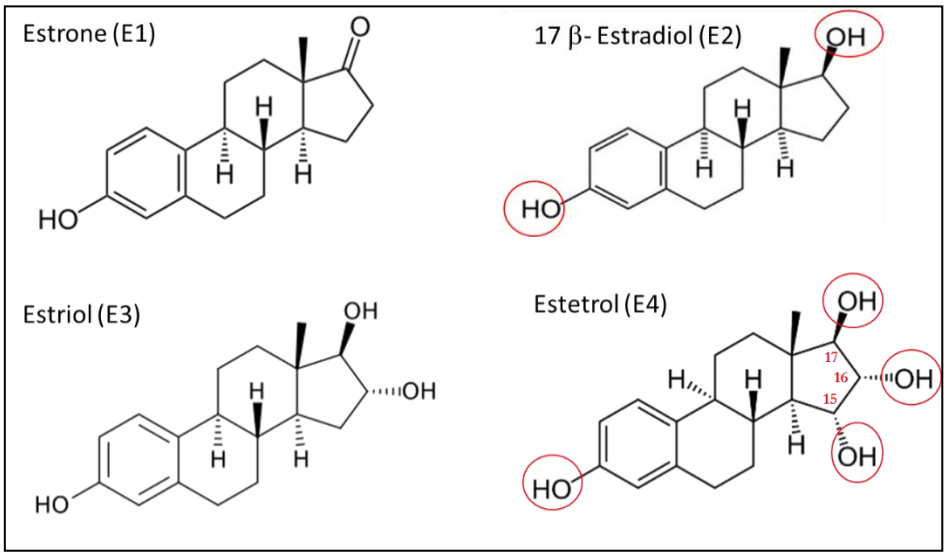

Figure 15. Molecular structure of the four naturally occurring estrogens in human: estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4).

7.2. Endogenous synthesis of E4

Endogenously, the synthesis of E4 requires the presence of two enzymes: the *15α-hydroxylase* which converts E3 in E4, and the *16α-hydroxylase* which, in combination to the 15α-hydroxylase, converts E2 in E4. In human, both hydroxylases are exclusively expressed by the fetal liver, explaining why E4 is only detected during pregnancy (Schwers, Eriksson, and Diczfalusy 1965; Schwers, et al. 1965; Schwers, et al. 1967; Mancuso, et al. 1968).

The biosynthesis of E4 starts with dehydroepiandrosterone-sulfate (DHEA-S) produced by both the maternal and fetal adrenals and converted to dehydroepiandrosterone (DHEA) in the

placenta (Figure 16). DHEA is then transformed in androstenedione by the 3β-hydroxysteroid dehydrogenase and the Δ5, 4 isomerase and in testosterone by 17-β-hydrosteroid dehydrogenase. Aromatase in the placenta then transforms these androgens in E1 and E2. Estradiol is then converted in E4 by the action of the 15-α-hydroxylase and 16-α-hydroxylase in the fetal liver. Estetrol may also be produced from E3. The principal precursor of E3 is fetal DHEA-S which is converted in 16-OH-DHEA-S by the 16-hydroxylase enzyme located in the fetal liver. The compound is then converted in E3 in the placenta by the action of desulfatase and aromatase enzymes. Under the action of 15α-hydroxylase in the fetal liver, E3 is converted into E4 (Smith 2001; Cantineau, et al. 1985).

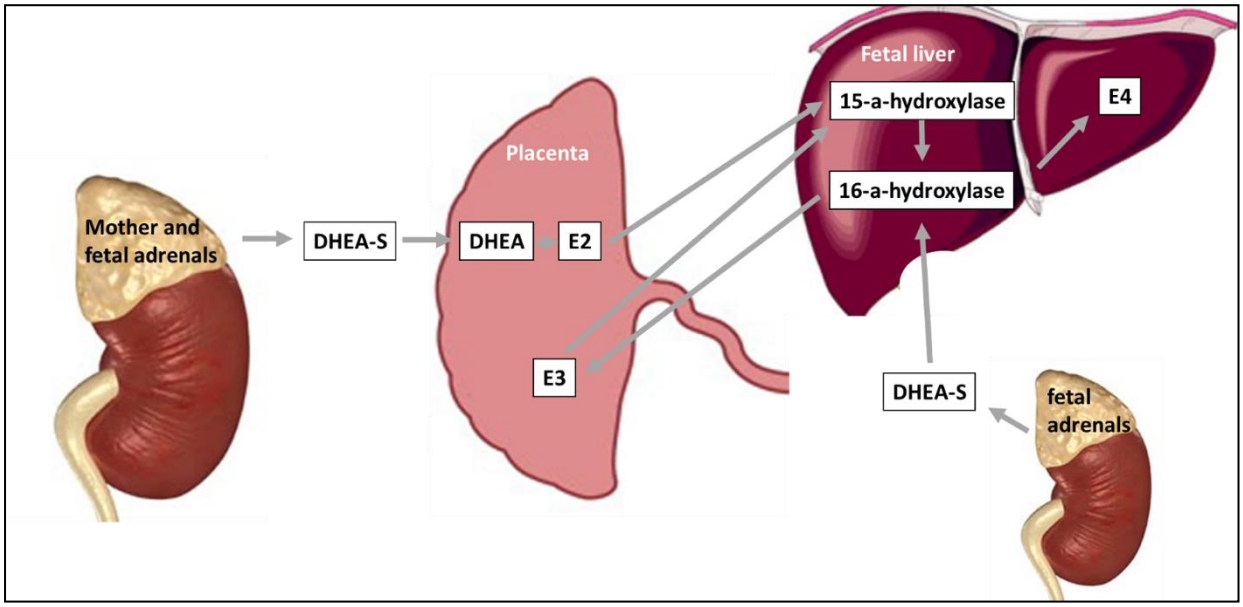

Figure 16. Schematic representation of endogneous synthesis of estetrol during pregnancy. DHEA, Dehydroepiandrosterone; DHEA-S, Dehydroepiandrosterone-sulfate; E2, estradiol; E3, estriol; E4, estetrol.

Data are available about the percentage of conversation of androstenedione to testosterone, E1 and E2 in pregnant women. These conversion rates increase during pregnancy as shown in Table 9 below (Belisle, Lehoux, and Brault 1980). Unfortunately, there is no similar data on the conversion of E4. However, a recent study evaluated the levels of E1, E2, E3 and E4 among 859 pregnant women and the results showed that values for all estrogens were strongly correlated, without difference in concentrations between male and female fetuses (Hickey, Hart, and Keelan 2014).

At the end of the pregnancy the conversation rate of DHEA into estrogens is 500-fold higher than in non-pregnant women (Belisle, Schiff, and Tulchinsky 1980). At the end of pregnancy, 35% of DHEA-S is converted into estrogens (Madden, et al. 1976).

Table 9. Production rate of androstenedione and conversion rate of androstenedione to testosterone, estrone and estradiol in non-pregnant and pregnant women.

	Non-pregnant women	Pregnant women	
		9-14 weeks	30-36 weeks
Production rate of Androstenedione in mg/day (±SE)	4.1 ± 0.7	9.8 ± 1.4	9.6 ± 1.4
Percentage conversion of:			
- ***Androstenedione → testosterone***	13.1 ± 1.2%	20.4 ± 3.2%	25.5 ± 6.4%
- ***Androstenedione → E1***	0.9 ± 0.2%	NR	4.3 ± 0.8%
- ***Androstenedione → E2***	2.8 ± 0.3%	7.9 ± 1.3%	49.7 ± 2.9%

SE, standard error; E1, estrone; E2, estradiol; NR, not reported.

Estetrol produced by the fetus crosses the placenta to reach the maternal circulation. It was detected in maternal urine from the 9th week of pregnancy onwards and concentrations increase constantly throughout the pregnancy, without diurnal variation. At pregnancy term, serum E4 concentrations greatly vary from one woman to another and from one fetus to another as shown in a quite recent study (2007) during which E4 was measured in maternal blood and umbilical cord at delivery using state-of-the-art methodology (liquid chromatography/mass spectrometry analysis) (F. Coelingh Bennink, et al. 2008). The results are reported in Table 10 and show that, among the 10 women tested, serum E4 concentration varied from 373 pg/ml to 1,207 pg/ml and from 4,474 pg/ml to 13,839 pg/ml in the fetuses.

Table 10. E4 plasma concentration (pg/ml) in mother and infant after delivery measured using liquid chromatography/mass spectrometry analysis (n=10) (F. Coelingh Bennink, et al. 2008).

	E4 plasma concentration (pg/ml)	
Subject number (Gestational Age in weeks)	**Mother**	**Child**
1 (40)	1,203	6,298
2 (40 + 6)	1,207	8,805
3 (40 + 5)	794	10,781
4 (38)	578	10,554
5 (42)	373	4,474
6 (40)	804	11,155
7 (37 + 6)	590	11,340
8 (39 + 6)	467	6,137
9 (40)	717	13,839
10 (41)	495	6,959
Mean (SD)	723 (290)	9,034 (2,968)
Median	654	9,680
Range	373-1,207	4,474-13,839

It is interesting to note that, in this study, there was a gradient of E4 concentrations between the fetal and maternal plasma levels. However the ratio of E4 concentrations varied considerably between individual pregnant mother-fetuses pairs, so that the highest E4 maternal plasma levels were not correlated with the highest fetal E4 plasma levels.

7.3. Physiological role of E4 and early researches

The physiological role of E4 remains currently unknown. As mentioned above, since its discovery in 1965, E4 was thought to be only produced by the human fetus. It was not found in the other species tested, namely, pregnant mares, rats, mice, rabbits (Coelingh Bennink, Holinka, and Diczfalusy 2008). However, very recent and currently unpublished data generated by a team of Dr Nicola Rose from the University of Leicester have also identified small amounts of E4 in the blood of pregnant cynomolgus monkeys, near term of pregnancy. The concentration of E4 in monkeys is about 1% of that seen in human at term. Given this new information, E4 is no longer considered as a human specific estrogen but rather as a *primate specific estrogen*. As said by the famous anatomist, anthropologist and neuroscientist, Sir Wilfrid Edward Le Gros Clark, the most distinctive trait of the Primates, wherein this order contrasts with all other mammalian orders in its evolutionary history, is the tendency towards the development of a brain which is large in proportion to the total body weight, and which is particularly characterized by a relatively extensive and often richly convoluted cerebral cortex (Barton 2006). Therefore, it is tempting to hypothesize that E4 could have been selected by Evolution for its neuroprotective capability, since its levels are correlated with the brain size.

Accordingly, several animal studies have been conducted in the last 7 years to evaluate the neuroprotective activity of E4 and confirm this hypothesis (see Section 7.9 below).
From its discovery to approximately mid-1980s, E4 was extensively studied in normal and complicated pregnancies. As it is produced by the fetus and easily detected in maternal urine and serum, it was hoped that E4 could be a useful marker of fetal well-being. However, the high inter-subject variability of E4 concentrations and the variability of E4 feto-maternal plasma concentrations ratios precluded its clinical use. Emergence of ultrasonography was shown to be considerably more precise, specific and clinically useful. Consequently, academic researches on E4 were stopped (Coelingh Bennink, Holinka, and Diczfalusy 2008; Heikkila and Luukkainen 1971; Tulchinsky, et al. 1975; Kundu and Grant 1976; Kundu, et al. 1981). In the early 2000s, the need to develop safer estrogens arose from the results of large epidemiological studies showing on one hand a higher VTE risk with EE-containing COCs and, on the other hand, a higher risk of cardiovascular events and mammary cancers among post-menopausal women treated with hormone replacement therapy (HRT) (Women Health Initiative 2020). Consequently, the natural estrogen E4 knew a revival of interest. It was no longer a question of using E4 as marker of fetal well-being but to evaluate its potential as therapeutic estrogenic compound, as suggested by Herjan Coelingh Bennink (Coelingh Bennink, Holinka, and Diczfalusy 2008).

7.4. Estetrol: Steroid receptors affinity and mechanism of action

7.4.1. Estrogen receptors

There are two types of ERs: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Both ERs belong to the nuclear receptor family of transcription factors. Each form of ER can be found either in the nucleus (nuclear ERα and ERβ) or at the membrane (membrane ERα and ERβ) (Heldring, et al. 2007).
After binding of a ligand to an ER, two ERs bind together. This process refers as the *dimerization of the receptors* and is necessary for the receptor activation.
Estrogen receptor α is encoded by the ESR1 gene and ERβ is encoded by ESR2 gene. Both ERα and ERβ show significant overall sequence homology, except in their N-terminal domains: they are both composed of five domains (A/B, C, D, E and F) ranged from the N- to C-terminus (Figure 17) (Marino, Galluzzo, and Ascenzi 2006).

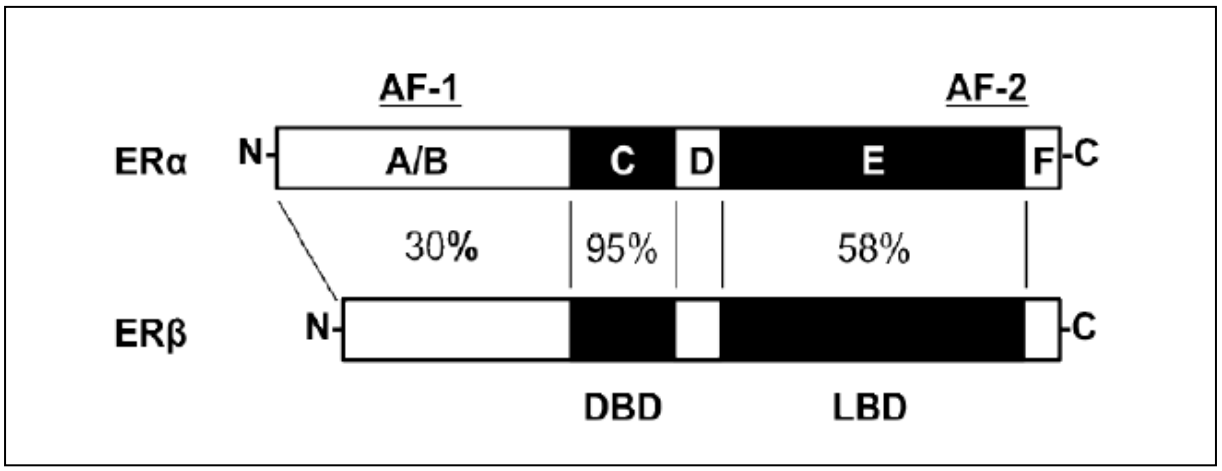

Figure 17. Domain organization of human estrogen receptors alpha and beta. The percentage indicates the homology between both receptors (Marino, Galluzzo, and Ascenzi 2006). ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; AF-1, activation function 1; AF-2, activation function 2; DBD, DNA-binding domain; LBD, ligand-binding domain.

The transcriptional activity of ER is activated by two regions called activation function 1 and 2 (AF-1 and AF-2). AF-1, located in the A/B domain is able to activate gene transcription in the absence of bound ligand (*e.g.*, the estrogen hormone). However, this activation is weak compared to the activation observed in the presence of a bound ligand and provided by the AF-2, located in the E domain.
Basically, the function of each ER domain can be summarized as follows (Figure 17):

- The A/B domain contains AF-1.
- The C domain is the DNA-binding domain which binds to the estrogen response element in the promotor region of target genes.
- The D domain is a hinge region, involved in the receptor dimerization process.
- The E domain contains the ligand binding domain and AF-2. It also contains binding sites for coactivator and corepressor proteins.
- The F domain function is not entirely clear and is variable in length.

7.4.2. Selective estrogen receptor modulators

Selective estrogen receptor modulators (SERMs) are synthetic ER ligands able to display an *agonistic activity in some tissues and an antagonistic activity in other tissues*. The molecular mechanism of action of SERMs is very complex and still not fully characterized. It relies on several mechanisms: first, different SERMs induce different conformational changes of the ER which in turn affect the ability of ER to interact with coregulators of genes transcription. Another mechanism of SERMs distinct profile relies on their different activity on ERα or ERβ but also on AF-1 and AF-2. Finally, the exact nature of estrogen-responsive element from the promotor may also affect SERM activity (Dutertre and Smith 2000). .

Table 11 summarizes the SERMs used in current clinical practice. Since the discovery of Clomifene, considered as the first SERM, many other SERMs have been developed with the main objective to discover the perfect SERM, *i.e.* a molecule with an anti-estrogenic activity on the breast and the endometrium but with an agonistic activity on bone, hot-flushes and vagina.

Table 11. SERMs and clinical applications.

SERM	Agonist activity	Antagonist activity	Clinical indication
Clomifene	-	Hypothalamus	Ovulation induction
Raloxifene	Bone	Breast Uterus	Osteoporosis Breast cancer
Tamoxifene	Bone Uterus	Breast	Osteoporosis
Ospemifene	Bone	Breast Uterus	Vulvovaginal atrophy
Bazedoxifene	Bone	Breast Uterus Brain	Osteoporosis (in association with E2)

7.4.3. Steroid receptors affinity

The affinity of a compound for a receptor is measured *in vitro* and reported using the *half maximal inhibitory concentration* (IC_{50}). To determine the IC_{50}, a dose-response curve is constructed using increasing dose of the agonist known to induce the maximum biological response on a given receptor. In the case of ER, diethylstilbestrol is the agonist of reference. Secondly, the effects of increasing doses of the tested compound on this dose-response curve are evaluated: if the compound has no affinity for the studied receptor, the dose-response curve will not be modified; in the contrary, if the compound tested has affinity for the tested receptor, the reference agonist will be progressively displaced at the receptor level by the tested compound (competitive agonist activity). This will shift the initial dose-response curve. Values of IC_{50} can be calculated for a given compound by determining the concentration needed to inhibit half of the response obtained with the reference compound. A low IC_{50} value corresponds to a high affinity of the tested compound for the receptor and inversely (Figure 18).

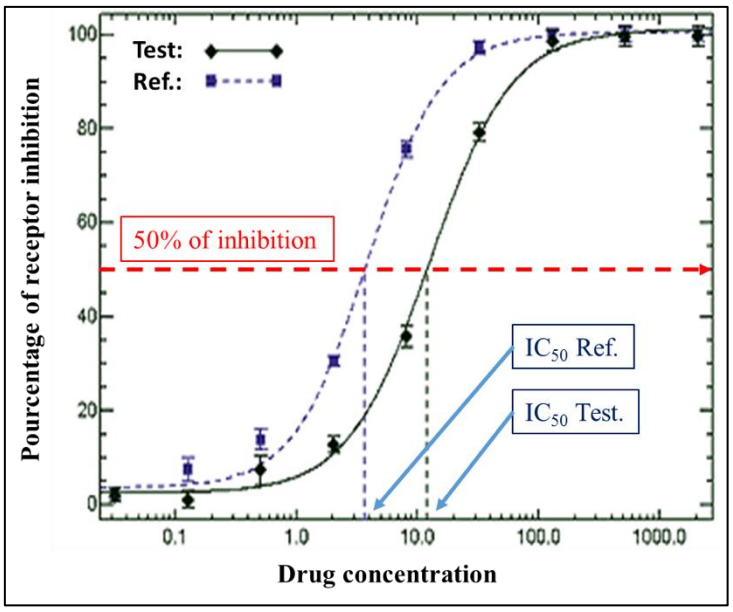

Figure 18. Example of how receptor affinity of a compound is determined using IC_{50}. IC_{50} represents the concentration of a drug necessary to inhibit half of the response obtained with the reference compound.

Figure 19 and Figure 20 show the dose-response curves of diethylstilbestrol alone or in presence of E4 at the ERα and ERβ level. The IC_{50} values of E4 for the ERα is 170 $x10^{-10}$ and for the ERβ is 910 $x10^{-10}$. This is in line with previously published data and demonstrates that E4 has a low affinity for the ERα and even lower for the ERβ (Visser, Foidart, and Coelingh Bennink 2008).

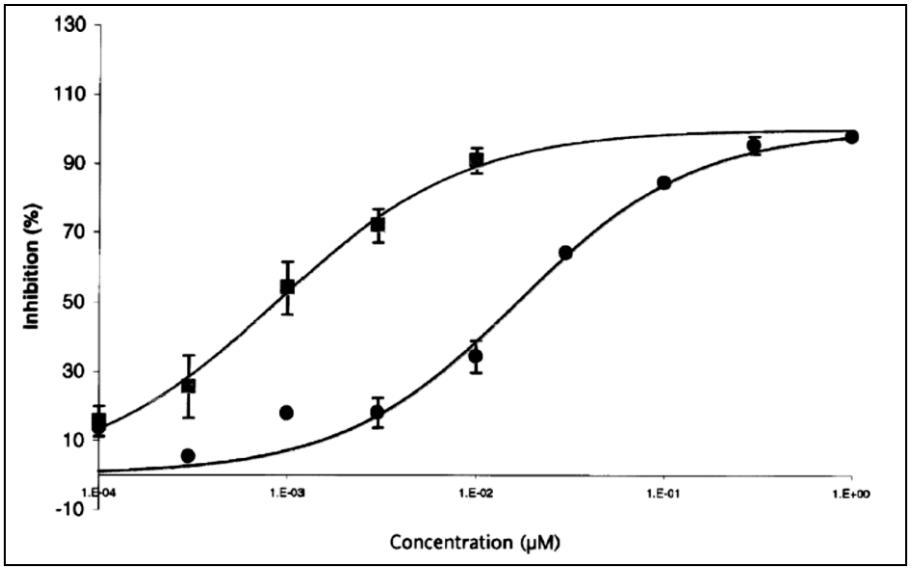

Figure 19. Binding of estetrol (circles) and the reference agonist diethylstilbestrol (squares) at the estrogen receptor alpha (Visser, Foidart, and Coelingh Bennink 2008).

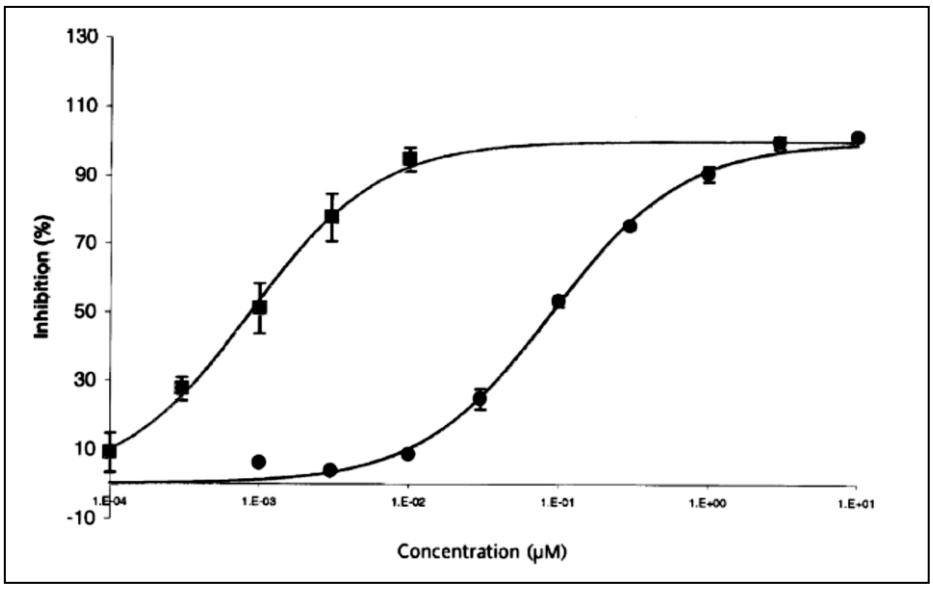

Figure 20. Binding of estetrol (circles) and the reference agonist diethylstilbestrol (squares) at the estrogen receptor beta (Visser, Foidart, and Coelingh Bennink 2008).

In comparison, the IC_{50} of E2 and EE for the ERα is 8.99 $x10^{-10}$ and 4.3 x 10^{-10}, respectively (Blair, et al. 2000). Because of this low ER affinity, E4 is classified among the "weak estrogens". This classification has certainly contributed to the fact that E4 was for a long time discarded as a candidate for therapeutic applications. However, as described below, this low affinity for the ER is counteracted by its PK profile.

At doses up to 10 fold the therapeutic doses resulting in 10 µmol, E4 does not significantly bind to the progesterone receptor, and does not bind to the androgen receptor, or the glucocorticoid receptor (Visser, Foidart, and Coelingh Bennink 2008).

7.4.4. Mechanism of action of E4 on the estrogen receptor alpha

Estrogen receptor α localizes to the cytoplasm, the nucleus and the membrane. In the absence of ligand, heat shock protein complex binds to ERα, maintaining the receptor inactive. When estrogenic ligands binds the ERα in the cytoplasm, the ligand-receptor complex detaches from heat shock protein, dimerizes, and is translocated into the nucleus. In the nucleus, a series of events occur leading to the binding of the receptor to specific sequences of DNA called estrogen response elements (Dhamad, et al. 2016). Once the binding to DNA has occurred, transcription of DNA into mRNA and subsequently of mRNA into proteins takes place.

The membrane-localized ERα is responsible for rapid signal transduction mainly via the activation of protein G (Levin 2009). This is also referred as the *membrane-initiated steroid signaling* (MISS) pathway. It was initially thought that the responses mediated by the activation of membrane ERα were non-genomic but *in vitro* studies have shown that it was also responsible for transcriptional activity (Abot, et al. 2014).

The action of an estrogen on a specific tissue seems to vary in function of the signaling pathway this estrogen uses (either nuclear or MISS pathway). Using transgenic mice with targeted mutations inactivating either the membrane or the nuclear form of ERα, it was established that uterine gene expression, uterine epithelial proliferation, and prevention of atheroma are mediated through nuclear ERα pathway while endothelial nitric oxide synthase (eNOS), acceleration of vascular endothelial-smooth muscle cells healing, and stimulation of migration and invasiveness of human breast carcinoma cells are mediated through membrane ERα (MISS pathway). The effect on the liver seems to be mixed, depending on both nuclear and MISS pathways.
Estetrol, as E2, is able to stimulate nuclear ERα as shown by its effects on the endometrium and atheroma prevention. However, E4 appears to block the MISS pathway: E4 had no effect on the endothelium and was able to antagonize the E2 promoting effects on breast cancer cells (Abot, et al. 2014; Adlanmerini, et al. 2014; Giretti, et al. 2014; Arnal, et al. 2017).
In conclusion, E4, in contrast to other estrogens like E2 and EE, activates the nuclear ERα but is an antagonist of the membrane ERα. As a consequence, the response in the presence of E4 is different from a tissue to another as a function of the predominant signaling pathway specific to that tissue. This could explain the weak effect of E4 on certain target tissues (*e.g.* the liver and the breast) while it displays a full estrogenic activity on other tissues (uterus, vagina, HPO axis). The mixed agonistic and anti-agonistic activity exerted by E4 as a function of the tissue is a characteristic that E4 shares with the synthetic SERMs. Therefore, E4 is sometimes seen as "the first natural SERM" (Arnal, et al. 2017). However, at the molecular level, their mode of action is clearly distinct since SERMs activities in tissues vary according to the repertoire of recruited co-activators and co-inhibitors while E4 activates the nuclear ERα and antagonizes the membrane form of this same receptor.

7.5. Plasma protein binding

In the bloodstream, 98-99% of sex hormones circulate bound to plasma protein, mainly to SHBG, albumin and corticosteroid binding globulin (CBG). Only the unbound fraction, called the *free fraction*, is biologically active. Regarding E2, 38% is bound to SHBG and 60% to albumin while 98% of EE is bound to albumin (Stanczyk, Archer, and Bhavnani 2013).
Human plasma analysis has shown that only 50% of E4 is bound to plasma protein, mainly to albumin (and to a very low extent to α-glycoprotein).
Competitive steroid binding assays using human SHBG have shown that E4 does not bind to SHBG.

Table 12 displays the relative binding affinity of the different sex hormones tested during the study expressed in percentage of the binding affinity for dihydrotestosterone (DHT), the molecule with the highest affinity for SHBG, used as standard reference. It clearly shows that E4 has no affinity for SHBG while EE has a 0.08% affinity and E2 a 6.80% affinity (Hammond, et al. 2008).

Table 12. Relative binding affinities of testosterone, estradiol, ethinylestradiol and estetrol for human SHBG in comparison to dihydrotestosterone (Hammond, et al. 2008).

Ligand tested	Relative binding affinity
DHT	100.00
Testosterone	40.00
Estradiol	6.80
Ethinylestradiol	0.08
Estetrol	0.00

DHT, dihydrotestosterone

This finding is of great clinical interest since it shows that, in the opposite to E2 and EE, a high fraction of the administered E4 remains free and therefore biologically active, compensating the relatively low affinity of E4 for the ER.

7.6. Estetrol: activity on estrogenic targets in animal

Different *in vivo* studies were conducted in validated animal models to determine the estrogenic activity of E4 on several tissues known to be estrogenic targets. These studies were also the occasion to compare the activity of E4 with that of the synthetic estrogen EE or the natural E2. The main goal of these studies were 1) to gather regulatory safety information in animals before performing the human studies, 2) to evaluate if E4 displayed the properties required for a therapeutic estrogen, 3) to precisely delineate the pattern of E4 specific activities in comparison to those of other natural and synthetic estrogens.

7.6.1. Utero-vaginal effects

In ovariectomized female rats, the vaginal epithelium becomes atrophic due to the lack in estrogens. In the opposite, administration of exogenous estrogens is known to restore the vaginal epithelium, a process called *vaginal cornification*. Vaginal cornification is highly specific of the response of the vaginal tissue to estrogen (Heegaard, et al. 2008).
To evaluate the estrogenic activity of E4 on the vagina, ovariectomized rats (8 per group) were exposed to increasing oral doses of E4 (0.1, 0.3, 1.0 and 3.0 mg/kg/day) or to EE (0.05 mg/kg/day) for seven days. The rats underwent daily vaginal lavage during the treatment. Presence of cornified epithelial cells in the vaginal washing attested of the vaginal cornification. Results showed that E4 displays estrogenic activity on the vaginal tissues in rats. The response was dose-proportional and 20-fold less potent than EE (Figure 21).

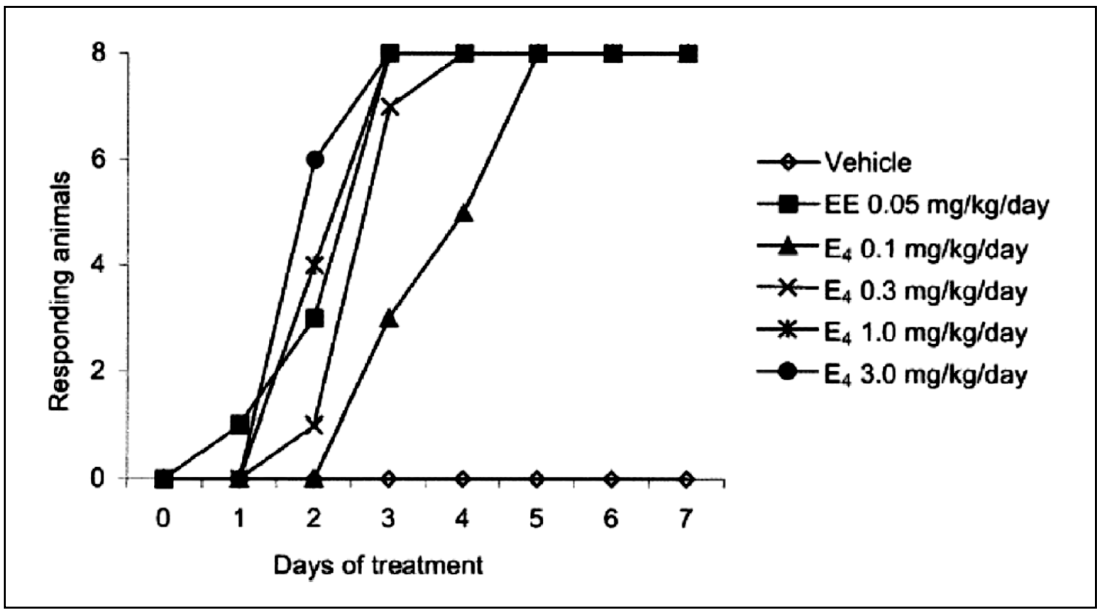

Figure 21. Number of rats with vaginal cornification over a 7-day treatment period with orally administered estetrol (0.1, 0.3, 1.0 and 3.0 mg/kg/day), 0.05 mg/kg/day ethinylestradiol or vehicle (n=8 animals per group). E4, estetrol; EE, ethinylestradiol (Heegaard, et al. 2008).

In the same study, uterine wet weight was also measured as marker of estrogenic activity on the uterus. Results demonstrated here also the estrogenic activity of E4 on the uterus. The effect was dose-dependent and 20-fold less potent than EE (Figure 22).

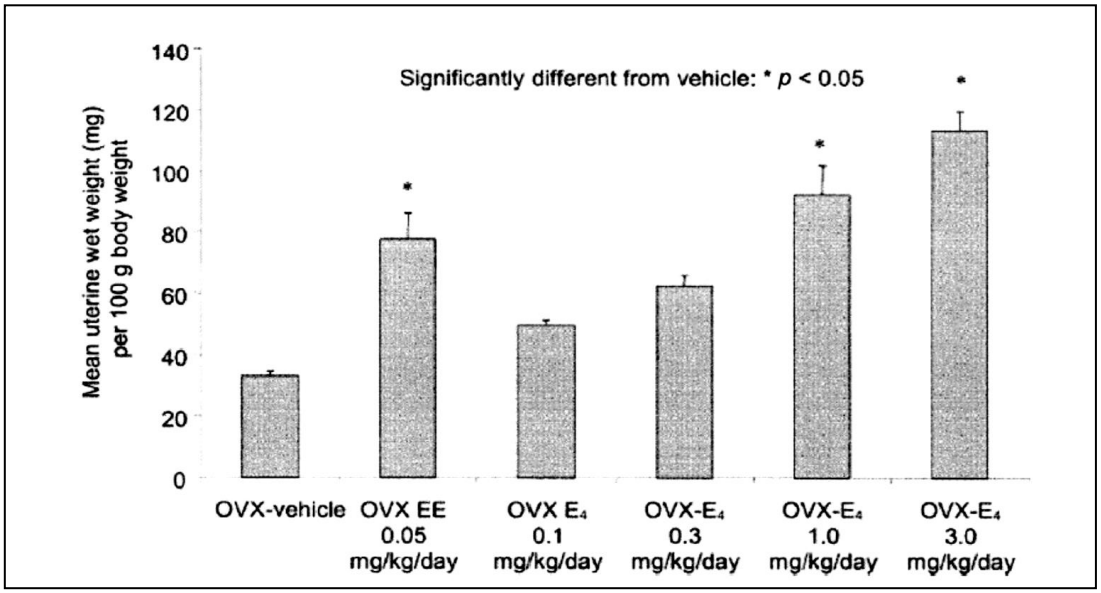

Figure 22. Mean (±SEM) uterine wet weight per 100g body weight after a 7-day treatment period with orally administered estetrol (0.1, 0.3, 1.0 and 3.0 mg/kg/day), 0.05 mg/kg/day ethinylestradiol or vehicle (n=8 animals per group). OVX, ovariectomized; E4, estetrol; EE, ethinylestradiol (Heegaard, et al. 2008).

It was recently demonstrated that the vaginal epithelial activation and lubrification of E4 was similar to that obtained with E2 in mice. It was further demonstrated, using ERα or ERβ knock-out mice and mice lacking the ERα membrane receptor, that the E4 action on this tissue can be entirely explained by its selective nuclear activation of ERα, accounting for its efficiency similar to that seen with corresponding dose of E2 (Benoit, et al. 2017).

7.6.2. Ovulation inhibition

The anti-ovulatory effect of E4 was evaluated in a validated rat model, in comparison to EE (considered as the most potent anti-ovulatory estrogen) (H. J. Coelingh Bennink, et al. 2008b). In this study, ovulatory rats (8 per treatment group) were administered increasing doses of E4 (0.03, 0.1, 0.3, 1.0 and 3.0 mg/kg) or EE (0.0003, 0.001, 0.003, 0.01 and 0.03 mg/kg) twice daily for four days, starting on the day of estrus. One group of 8 rats did receive vehicle for comparison.
Results showed that E4 was able to inhibit ovulation in rats in a dose-dependent manner, starting with the administration of 0.1 mg/kg twice daily. Same results were observed with EE from 0.003 mg/kg onwards (Table 13).

Table 13. Inhibition of ovulation in 4-day cycling rats treated twice daily with estetrol, ethinylestradiol or vehicle (H. J. Coelingh Bennink, et al. 2008b).

Treatment groups	Number of ovulating rats/ number of treated rats
Vehicle control	8/8
E4 0.03 mg/kg/day	8/8
E4 0.1 mg/kg/day	6/8
E4 0.3 mg/kg/day	3/8
E4 1.0 mg/kg/day	1/8
E4 3.0 mg/kg/day	0/8
EE 0.0003 mg/kg/day	7/7
EE 0.001 mg/kg/day	8/8
EE 0.003 mg/kg/day	7/8
EE 0.01 mg/kg/day	4/8
EE 0.03 mg/kg/day	0/8

E4, estetrol; EE, ethinylestradiol

From these results it was calculated that the oral anti-ovulatory potency of EE in rats was approximately 18 times higher than E4.

7.6.3. Hot flushes

To evaluate the effect of E4 on vasomotor symptoms, a rat model was used. For this experiment, ovariectomized female rats (6 per group) were induced a morphine dependency using subcutaneous implant of morphine (Holinka, Brincat, and Coelingh Bennink 2008). After 5 days of morphine treatment, the rats were injected with the opioid antagonist Naloxone. This induced a sudden morphine withdrawal characterized by a short phase of heat dissipation which was measured by monitoring of tail skin temperature (TST). In parallel to this increase in TST, rats present an increase in heart rate and hypersecretion of LH. These symptoms are the same as those experienced by post-menopausal women presenting hot flushes. It has been demonstrated that the use of estrogen therapy decreases the rise in TST and consequently, this rat model is considered an appropriate tool for the study of vasomotor symptoms.
In this study, beside the vehicle group and the control group treated with EE 0.3 mg/kg/day, four groups were treated with E4 at the dose of 0.1, 0.3, 1 or 3 mg/kg/day. Each treatment group included 6 animals. A representation of the results is given in Figure 23. There was a dose-dependent decrease in TST with E4 with the effect of the highest E4 dose (3 mg/kg/day) similar to dose of 0.3 mg/kg/day EE. It was concluded that E4 displays estrogenic activity in the vasomotor symptoms rat model and that the potency of EE was at least 10 times higher than that of E4.

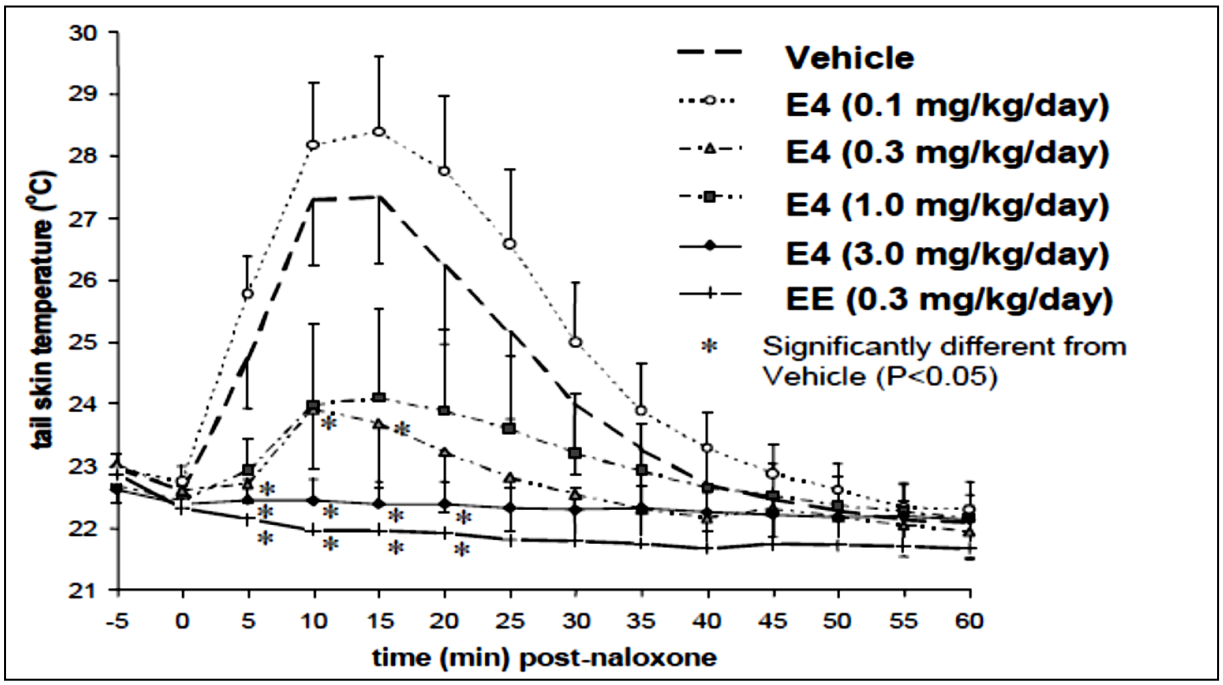

Figure 23. Effect of estetrol and ethinylestradiol on the increase in tail skin temperature observed after naloxone injection in morphin dependent ovariectomized rats (hot flushes model) (n=6 rats per group). E4, estetrol; EE, ethinylestradiol (Holinka, Brincat, and Coelingh Bennink 2008).

7.6.4. Bone mass density

The mechanisms by which estrogens interfere with bone metabolism are complex and ongoing research to elucidate them is still in progress. The onset of osteoporosis in case of estrogen depletion (*e.g.* post-menopausal stage, anorexia nervosa) attests that estrogens play a major role in bone density regulation. In parallel, estrogen replacement therapy is one of the most efficient therapy to maintain (and even restore) bone density (Rossouw, et al. 2002).

Using a rat model of osteoporosis, the bone effects of E4 in comparison to EE were evaluated (H. J. Coelingh Bennink, et al. 2008a). Female adult rats (10 per group) underwent bilateral ovariectomy (except a group of the sham-operated animals which underwent placebo surgery). The ovariectomized rats were assigned to one of the following treatments: vehicle, E4 (0.1, 0.5 or 2.5 mg/kg/day) or EE 0.1 mg/kg/day.

The estrogen depletion consecutive to ovariectomy was responsible of a 20% increase in osteocalcin levels, a marked decline in bone mineral density (BMD) in the lumbar vertebrae and proximal tibiae, and a decrease of mechanical strength at the distal femora (measured with an indentation testing). As expected, treatment with EE restored all these parameters to values identical to sham-operated animals. The effect of E4 were dose-dependent and globally the highest dosage tested (2.5 mg/kg/day) gave similar results as 0.1 mg/kg/day EE (Figure 24). This demonstrated the positive effect of E4 at preventing development of osteoporosis induce by estrogen deficiency.

It was recently demonstrated that both nuclear and membrane ERα are necessary to obtain the full beneficial effects of E2 on a mice osteoporotic model. Estradiol is known to dramatically decrease the number of osteoclast, a feature that does not depend on the MISS pathway. At the opposite, the known proliferative activity of E2 on osteoblast was abrogated in mice lacking membrane ERα, suggesting the role of the MISS pathway on this target (Vinel, et al. 2016). However, mice lacking membrane receptor do not present osteoporosis if they are not ovariectomized. One may therefore conclude that circulating estrogen levels are sufficient to overcome the lack of membrane receptor.

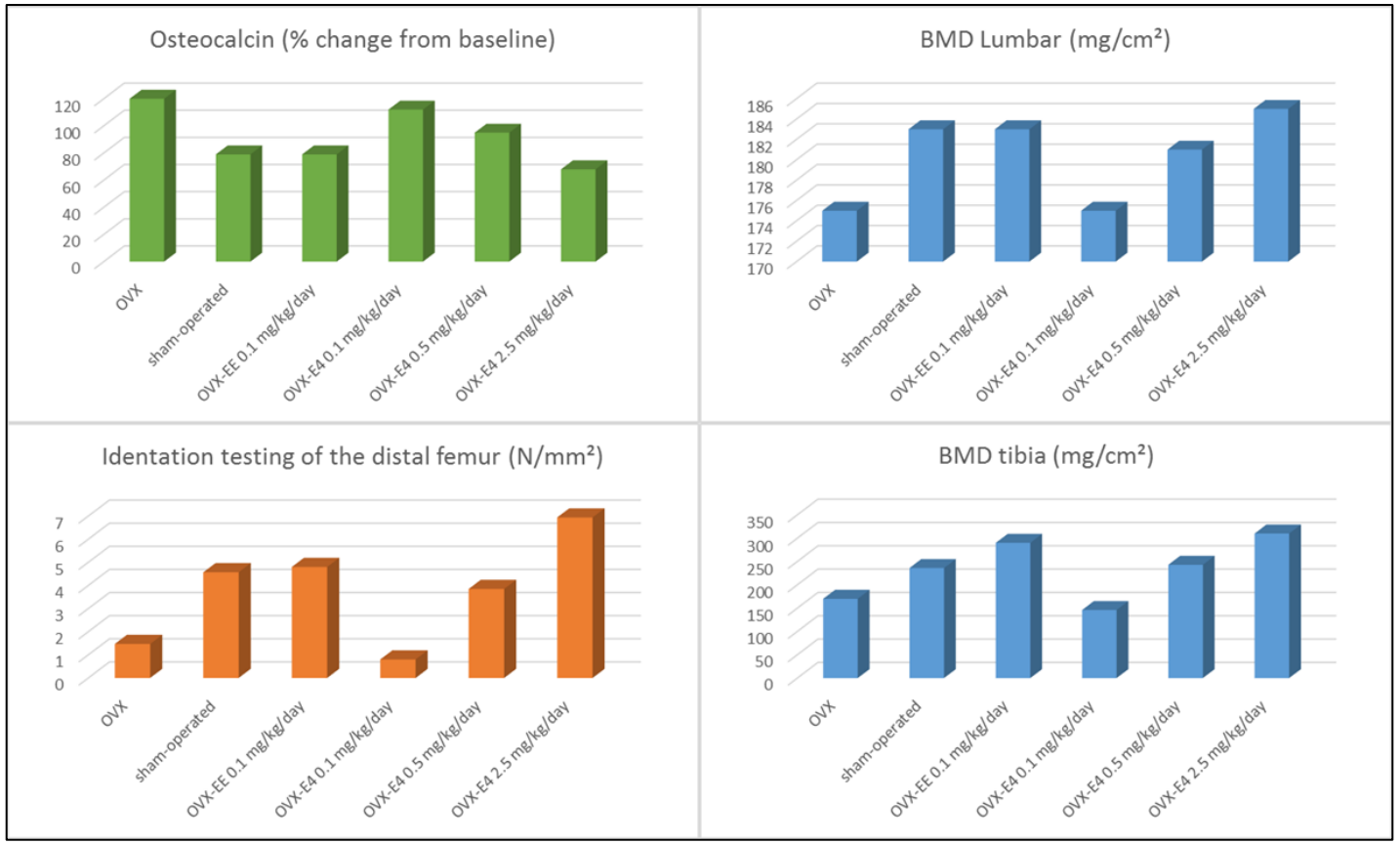

Figure 24. Effect of estetrol in comparison to ethinylestradiol on osteoporosis in a rat model. A. Percentage change from baseline in osteocalcin level; B. Indentation testing (mechanical strength of the bone); C. Bone mineral density at the lumbar vertebrae; D. Bone mineral density at the proximal tibia. BMD, bone mineral density; N, Newton; OVX, ovariectomized; E4, estetrol; EE, ethinylestradiol (Heegaard, et al. 2008).

7.7. Estetrol and mammary tissue (normal and cancer)

Estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. Their role as breast carcinogens has long been suspected in clinical settings and has been confirmed by epidemiological studies (Russo and Russo 2006). The cumulative estrogen "dose" to the breast epithelium seems to be one of the major risk factors for breast cancer. Early menarche and late menopause maximize consequently the risk of developing breast cancer as these conditions are associated with a high number of ovulatory cycles. In general, each year menarche is delayed results in a decrease of about 20% in breast cancer risk , and women who experience natural menopause before age 45 are estimated to have only half the breast cancer risk of those whose menopause occurs after age 55 (Feigelson and Henderson 1996).

Even if the results of epidemiological studies on the subject are controversial, it seems that the administration of exogenous estrogens in COCs may be associated with an increased breast cancer risk (Burkman, Schlesselman, and Zieman 2004). Regarding the risk of breast cancer and HRT, strong debates are still ongoing. Estrogen alone therapy is safe while administration of combined treatment (estrogen + progestin) is associated with a significant increased breast cancer incidence in post-menopausal women (Anderson, et al. 2004). However, the risk seems to differ in function of the progestin type used, natural progesterone and dydrogesterone being safer than synthetic progestins (Fournier, Berrino, and Clavel-Chapelon 2008).

Developing safer estrogen for the breast is therefore an important target.

7.7.1. In vitro and in vivo studies on human breast epithelial cells

In vitro studies using normal human breast epithelial cells and *in vivo* studies in mice showed that the proliferative effect of E4 on normal breast tissue is 100-fold lower than E2. Interestingly, when E4 was co-administered with E2, the proliferative action of E2 was decreased proportionally to the E4 dose. This demonstrates that E4 exhibits antagonistic properties toward the proliferative effect of E2 on breast epithelial cells Figure 25 (Gerard, et al. 2015a).

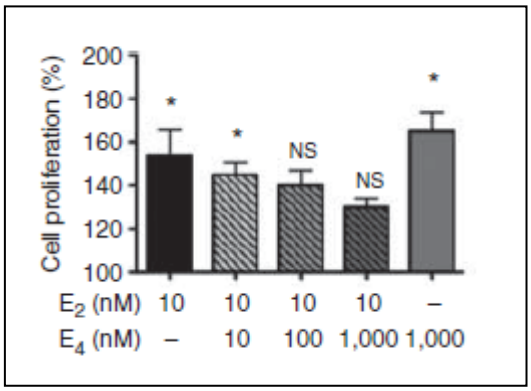

Figure 25. Quantification of the proliferative effect of estetrol and estradiol on normal human breast epithelial cells. Taken separately, each estrogen displays a proliferative activity but E4 is much less potent than E2. Combined together, E4 antagonizes the proliferative action of E2 in a dose-dependent way. E4, estetrol; E2, estradiol (Abot, et al. 2014).

7.7.2. In vitro studies in human breast cancer cell lines

For cancer spread, invasion and metastasis, cytoskeleton remodeling is necessary in order to allow cancer cells movement. Estrogens administered to breast cancer cells stimulate this cytoskeletal remodeling and, consequently, promote invasion and migration of the tumor. The effect of E4 on breast cancer cell invasion was studied by Giretti and co-workers using the human breast carcinoma cell line T47-D (Giretti, et al. 2014). They first demonstrated that, as opposed to E2, E4 weakly stimulated T47-D cells migration and invasion. Interestingly, in the presence of E2, E4 inhibited the cancer cells migration and invasion, demonstrating again an antagonistic activity of E4 on E2 (Figure 26).

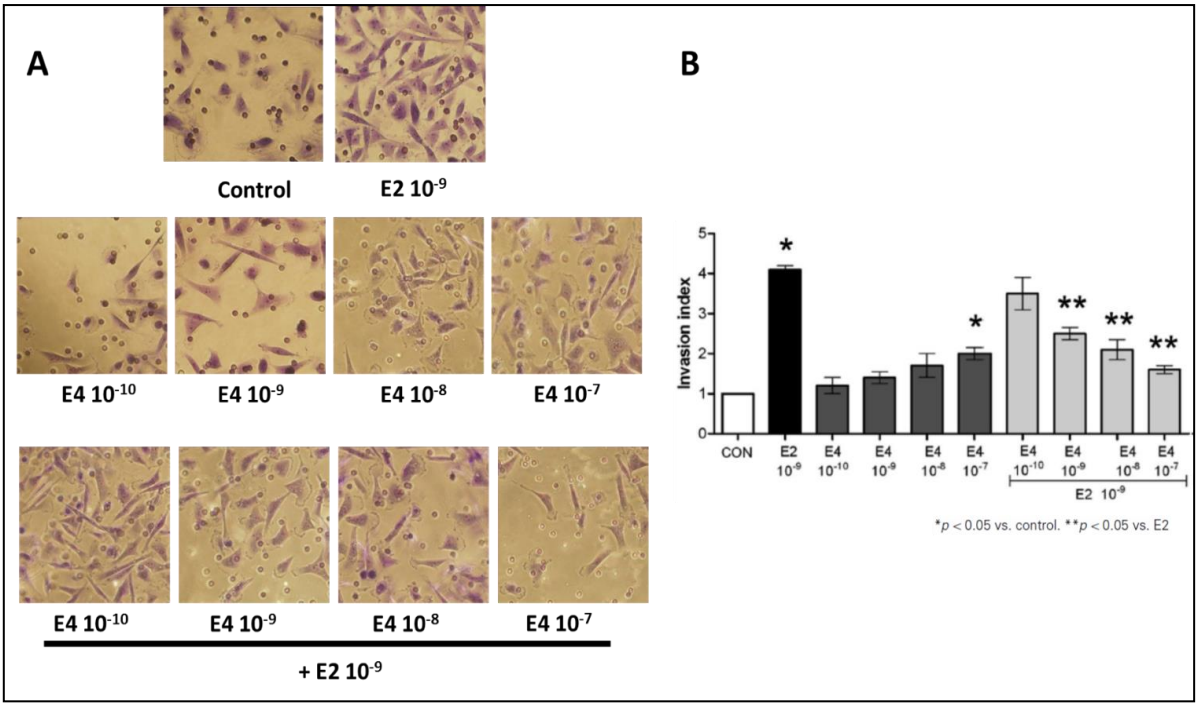

Figure 26. T47-D cells were exposed to E2 10^{-9} M or to increasing concentrations of E4, without and with adjunction of E2 10^{-9} M. (A) Pictures of cells migration through matrigel, (B) Quantification of breast invasion. E2, estradiol; E4, estetrol; CON, control (Giretti, et al. 2014).

7.7.3. In vivo studies in breast cancer animal models

The first animal data on the impact of E4 on breast cancer were generated in the so called *DMBA rat model* (Visser, Kloosterboer, and Bennink 2012). Female rats were exposed to a single oral dose of 7,12-Dimethylbenz(a)anthracene (DMBA), a carcinogenic agent known to promote the development of estrogen related tumors. The first part of the study evaluated the preventive action of E4 on breast tumor development. For this purpose, after having received DMBA, rats were exposed for 8 weeks to increasing doses of E4 (0.5, 1, 1.5, 2, 2.5 or 3 mg/kg/day), to tamoxifen as positive control (0.5, 1.5 or 3 mg/kg/day) or to vehicle as negative control. Mean number of mammary tumors in each group is reported in Figure 27: increasing doses of E4 had a significant protective effect on the number and growth of mammary tumors in rat; from 2.5 mg/kg/day onwards, the protective effect of E4 was identical to that of tamoxifen.

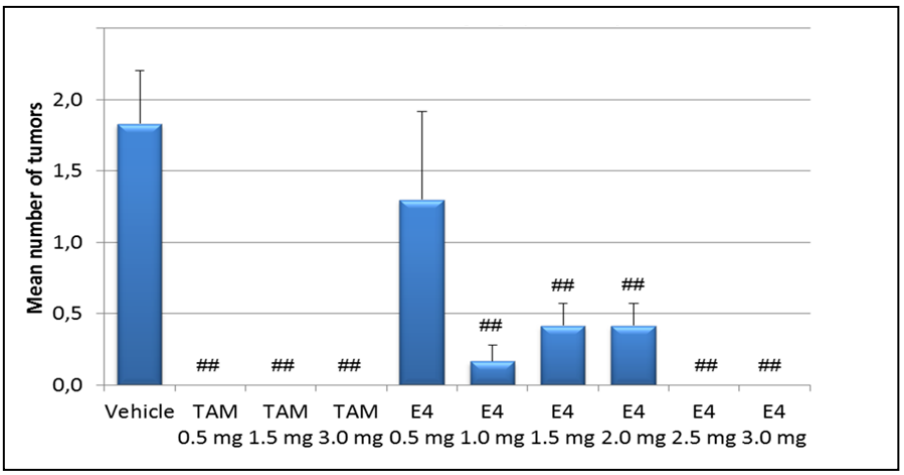

Figure 27. Mean number of mammary tumors in rat exposed to tamoxifen (TAM), estetrol (E4) or to vehicle for 8 weeks after tumor initiation by DMBA. Results suggest a protective action of E4 on tumor development in this model. ##, significantly different from vehicle (Visser, Kloosterboer, and Bennink 2012).

The second part of the experiment evaluated the therapeutic potential of E4 on already present mammary tumor (Figure 28). Eight to 11 weeks after DMBA exposure, rats were either ovariectomized (first positive control), or received one of the following oral treatments for 4 weeks: EE (0.15 or 0.5 mg/kg/day), E4 (0.5, 1, 3 or 10 mg/kg/day), tamoxifen 1 mg/kg/day as positive control, or vehicle as negative control. Results demonstrated that increasing doses of E4 had a curative activity on mammary tumors. The best improvement was seen in the E4 10 mg/kg/day group which showed identical results as ovariectomized animals.

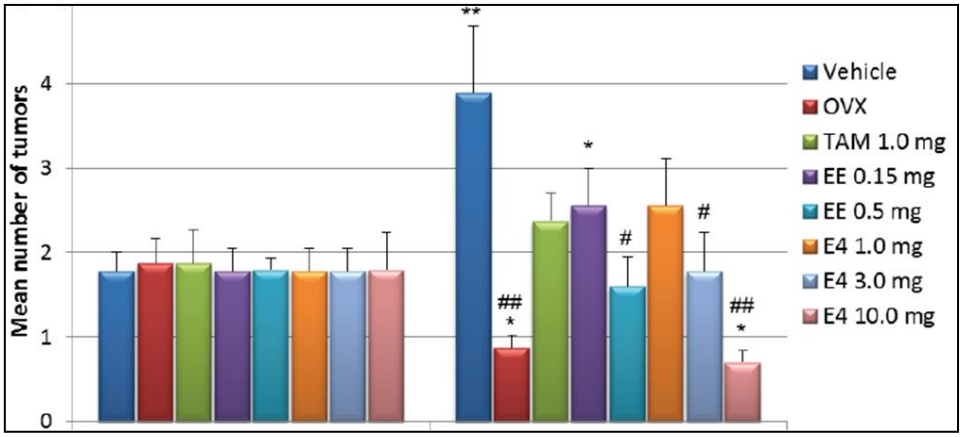

Figure 28. Mean number of mammary tumors in rat 8-11 weeks after having been exposed to DMBA and after 4 weeks of ovariectomy, oral treatment with to tamoxifen (TAM), ethinylestradiol (EE), estetrol (E4) or vehicle. Results suggest a curative action of E4 on tumor development in this model. *, significantly different from baseline; ##, significantly different from vehicle (Visser, Kloosterboer, and Bennink 2012).

The next *in vivo* data were generated by Gerard et al. in ovariectomized immunodeficient mice implanted with human breast cancer cells of the MCF-7 line (Gerard, et al. 2015b). In the first experiment, mice were treated with a daily oral treatment of E4 (0.5, 1, 3 or 10 mg/kg/day) or E2 (3 mg/kg/day). In the second experiment they were first implanted with an E2 pellet and they received in parallel E4 (0.5, 1, 3 or 10 mg/kg/day). After 5 weeks of treatment, animals were sacrificed and tumors were weighted. Results are reported in Figure 29. The first experience showed that E2 significantly promoted tumor growth (in this group, tumor weights were 5-fold increased compared to the untreated group) while E4 had a weaker proliferative activity on tumor than E2. The lowest E4 dose was not able to significantly increase tumor weight in comparison to the untreated control group. E4 was as effective as E2 in promoting tumor growth only at the dose of 10 mg/kg/day. The second study showed that tumor weight was significantly decreased when E4 was added to the E2 treatment, demonstrating that the antagonistic activity of E4 on E2-induced tumor growth is also present in *in vivo* models.

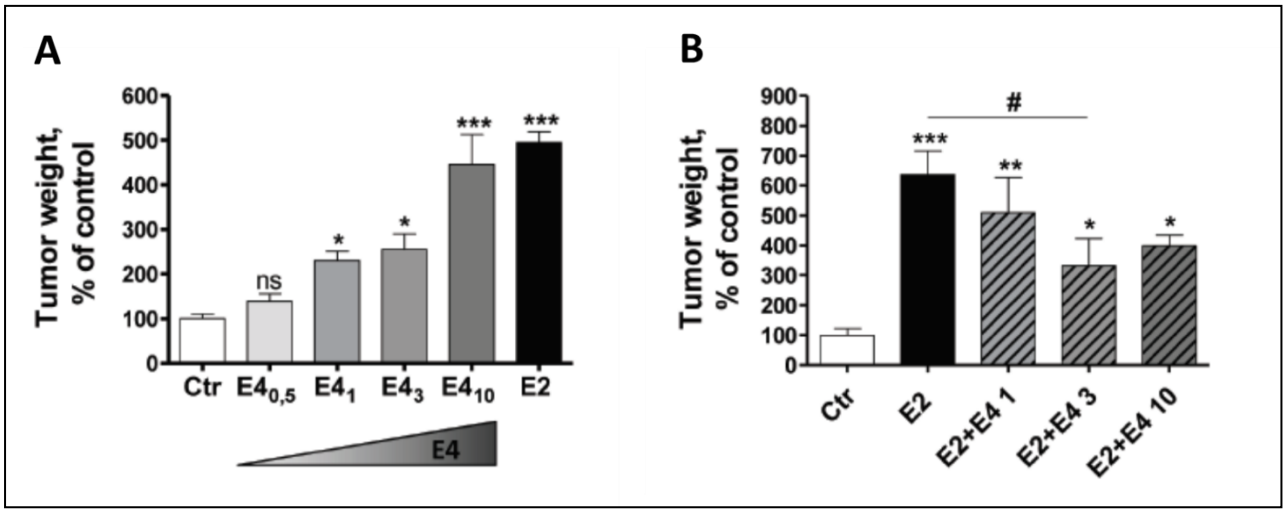

Figure 29. In this mice model, ovariectomized mice, implanted with MCF-7 cells, were treated with either estrogen alone therapy (estradiol or increasing doses of estetrol) (A) or with combined estrogen therapy (estradiol + increasing doses of estetrol) (B). After 5 weeks, tumor growth was evaluated in each group. Estetrol has a weak tumor promoting activity in comparison to estradiol (A) and estetrol antagonizes the estradiol-induced tumor promoting activity (B). Ctr, control; E4, estetrol; E2, estradiol (Gerard, et al. 2015b).

7.7.4. First human study in breast cancer patients

Given the promising anti-estrogenic activity of E4 on breast cancer observed *in vitro* and in animal models, a first-in-human study was conducted with 30 pre-and post-menopausal women diagnosed with ER-positive early breast cancer. The women were randomly assigned to E4 20 mg/day or to placebo. They received treatment for 14 days before cancer surgery (Singer, et al. 2014).

In the tumor, E4 had a significant pro-apoptotic effect in comparison to the placebo. However, there was no change in the expression of the proliferative marker Ki67 (Figure 30).

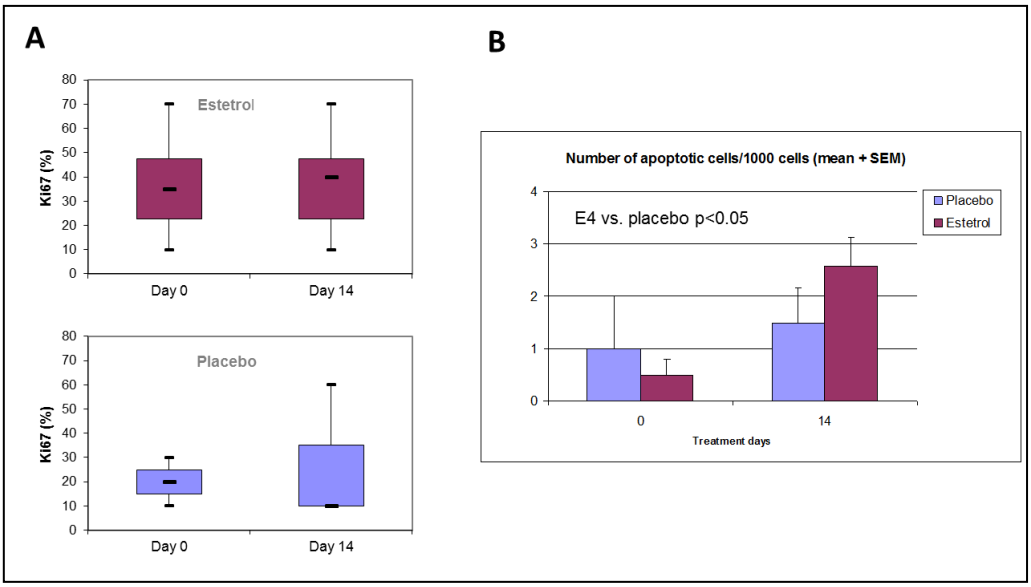

Figure 30. Change from baseline (Day 0) and end of a 14-day treatment with estetrol 20 mg/day or with placebo in the proliferative marker Ki67 (A) and in the number of apoptotic cells (B) in each group. E4, estetrol (Singer, et al. 2014).

Interestingly, expression of ERα was significantly lower in the tumor exposed to E4 while ERβ expression tended to increase with E4; surexpression of ERβ has been previously shown to have an antiproliferative activity (Figure 31) (Pettersson, Delaunay, and Gustafsson 2000).

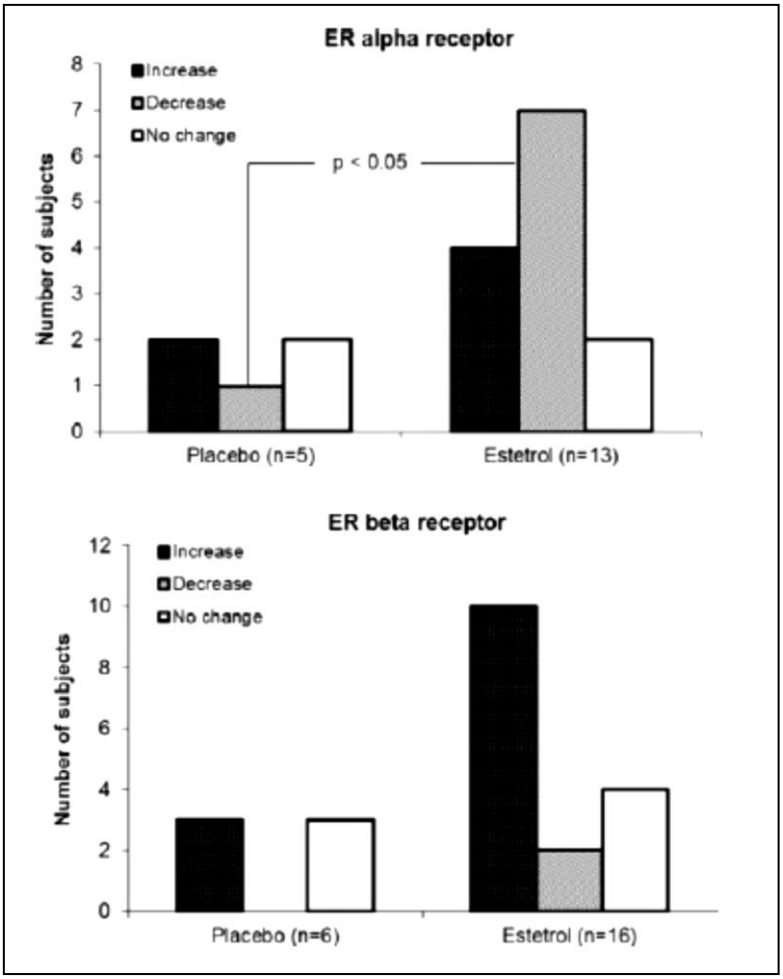

Figure 31. Increase (black bar), decrease (grey bar) or no change (white bar) in intra-tumoral expression of estrogen receptor alpha and estrogen receptor beta in response to 14 days of oral treatment with either 20 mg estetrol or placebo. ERα, estrogen receptor alpha; ERβ, estrogen receptor beta (Pettersson, Delaunay, and Gustafsson 2000).

Altogether, the pre-clinical and the small clinical data obtained so far with E4 on breast cancer suggest that E4 displays a different pattern on the breast than E2 or EE. An extended research program is currently ongoing in partnership with different universities and research centers to further characterize the potential of E4 on breast cancer.

7.8. Cardiovascular impact of estetrol

The studies on the cardiovascular impact of E4 covers two different aspects: first the cardioprotective activity of E4 (*i.e.* the impact of the molecule on the heart itself) for which the preclinical studies are currently ongoing and, secondly, the vasoprotective activity of E4 (*i.e.* the impact of the molecule on the blood vessels) for which a couple of pre-clinical studies have been conducted so far and that may be divided into three different aspects: impact on atherosclerosis, on myo-intimal protection, and on vasodilatation.

7.8.1. Impact of E4 on atherosclerosis

To evaluate the impact of E4 on atherosclerosis development, a well-recognized animal model to evaluate atheroprotective activity of estrogens was used: LDL receptor knockout mice (LDLr -/-), either ERα knockout (ERα-/-) or not (ERα+/+), were ovariectomized and received a high-cholesterol diet in parallel to an E4 treatment (0.6 and 6 mg/kg/day) or not (control) (Abot, et al. 2014). Lipid deposition in the aorta was measured. Results are presented in Figure 32 and showed that E4 displays an atheroprotective activity which is dose-dependent and this activity is mediated by the ERα since the effect was abolished in mice that did not express ERα. The highest E4 dose was associated with a decrease in atheroma deposit by up to 80%, a level of protection similar to that obtained using a high dose of E2 (80 µg/kg/jour) (Billon-Gales, et al. 2009).

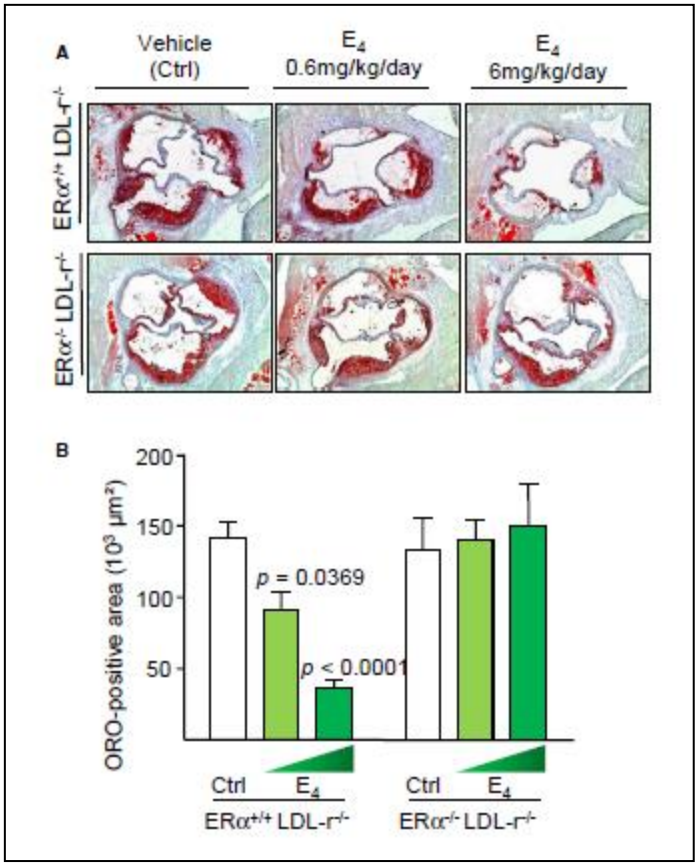

Figure 32. Four-week-old ovariectomized ERa+/+LDL-r-/- or ERa-/-LDL-r-/- mice were switched to atherogenic diet from the age of 6–18 weeks added with placebo (Ctrl) or estetrol (0.6 or 6 mg/kg/day). A, B: Representative micrographs of Oil red-O (ORO) lipid-stained cryosections of the aortic sinus (A) and quantification of lipid deposition (B) are represented. Ctrl, control; E4, estetrol (Abot, et al. 2014).

7.8.2. E4 and myo-intimal protection

After percutaneous coronary interventions, mainly ballooning (angioplasty) and stenting, vascular smooth muscles cells from the media can proliferate and migrate through the internal elastic lamina of the arteries. This process, called neo-intimal hyperplasia, creates a thickening of the arterial walls which leads to a narrowing of the arterial lumen (*restenosis*) (Purcell, Tennant, and McGeachie 1997).

Estradiol has been shown to decrease neointimal hyperplasia by two mechanisms: first, it accelerates the endothelial regrowth and secondly, it inhibits the proliferation of vascular smooth muscle cells. Using transgenic mice, in a model of femoral arterial injury, Arnal and colleagues demonstrated recently that neointimal hyperplasia is also prevented by E4, in the same extent as E2 (Figure 33) (Smirnova, et al. 2015).

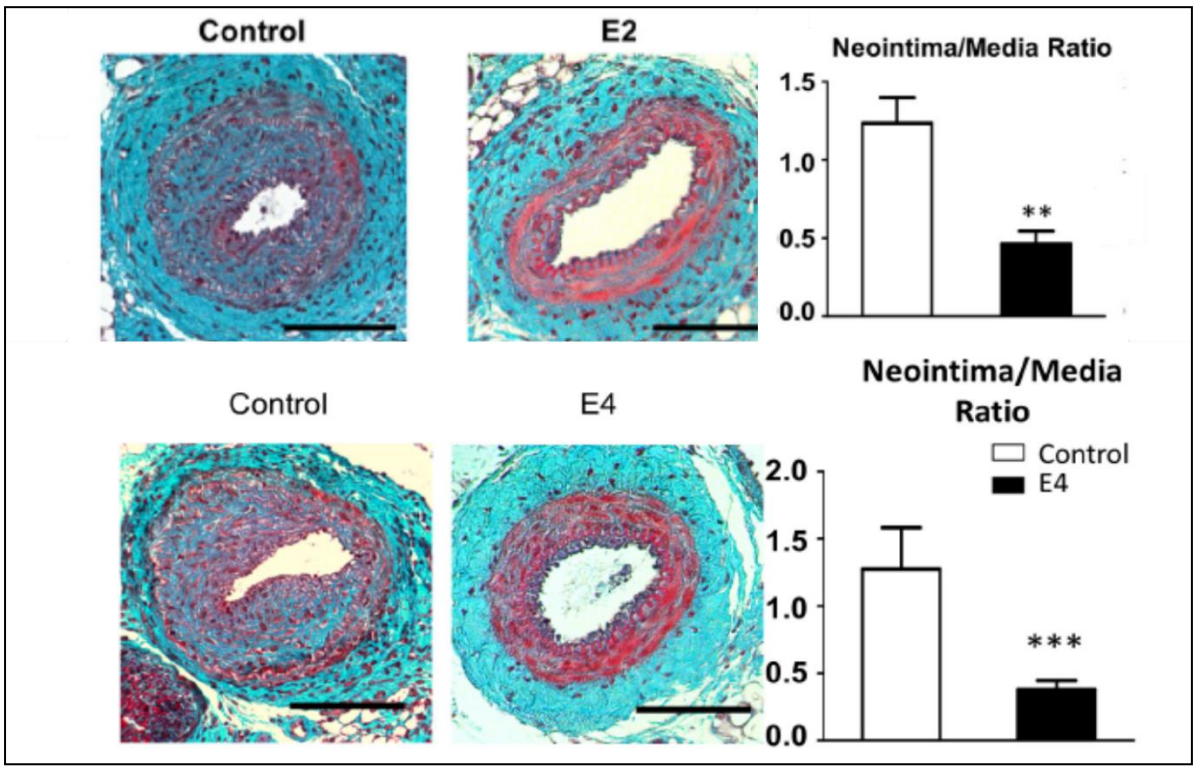

Figure 33. Four-week-old wild-type female mice were ovariectomized and subcutaneously implanted with placebo (control) or with estradiol (above) or estetrol (below). Two weeks later, animals were submitted to mechanical injury of the femoral artery. Arteries were harvested 28 days after the injury for morphometric analysis. Left, Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Right, Quantitative analysis of neointima/media ratio of indicated mice. Values are presented as mean±SEM (n=7–15 mice per group), and statistically compared with Mann–Whitney U test. ***P<0.001. E2, estradiol; E4, estetrol (Smirnova, et al. 2015).

7.8.3. Impact of E4 on vasodilatation

The effect of E4 on eNOS and subsequent production of nitric oxide (NO), considered as a key player in the vascular function, is still unclear and necessitates further studies. In a study done by Abot and colleagues on endothelial cells from mice, aorta did not show any modulation of eNOS in the presence of E4, in the opposite of E2 (Abot, et al. 2014). On the other hand, the team of Simoncini and co-workers have addressed the effects of E4 on the activity and expression of the eNOS in cultured human umbilical vein endothelial cells. It appeared that E4 stimulated the activation of eNOS and NO secretion, in significantly lower extent than E2. In the opposite to what was expected, no concentration-dependent effect was found as higher amounts of E4 were not associated with the higher effect. Associated to E2, given in concentrations seen during pregnancy, E4 antagonized NO synthesis while it did not when it was associated to E2 given in concentrations seen during menopause (Montt-Guevara, et al. 2015). Finally, Hilgers et al. assessed the vasorelaxing effect of E4 in comparison to E2 in height arterial beds (uterine, thoracic aortic, carotid, mesenteric, pulmonary, renal, middle cerebral and septal coronary arterial segments). They demonstrated that E4, like E2, is able to induce a vasorelaxing effect. The vasorelaxing effect of E4 was not NO dependent but involved guanylate cyclase and blockade of smooth cells Ca2+ entry (Hilgers, et al. 2012). Altogether, these data demonstrate that E4, like E2, induces a vasodilatation but the underlying mechanisms are still under evaluation.

7.9. Neuroprotective activity of estetrol

Hypoxic-ischemic encephalopathy (HIE) is still of concern in perinatal medicine. Two recent trials provided updated information on mortality and the neurodevelopmental outcomes in infants with moderate and severe HIE as follows: 23-27% of infants died prior to discharge from the neonatal IC unit, whereas 37-38% died at follow up 18-22 months later, and the neurodevelopmental outcome at 18 months included: mental and psychomotor development retardation, cerebral palsy, epilepsy, blindness and hearing impairment (Gluckman, et al. 2005; Shankaran, et al. 2005). Physiopathology of perinatal HIE can be multifactorial: maternal causes (*e.g.* impaired oxygenation due to maternal disease, impaired perfusion of the placenta due to pregnancy and/or placental pathologies), fetal causes, and/or combination of both.
Main principles of HIE treatment, following initial resuscitation and stabilization, include neuroprotective strategy, support of adequate ventilation and perfusion, careful fluid management, avoidance of hypo- and hyper-glycaemia, avoidance of hypotension (a mean blood pressure above 35-40 mm Hg is necessary to avoid decreased cerebral perfusion) and treatment of seizures (Shankaran 2002; Stola and Perlman 2008). At present, therapeutic hypothermia, started within the first hours after delivery and maintained for 72 hours at 33 to 35°C, is considered the best neuroprotective strategy (Azzopardi, et al. 2009; Gluckman, et al. 2005; Shankaran, et al. 2005).However, neurodevelopmental deficits persist in 40–50% of patients even after hypothermia (Shankaran, et al. 2005). So far, no specific medical treatment provides important neuroprotection against HIE neither in near-term and term new-borns.

Several studies have demonstrated the potential benefit of E4 in the attenuation of neonatal HIE. To study the neuroprotective and therapeutic effects of E4 *in vivo*, a first study was conducted with a neonatal hypoxic–ischemic encephalopathy model of 7-day-old newborn rat pups. The neuroprotective and therapeutic effects of E4 before and after hypoxic–ischemic insult was studied in 1 mg/kg/day, 5 mg/kg/day, 10 mg/kg/day, 50 mg/kg/day E4 pretreated and treated groups and compared with the sham and the vehicle treated groups. The body temperature of the rat pups was examined along with their body and brain weights. Brains were studied at the level of the hippocampus and cortex. Intact cell counting and expression of microtubule-associated protein-2, doublecortin and vascular-endothelial growth factor were evaluated by histo- and immunohistochemistry. Concentrations of brain damage markers (S100B and glial fibrilary acidic protein) were defined in blood samples. The results revealed for the first time that E4 significantly decreased the early gray matter loss and promoted neuro- and angiogenesis *in vivo* (Tskitishvili, et al. 2014). Further studies tested the effect of different combinations of E4 with progesterone and/or E2 and compared the results with the use of E4 alone. *In vivo* pre-treatment (before the experimental hypoxic-ischemic injury) or treatment (following the hypoxic-ischemic insult) by different combinations of E4 with other steroids had unlike effects on body and brain weight, neuro- and angiogenesis, and glial fibrillary acidic protein expression in blood. It appeared that combined use of E4 with other steroids did not show any beneficial effect over the single use of E4 (Tskitishvili, et al. 2016).

7.10. First exogenous administration of estetrol in human

The first in-human administration of exogenous E4 was carried out in 2003 and the results were reported in an article published in Climacteric in 2008 (Visser, Holinka, and Coelingh Bennink 2008). For ethical reason, the study was conducted with healthy post-menopausal women: in each treatment group, 6 women received E4 while two women received a placebo. The following doses of E4 were administered: 0.1 mg E4, 1 mg E4, 10 mg E4 and 100 mg E4. The primary objective of the study was to evaluate the safety and tolerability of the compound. Beside this, the PK profile of E4 and its impact on gonadotropins secretion (LH and FSH) were also evaluated.

7.10.1. Safety profile

As a precaution, the dosing was done sequentially: the safety and PK data of the lowest dose were analyzed before starting the dosing of the next dose. The results showed that single doses of E4 up to the dose of 100 mg were safe and well-tolerated. The only remarkable safety event during the study occurred in a woman from the 100 mg dose group who developed an urticarious rash on face and chest shortly after the administration. The woman recovered rapidly with antihistaminic treatment. In order to determine if the allergic reaction was due to E4, skin prick tests using several dilutions of E4 were carried out but did not provoke an allergic reaction.

7.10.2. Pharmacokinetics

No PK data could be obtained for the lowest dose (0.1 mg E4) since this dose gave concentrations below the lowest level of quantification. The PK profile obtained with the three other doses is displayed in Figure 34. It appeared that the PK parameters were dose-proportional:

- Mean T_{max} (SD) expressed in hours: 0.253 (0.0082), 0.333 (0.1291) and 0.542 (0.2458) in the 1, 10 and 100 mg dose group, respectively.
- Mean C_{max} (SD) expressed in ng/ml: 0.5897 (0.2837), 8.928 (2.731) and 162.2 (39.99) in the 1, 10 and 100 mg dose group, respectively.
- $AUC_{0\text{-last}}$ (SD) expressed in h.ng/ml: 4.081 (2.2510), 42.27 (10.170) and 661.9 (205.18) in the 1, 10 and 100 mg dose group, respectively.

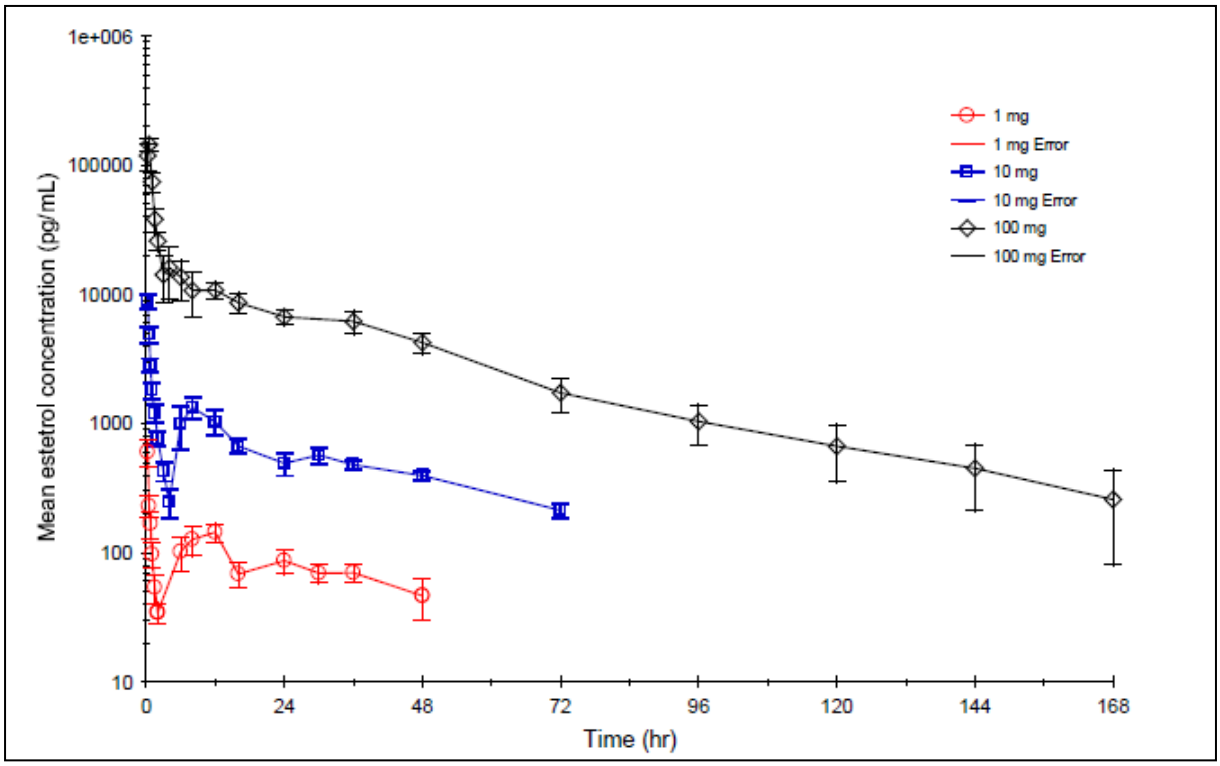

Figure 34. Mean estetrol concentration in plasma after oral intake of a single dose of 1 mg estetrol (measurements of estetrol done over 48h), 10 mg estetrol (over 72h) and 100 mg estetrol (over 168h) in post-menopausal women (n=6) (Visser, Holinka, and Coelingh Bennink 2008).

The mean terminal half-life was 28 hours in both the 10 and 100 mg E4 groups (not determined for the 1 mg group).

As shown in Figure 34, in each dose group, the general PK profile was characterized by a very steep increase followed by sharp decline. It is to notice that a secondary peak was seen in all groups and even a third peak was visible in the 1 and 10 mg E4 groups. This suggests an

enterohepatic recirculation of E4, a phenomenon also described with other estrogens (Sher and Rahman 2000).

7.10.3. Impact on LH and FSH

Serum LH level was not impacted by the administration of 0.1 mg and 1 mg E4 while it decreased proportionally to the E4 concentration in the two other groups. This was particularly visible in the highest dose group (100 mg E4): after 4 hours, maximum mean decrease in LH level was 48%. The level subsequently increased and once again decreased (maximum mean decrease of 37%) 12 hours after dosing, short after the second peak of E4 in blood (Figure 35). LH values returned to baseline after 72 hours.

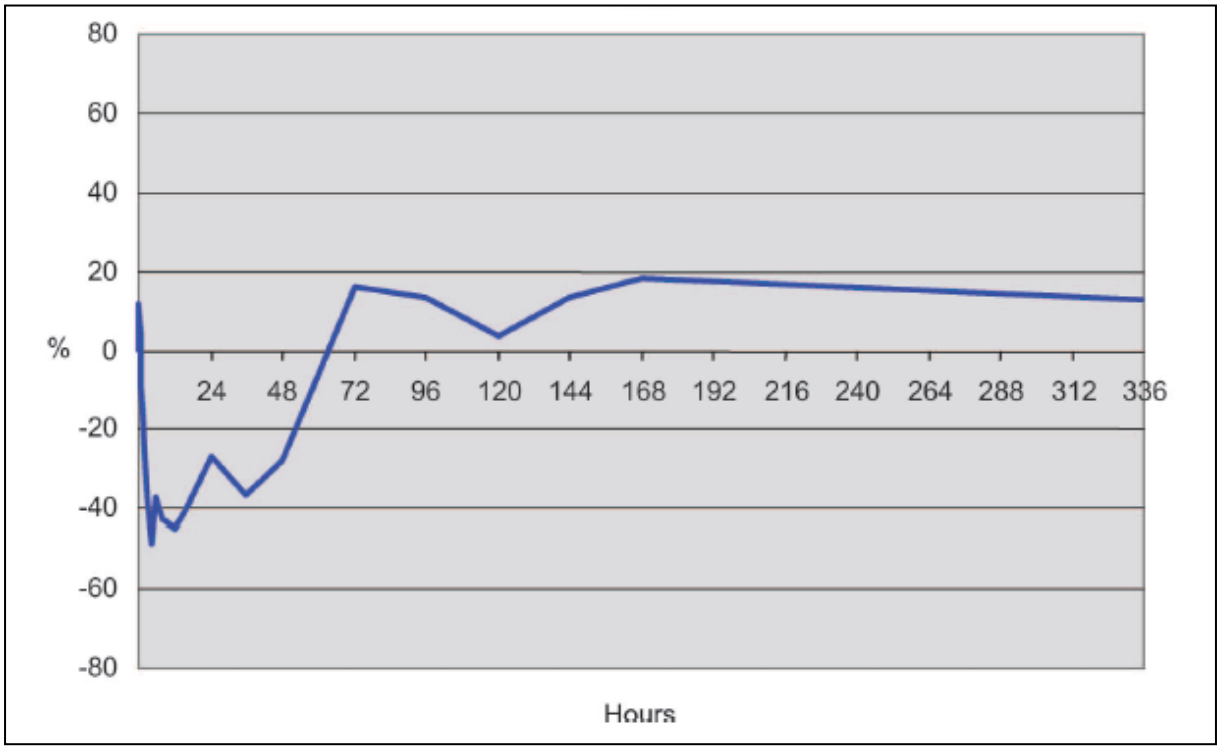

Figure 35. Mean percentage change from baseline in luteinizing hormone (LH) levels after administration of a single oral dose of 100 mg estetrol in post-menopausal women (n=6) (Visser, Holinka, and Coelingh Bennink 2008).

Serum FSH was only measured in the 100 mg E4 group and displayed a different profile than LH (Figure 36): FSH seemed more profoundly and sustainably decreased than LH, with no correlation with E4 blood concentration. Maximum mean decrease in FSH level was seen at 48 hours (-41%) and FSH level returned to baseline in 168 hours.

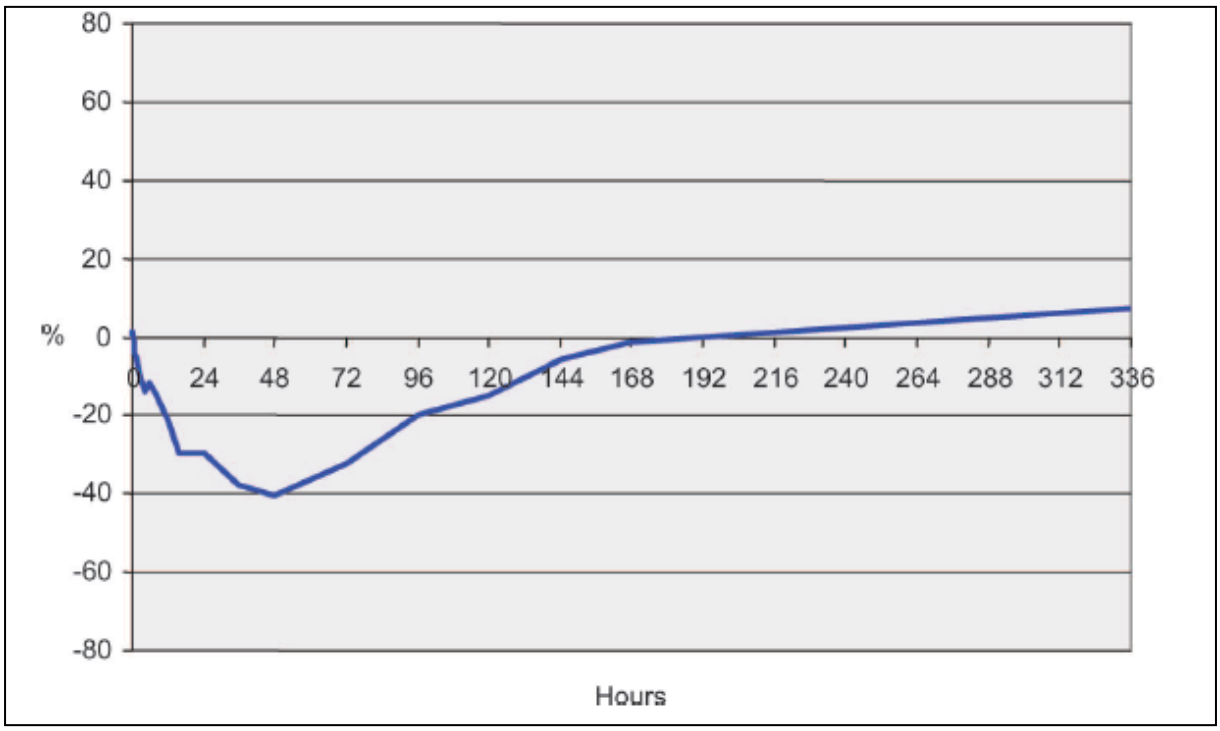

Figure 36. Mean percentage change from baseline in follicle-stimulating hormone (FSH) levels after administration of a single oral dose of 100 mg estetrol in post-menopausal women (n=6) (Visser, Holinka, and Coelingh Bennink 2008).

The good safety data gathered during the first-in-human study allowed to continue the development and a second phase 1 study was designed to evaluate the impact of E4 administered for 28 days. The results of this study are presented below.

7.11. Second human study with exogenous E4

In this second phase 1 study, 49 healthy post-menopausal women were randomly assigned to one of the following treatment groups: 2 mg E4 (n=10), 10 mg E4 (n=10), 20 mg E4 (n=10) and 40 mg E4 (n=9). A fifth group received 2 mg E2V as control treatment (n=10). The treatments were administered for 28 consecutive days. Following the same principle of precaution as in the first trial, the treatments were given sequentially: only once the safety and pharmacodynamic properties of an E4 dose were analyzed, the next dose was started.
Here also the primary objective was the evaluation of the safety profile of E4. Beside the safety, the multiple dose administration used in this study allowed to evaluate the impact of E4 on several pharmacodynamic parameters known to be affected by estrogens: vaginal cytology, endometrium, gonadotrophins levels, hemostasis parameters, lipids and glucose parameters. Finally, a preliminary assessment of the impact of E4 on vasomotor symptoms (hot flushes) was also carried on.

The subjects included in the highest E4 dose groups (20 mg and 40 mg) were all hysterectomized while the others had an intact uterus. At the end of the trial, these received 14 days of progestogen for endometrial protection.
The results of this study were reported in three publications and are described below (Coelingh Bennink, et al. 2016; Coelingh Bennink, et al. 2017b; Coelingh Bennink, et al. 2017a).

7.11.1. Safety profile

In this study, E4 given for 28 days up to 40 mg appeared safe and well-tolerated. The adverse events reported by the participants were all expected for an estrogen therapy. The most frequent drug-related adverse events were nipple tenderness, headache, abdominal pain and vaginal discharge. No dose-dependency was observed in the occurrence of adverse events except for nipple tenderness which seemed to be related with the E4 dose. There was no clinically significant change in laboratory parameters, electrocardiograms and vital signs (Coelingh Bennink, et al. 2016).

7.11.2. Pharmacokinetics

This multiple dosing study confirmed that the PK parameters of E4 are dose-proportional. Estetrol concentrations on day 28 were slightly higher compared to day 1, indicating some accumulation (Table 14).

Table 14. Pharmacokinetic parameters on Day 1 and Day 28 for the 2 mg, 10 mg, 20 mg and 40 mg estetrol groups (Coelingh Bennink, et al. 2017b).

		2 mg E4	10 mg E4	20 mg E4	40 mg E4
Day 1					
C_{max} *(ng/ml)*	*GM*	0.85	7.51	NC	NC
	CV% GM	32.71	39.09	NC	NC
T_{max} *(h)*	*Mean*	0.59	0.36	NC	NC
	SD	0.32	0.13	NC	NC
Day 28					
C_{max} *(ng/ml)*	*GM*	1.27	9.18	21.94	72.27
	CV% GM	18.61	28.55	39.94	61.57
T_{max} *(h)*	*Mean*	0.54	0.41	0.22	0.22
	SD	0.28	0.16	0.08	0.12
$AUC_{0\text{-}24}$ *(h*ng/ml)*	*GM*	NC	NC	76.85	187.50
	CV% GM	NC	NC	24.80	26.29

E4, estetrol; GM, geometric mean; CV%, coefficient of variation; NC, not calculated.

Steady state was reached between Day 7 and Day 14 of treatment (in this study there was no measurement of E4 concentration done between Day 7 and Day 14 to more precisely define the day of steady state) (Figure 37).

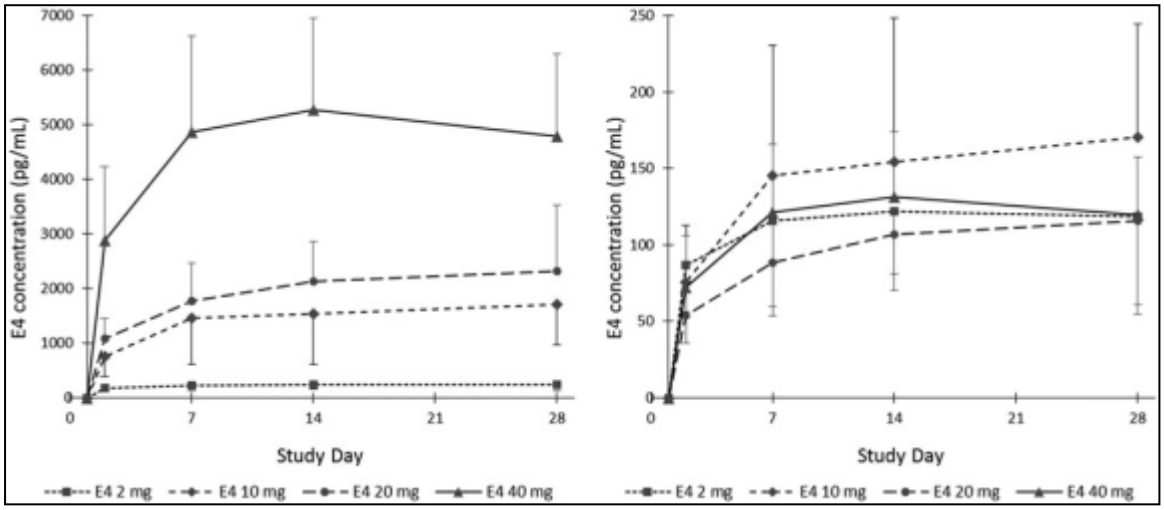

Figure 37. Mean estetrol trough plasma concentrations (left) and mean trough plasma concentrations normalized for dose (right) for the 2 mg, 10 mg, 20mg, and 40 mg estetrol groups. E4, estetrol (Coelingh Bennink, et al. 2017b).

A robust PK assessment done after the administration of E4 on Day 28 showed that E4 PK profile is characterized by a very fast absorption, followed by a multiphasic elimination with an initial rapid decline, gradually continuing with a slower elimination (Figure 38). The terminal half-life was not calculated in this study.

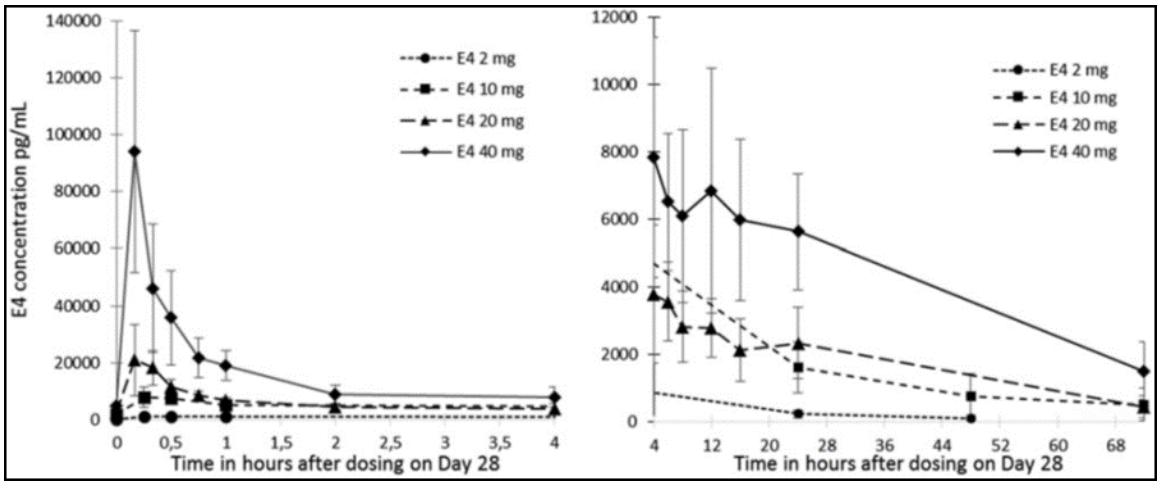

Figure 38. Mean estetrol plasma concentration time plots from 0 to 4 (left) and from 4 to 72 h post dose (right) on day 28 for the 2 mg, 10 mg, 20 mg and 40mg E4 groups (note the different scales on the x- and y-axes for the left and right figures). E4, estetrol (Coelingh Bennink, et al. 2017b).

7.11.3. Change in vaginal cytology

Vaginal cytology was evaluated using the vaginal maturation index performed at baseline and at the end of the estrogen treatment. The vaginal maturation index is an evaluation of the proportion of the different vaginal epithelial cells (reported in percentages): superficial, intermediate and parabasal cells. Post-menopausal status is characterized by vulvo-vaginal atrophy which translates into a decrease in both superficial and intermediate cells and an increase in parabasal cells (Mac Bride, Rhodes, and Shuster 2010). In average, the percentage of the different vaginal cells types of the study participants at baseline demonstrated vulvo-vaginal atrophy. To note, there was a high inter subject variability probably due to the heterogeneity in the duration of menopause across the study population.
Table 15 displays the results. After 28 days of E4 treatment, an improvement in the vaginal maturation index was seen in all groups without real dose-proportionality, except for the proportion of superficial cells which appeared to increase with the E4 dose. In the control group, an improvement in-between the 2 mg E4 and 10 mg E4 group was observed (Coelingh Bennink, et al. 2016).

Table 15. Percentage of vaginal epithelial cells types (parabasal, intermediate and superficial) expressed in mean percentage (range) before treatment and after 28 days of estrogenic treatment with 2, 10, 20 or 40 mg estetrol or with 2 mg estradiol valerate in post-menopausal women (Coelingh Bennink, et al. 2016).

	Parabasal cells Mean% (range)		**Intermediate cells Mean% (range)**		**Superficial cells Mean% (range)**	
Treatment groups	***Screening***	***Day 28***	***Screening***	***Day 28***	***Screening***	***Day 28***
2 mg E2V (n=10)	32 (0-93)	0 (0-3)	64 (5-99)	74 (51-92)	4 (0-19)	26 (9-49)
2 mg E4 (n=10)	40 (0-99)	3 (0-15)	47 (1-92)	81 (53-94)	13 (0-53)	18 (6-47)
10 mg E4 (n=10)	51 (1-99)	8 (0-17)	46 (0-98)	45 (23-78)	3 (1-10)	47 (5-77)
20 mg E4 (n=10)	29 (0-99)	0 (0)	65 (1-99)	60 (17-88)	6 (0-21)	40 (12-83)
40 mg E4 (n=9)	65 (0-95)	0 (0-2)	30 (4-92)	46 (26-71)	5 (0-24)	53 (29-74)

E4, estetrol; E2V, estradiol valerate

This showed that orally administered E4, even at low dose, is able to exert a strong estrogenic activity on the vaginal epithelium. These data confirmed the animal data gathered in rats and presented in section 7.6 (Heegaard, et al. 2008).

7.11.4. Changes in endometrium

Endometrial assessments could be done in the 2 mg E2V, 2 mg E4 and 10 mg E4 (non-hysterectomized women) (Coelingh Bennink, et al. 2016).
First, endometrial thickness was measured by ultrasonography before treatment, after 14 days and 28 days of treatment and after progestogen treatment. Results are depicted in Figure 39: mean endometrial thickness was quite stable throughout the treatment in the group receiving 2 mg E4 while it increased over time in the control group (2 mg E2V) and the 10 mg E4 group.

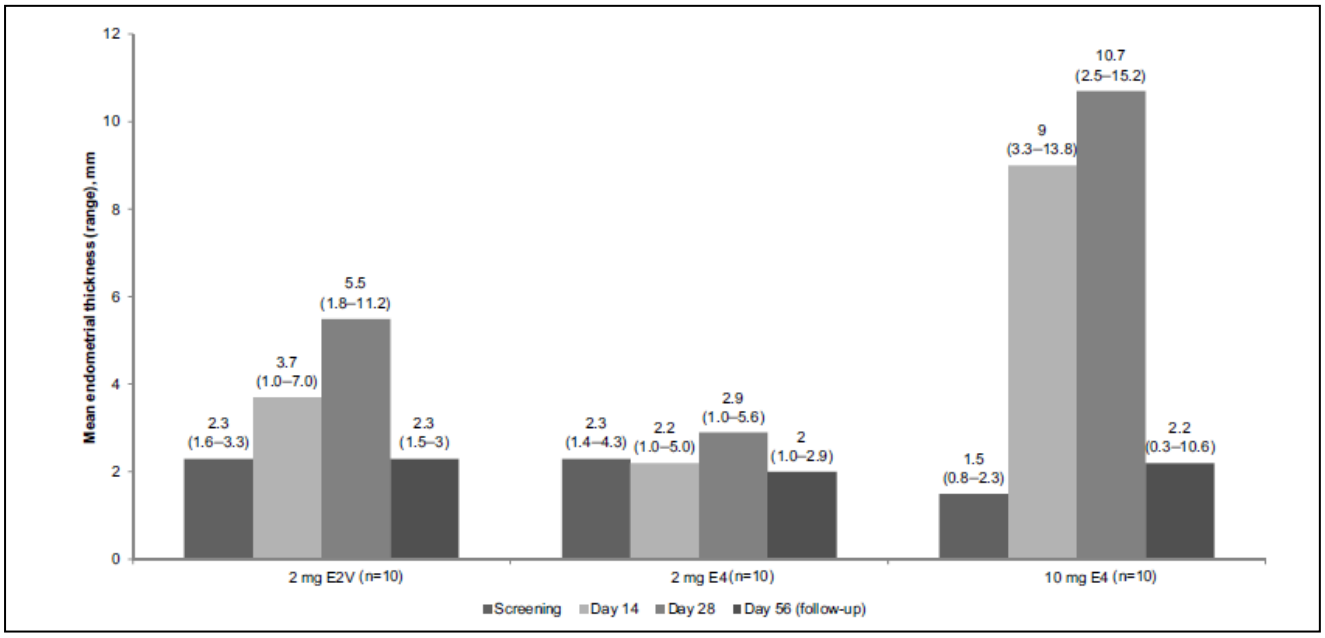

Figure 39. Mean endometrial thickness before treatment (screening), at treatment Day 14, at treatment Day 28 and at follow-up (Day 56) in post-menopausal women treated for 28 days with 2 mg estradiol valerate, 2 mg estetrol or 10 mg estetrol (n=10). E2V, estradiol valerate; E4, estetrol (Coelingh Bennink, et al. 2016).

Secondly, histological assessment of the endometrium was performed. All participants with an intact uterus underwent an endometrial biopsy at baseline. In line with the post-menopausal status, the samples obtained at this stage were classified as inactive. A second biopsy was performed in women presenting a doubling of the endometrial thickness at the end of the 28 days of treatment: 8, 3 and 7 subjects from the 2 mg E2V, 2 mg E4 and 10 mg E4 groups, respectively. All samples were classified as proliferative.

7.11.5. Gonadotrophins levels

Both serum LH and FSH levels were decreased by E4, confirming the data from the preceding study. The decreases in gonadotrophins were dose-proportional with the E4 dose (ranging from 20% in the 2 mg E4 group to 80% in the 40 mg E4 group) and more pronounced on FSH than

on LH, confirming here also the data from the first study (Figure 40). The decrease observed with the control treatment 2 mg E2V was between that seen with 2 mg and 10 mg E4. All values had returned to baseline values at follow-up (28 days after the last day of treatment) (Coelingh Bennink, et al. 2017a).

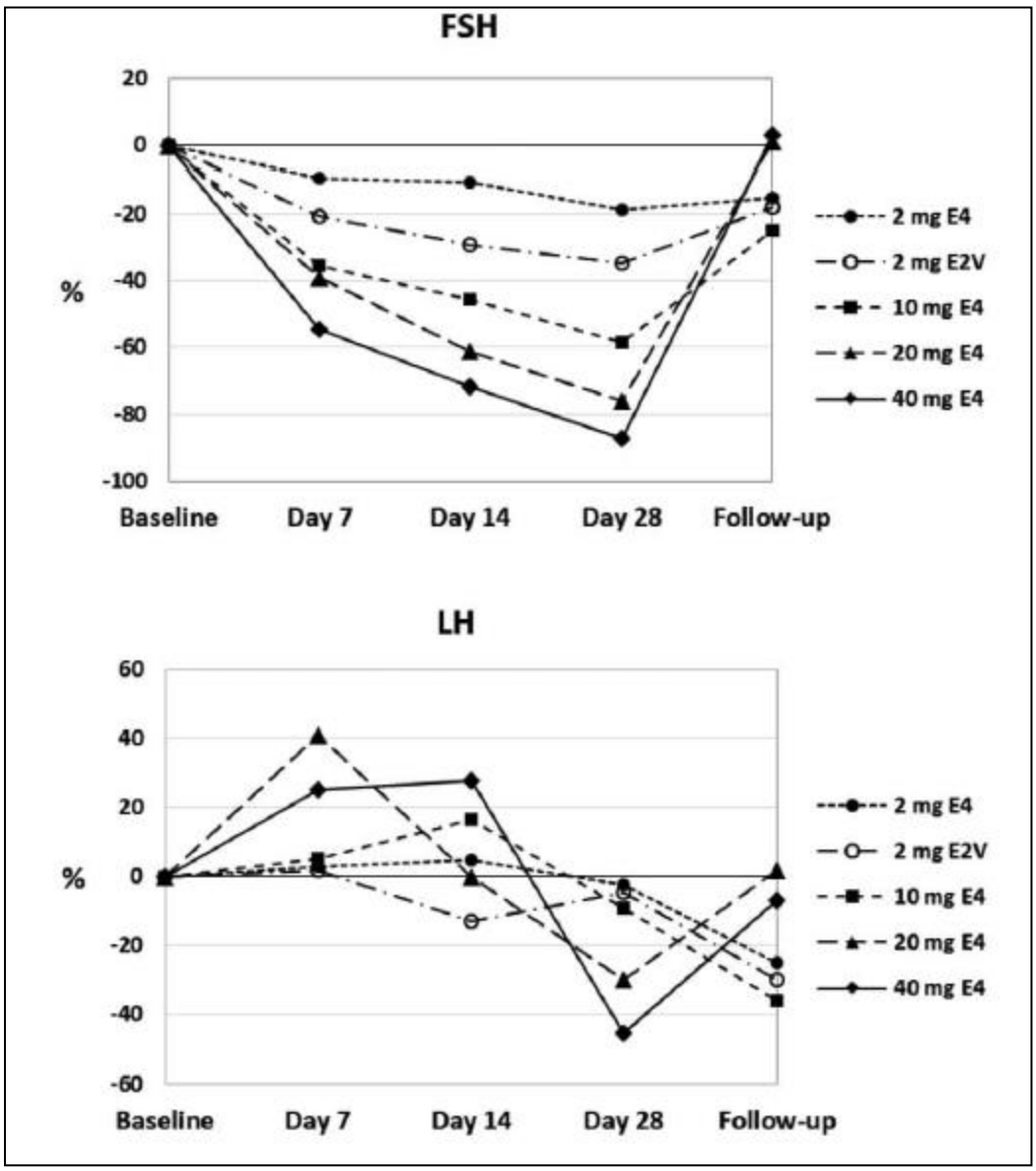

Figure 40. Mean percentage change from baseline in follicle-stimulating hormone and luteinizing hormone serum concentrations in post-menopausal women treated for 28 days with 2, 10, 20 or 40 mg estetrol or 2 mg estradiol valerate. FSH, follicle-stimulating hormone; LH, luteinizing hormone; E4, estetrol; E2V, estradiol valerate (Coelingh Bennink, et al. 2017a).

7.11.6. Hemostasis parameters

Sex hormone binding globulin levels increased dose-proportionally with the E4 dose: changes from baseline in SHBG level were 13%, 59%, 148% and 179% in the 2 mg E4, 10 mg E4, 20 mg E4 and 40 mg E4. Mean increase in SHBG level with E2V was 62%, *i.e.* close to that seen with 10 mg E4.
Prothrombin F1+2 levels were not affected neither by E4 nor by E2V (Coelingh Bennink, et al. 2017a).

7.11.7. Lipids and glucose parameters

Table 16 displays the data on lipids and glucose parameters (Coelingh Bennink, et al. 2017a).
Mean HDL cholesterol tended to increase with the E4 dose, except for the 10 mg E4 dose where no change from baseline in HDL cholesterol was seen. HDL cholesterol increased with 2 mg E2V to the same extent as 20 mg E4.
Mean LDL cholesterol tended to decrease with the E4 dose, except with the lowest dose where no change from baseline was observed. Mean change from baseline with 2 mg E2V was similar to that seen in the 40 mg E4 group.
Triglycerides levels were minimally affected by E2V and the different E4 doses.
Fasting glucose was mainly unchanged or slightly decreased in all treatment groups without dose-effect.

Table 16. Mean actual values (standard deviation) in lipids and fasting glucose before treatment and after 28 days of estrogenic treatment with 2, 10, 20 or 40 mg estetrol or with2 mg estradiol valerate in post-menopausal women (Coelingh Bennink, et al. 2017a).

	LDL cholesterol mmol/l Mean (SD)		**HDL cholesterol mmol/l Mean (SD)**		**Triglycerides mmol/l Mean (SD)**		**Fasting glucose mmol/l Mean (SD)**	
Treatment groups	***Screening***	***Day 28***	***Screening***	***Day 28***	***Screening***	***Day 28***	***Screening***	***Day 28***
2 mg E2V (n=10)	3.7 (0.8)	3.0 (0.7)	7.4 (11.8)	3.3 (1.3)	1.0 (0.4)	1.2 (0.4)	6.8 (3.6)	5.1 (0.3)
2 mg E4 (n=10)	3.7 (0.9)	3.7 (1.2)	3.6 (1.4)	3.3 (1.1)	1.1 (0.4)	1.1 (0.4)	5.2 (0.5))	5.1 (0.4)
10 mg E4 (n=10)	3.7 (0.9)	3.1 (0.8)	3.2 (0.8)	2.9 (0.7)	1.1 (0.5)	1.1 (0.5)	5.5 (0.8)	5.3 (0.7)
20 mg E4 (n=10)	3.9 (1.0)	3.4 (1.0)	3.9 (1.0)	3.2 (0.8)	1.4 (0.5)	1.5 (0.6)	5.5 (0.5)	5.4(0.5)
40 mg E4 (n=9)	3.2 (1.1)	2.4 (0.8)	3.4 (1.1)	2.6 (0.9)	1.4 (0.3)	1.5 (0.6)	5.3 (0.2)	4.9 (0.3)

E4, estetrol; E2V, estradiol valerate

Altogether, these data suggest that E4 modifies the lipid/glucose profile in a cardioprotective way characterized by an increase in HDL/LDL cholesterols ratio and no impact on glucose tolerance. Very interestingly, the change in triglyceride levels was much lower with E4 than with E2, even when a high dose of E4 (40 mg) was administered.

7.11.8. Number of hot flushes

The impact of a treatment on vasomotor symptoms is classically evaluated in a 12-week study, however, the current 4-week study was a good opportunity to gather some preliminary data on E4 in terms of hot flushes reduction (Coelingh Bennink, et al. 2016). The volunteers participating in the 2 mg E4 and 2 mg E2V groups were not selected based on the number of hot flushes they had and only those who reported hot flushes at baseline were asked to fill in a diary to count the number of hot flushes they had during the treatment period. When it appeared that 2 mg E4 had a positive impact on hot flushes, it was decided to include only subjects with hot flushes in the 10 mg E4 group in order to characterize the E4 potential in this indication on a larger number of subjects. Data on hot flushes were not gathered in the two subsequent dosing groups (20 mg and 40 mg E4).
Results are reported in Figure 41 below and only include the data from the subjects presenting at least 35 hot flushes at baseline: 4 subjects in the 2 mg E2V group, 5 subjects in the 2 mg E4 group and 8 subjects in the 10 mg E4 group

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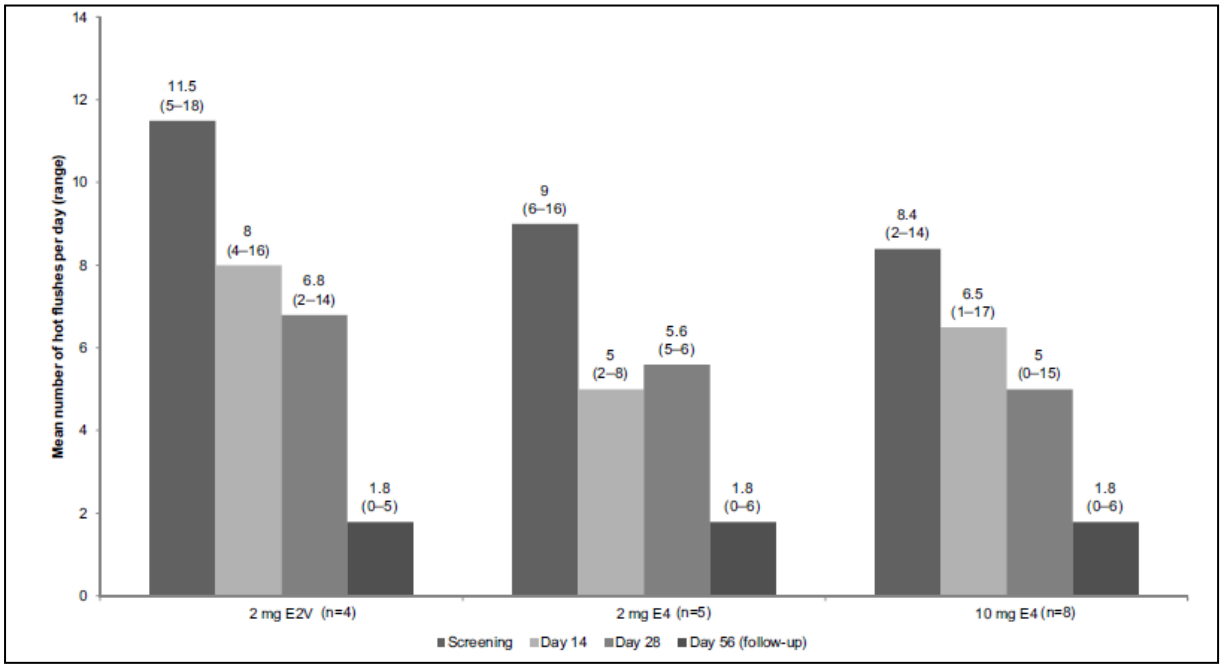


Figure 41. Mean number of hot flushes (range) in post-menopausal women who reported at least 35 hot flushes per week before treatment and who were treated with 2 mg estradiol valerate, 2 mg estetrol or 10 mg estetrol for 28 days. E2V, estradiol valerate; E4, estetrol (Coelingh Bennink, et al. 2016).

From these preliminary data, it appeared that E4 is able to improve vasomotor symptoms. In the opposite to the other pharmacodynamic parameters evaluated during this study (and described in the sections above), no E4 dose-dependency was observed as 10 mg E4 once daily seemed to achieve the same reduction in hot flushes as 2 mg E4. Results were similar to those observed in the control group (2 mg E2V). The fact that both 2 mg and 10 mg E4 doses had a similar impact on hot flushes reduction may be at least partially explained by the small number of subjects included in the groups. Evaluation on a larger population is currently ongoing in order to correctly characterize the impact of E4 for the relief of vasomotor symptoms.

8. The Estelle project

The Estelle project refers to the development of a new COC containing E4 as estrogen compound, in association with an already commercialized progestin compound.
The decision to further evaluate E4 in the contraceptive indication was supported by the clinical data obtained across the two studies conducted with post-menopausal women presented in sections 7.10 and 7.11. These data showed that E4 has a good therapeutic potential:

- E4 has a long half-life allowing for a single daily administration. This long half-life would also enhance the contraceptive efficacy, particularly in case of missed pill.
- E4 was shown to be safe and well tolerated up to 100 mg as a single dose and up to 40 mg in daily administration for 28 days.
- E4 also clearly demonstrated the required estrogenic activity for a contraceptive estrogenic compound: centrally, it inhibits the synthesis and release of gonadotropins (LH and FSH) and peripherally, it displays a proliferative action on the endometrium.
- E4 appeared different from EE by its less negative impact on the liver synthesis as attested by the much lower increase in SHBG and lower increase in triglycerides.

Estetrol is currently not available in any approved drug, consequently it is considered a *new chemical entity*. A complete pharmaceutical development and characterization are therefore required before obtaining the marketing authorization by the health authorities.

8.1. Global process for the development of a new drug

The development of a new drug follows a complex process which may be schematically divided into three different aspects: the clinical development, the non-clinical development and the chemistry, manufacturing and control (CMC) development. The clinical development involves all the trials conducted in humans. In the opposite, the non-clinical development involves the data generated in *in-vitro* and in animal settings. Finally, the CMC development involves the characterization of the drug in terms of physicochemical properties (chemical structure, stability, and solubility), improvement of the manufacturing process (essentially to scale up from a small production to manufacturing on the kilogram or ton scale) and packaging (capsules, tablets, intramuscular injection, etc).
The three aspects (clinical, non-clinical and CMC) are developed in parallel and are only virtually separated because, in practice, each aspect influences the progress of the others. For example, a certain amount of toxicological data must be gathered in animals before the first trials in humans may be started. Another example is that the CMC development is adapted on the requirements formulated by the clinical department in order to deliver the most suitable drug for the population intended to be treated and for the indication of the new drug: the CMC department may consequently adapt the formulation of the drug in order to increase or decrease the delivered dose of the active compound or to facilitate its absorption using different ways (sub-lingual administration, depot administration or administration using a device, for examples).
Each aspect of the drug development is strictly submitted to international and national laws and regulations intended to protect the subjects participating in the studies and to insure the launch of the safest drug. This aspect of the drug development commonly refers to the

"regulatory" aspect. For example, in Europe, the European Medicines Agency (EMA) publishes scientific guidelines that are harmonized between Europe, Japan and the USA by the International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (also commonly referred as *ICH*). Any new drug intended for the European market must have been developed in accordance with the EMA guidelines to receive marketing authorization.

Regarding the development of a new COC, EMA has developed a specific guideline delineating the required and recommended information to gather during the development of any new hormonal contraceptive. This document is entitled "Guideline on clinical investigation of steroid contraceptives in women" and was last updated in 2006 (EMA 2006). The FDA issued a guidance on this topic in 2019 (FDA 2019). Therefore, and for many years, in the absence of American guidance, the European guideline was the one that was followed, even in the USA.

The clinical development classically follows the below described steps:

- Phase 1 studies: These studies are generally conducted with a small group of healthy volunteers (20 to 80 subjects) on a short period of time. The main goal of the phase 1 studies is to establish the safety of the drug by evaluating the dug's most frequent side effects. It is also a good occasion to evaluate PK properties of the drug (absorption, distribution, metabolism and excretion).
- Phase 2 studies: These studies are conducted with a larger number of subjects presenting the condition for which the drug is developed (typically several hundred subjects). The duration of phase 2 studies is generally longer than in phase 1 studies (several months). The main goal is to assess the efficacy of the drug in the condition for which it is developed and also to define the minimal effective dose. Beside the efficacy assessment, the side effects are further evaluated.
- Phase 3 studies: these studies are the main source of information regarding the efficacy and the safety of the drug. They are conducted in a larger population and for a longer period of time than phase 2 studies (typically several thousand subjects for one year). This is also the occasion to evaluate drug-drug interactions and eventual differences between populations (for example, obese versus non-obese or smokers versus non-smokers).

Phase 4 studies may also be required by the Authorities at the moment of obtaining the commercialization authorization. Phase 4 studies are conducted when the product is already commercialized because this type of study necessitates a very large population (several hundred thousand subjects). They are typically intended to evaluate the incidence of a rare adverse event.

The non-clinical development covers the studies done before testing a drug in humans. Indeed, an evaluation of the drug's potential toxicity is required in *in vitro* and *in vivo* (animals) studies. Beside the evaluation of the drug's toxicity on organs targeted or not by that drug, it is also necessary to evaluate long-term carcinogenic effects or toxic effects on mammalian reproduction. Importantly, the regulatory guidelines of FDA, EMA, and other similar

international and regional authorities usually require safety testing in at least two mammalian species, including one non-rodent species, prior to human trials authorization.
In addition to follow the regulatory requirements, during the development of a new drug, the Sponsor may also seek direct advice from the health authorities of the countries or regions in which the drug is intended to be commercialized. This process, also called “scientific advice”, allows for a direct discussion of the results and of the development plan with the authorities. This is generally done before moving from the phase 2 to the phase 3 program in order to get the feedback of the authorities on the selection of the minimal effective dose intended to be tested in phase 3. Scientific advice may also be needed when a finding during the drug development has not been foreseen in the regulatory guidelines and, consequently, necessitates to elaborate an action plan agreed by the health authorities.

9. Objective of this thesis

The goal of this thesis is to describe the clinical phase 2 program of the Estelle project and the rationale for the choice of the dose of E4 chosen for phase 3 studies as well as the type of progestin to associate in a COC. In addition, the next clinical studies necessary to complete the dossier are also briefly described.

CHAPTER 2: RESULTS

The data obtained during *the phase 2 dose-finding program* of the Estelle project have been reported in six articles published in peer reviewed scientific journals. These articles are described in details in this chapter. An additional article from the phase 3 program has recently been submitted and is also reported here. The publication status of this large clinical program is summarized in Table 17 below.

Table 17. Overview of the publication status of the Estelle program.

Study Phase	Short Study Title	Publication Status
Phase 1 program	Single dose administration of 0.1, 1, 10 and 100 mg E4 in post-menopausal women	Article published and described in Chapter 1, Section 7.10
	Multiple dose administration of 2, 10, 20 and 40 mg E4 versus 2 mg E2V in post-menopausal women	Articles publisehd and described in Chapter 1, Section 7.11
Phase 2 dose-finding program	1st dose-finding study assessing the impact of E4 (5 or 10 mg)/DRSP and E4 (5, 10 or 20 mg)/LNG on: 1. Ovulation ihibition 2. Hemostasis parameters 3. Carrier proteins, Lipids, Grwoth endocrinology, and PK	Articles published 1. Described in Chapter 2, Section 1 2. Described in Chapter 2, Section 2 3. Described in Chapter 2, Section 3
	2nd dose-finding study assessing the impact of E4 (15 or 20 mg)/DRSP and E4 (15 or 20 mg)/LNG on: 1. Bleeding Pattern 2. Quality of life	Articles published 1. Described in Chapter 2, Section 4 2. Described in Chapter 2, Section 5
Phase 3 program	A study to evaluate the impact of 15 mg E4/DRSP on hemostasis parameters.	Article published. Described in Chapter 2, Section 6.
	Ovulation inihbition with 15 mg E4/DRSP.	Article in preparation.
	Efficacy Study in Europe/Russia	Article in preparation.
	Efficacy Study in the USA	Article in preparation

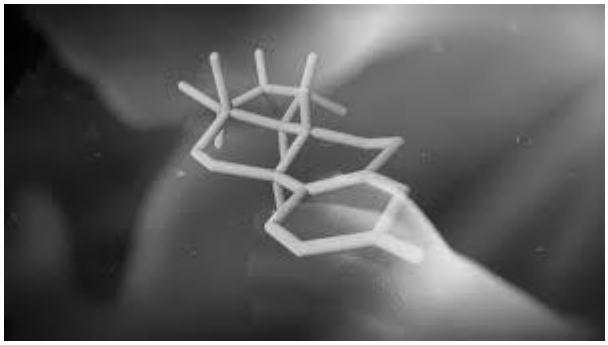
E2V, estradiol valerate; E4, estetrol; DRSP, drospirenone; LNG, levonorgestrel; PK, pharmacokinetic; USA, United States of America.

The phase 2 dose-finding program consisted in two clinical studies designed to select the dose of E4 and the progestin to be combined with E4 to form an effective and well-tolerated COC. A dose-finding is a necessary step when developing a *new chemical entity*, which is the case of E4, as E4 has never been approved as commercialized drug before.

The E4 doses tested ranged from 5 to 20 mg. Combined to E4, two different progestins were evaluated, namely 3 mg DRSP and 150 mcg LNG. The intention was to get marketing authorization for the new E4-based COC in Europe and in the USA. Consequently, evaluating a progestin already approved by both the FDA in the USA and by the European countries was required. This was only the case of DRSP and of LNG at the time of the beginning of this clinical program (2007). However, due to its androgenic activity, LNG is often associated with a less favorable tolerability profile than DRSP (leading to higher incidence of acne, oily hair, weight gain, lipid profile impairment) and controversy exists regarding a higher risk of VTE associated with the use of COCs containing DRSP in comparison with those containing LNG. All these data are well studied when LNG and DRSP are combined to EE but no information exist when these progestins are combined to another estrogen. Therefore, our clinical program was an excellent occasion to gather new comparative data on these progestins when they are combined to a new estrogen.

As described in the introduction of this work, different authors have demonstrated that the contraceptive efficacy of a given COC and its tolerance in terms of bleeding pattern, headache and breast tenderness were improved with a shorter pill-free interval (see Chapter 1, Section 5 for more details). Accordingly, it was chosen to administer the E4-containing combinations in a 24/4 day regimen instead of the classical 21/7 day regimen.

At the end of the phase 2 program, the exact composition of the COC to be evaluated in the phase 3 program had to be known, with the intention to launch this combination on the market when the full development is completed.



1st Publication

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

The European Journal of Contraception and Reproductive Health Care, 2015; 20: 476-489

Duijkers IJ, Klipping C, Zimmerman Y, Appels N, Jost M, Maillard C, Mawet M, Foidart JM, Coelingh Bennink HJ

1. First publication

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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1.1. Introduction

The first step when developing a COC is to ensure that the combination achieves a sufficient HPO axis inhibition and adequately blocks the onset of ovulation. This has been evaluated in the first phase 2 study entitled: "*A dose-finding study with active control group YAZ® to assess the contraceptive efficacy and the effect on liver function of 5 or 10 mg estetrol combined with either 3 mg drospirenone or 150 mcg levonorgestrel, or 20 mg estetrol combined with 150 mcg levonorgestrel, by daily oral administration to healthy female volunteers for 3 cycles of 24 days each followed by a 4-day treatment pause*".

Five different E4 combinations were tested in this study: two E4 doses (5 and 10 mg) combined with 3 mg DRSP and three E4 doses (5, 10 and 20 mg) combined with 150 mcg LNG. The choice of the E4 doses to be tested was based on the data gathered in the phase 1 study conducted in post-menopausal women in which the use of 2, 10, 20 and 40 mg E4 administered daily for 28 days was shown to be safe and well-tolerated (see Chapter 1, Section 7.11 for all the details on this study) (Coelingh Bennink, et al. 2016; Coelingh Bennink, et al. 2017a). The results also showed that 10 and 20 mg E4 had pharmacodynamic activities close to that of 2 mg E2 used as comparator, notably on the reduction of hot flushes and night sweats, and on vaginal histology. Based on these findings, it was reasonable to choose doses of 5, 10 and 20 mg E4 for this first phase 2 trial.

The population selected in the trial was healthy women, between 18 and 35 years of age. To be allowed to participate, during the spontaneous menstrual cycle preceding the start of the treatment, the candidates had to be good ovulating women (defined as the presence of ultrasonographic sign of ovulation between day 9 and day 24 of the cycle, followed by a rise in progesterone level ≥16 nmol/l) with a luteal phase of at least 6-day of duration. To document their ovulatory status, ovarian follicular growth was monitored every 3 days by ultrasonographic imaging during the spontaneous menstrual cycle before start of study medication. Ovulation suspected on visualization of a follicle rupture was confirmed by a serum progesterone level > 16 nmol/L in at least one measurement after follicle rupture. In spontaneous cycles, progesterone levels normally exceed 16 nmol (Landgren, Unden, and Diczfalusy 1980). If it is not the case, the luteal phase is insufficient and the subject usually starts menstruating within 6 days after follicle rupture. Practically, these women will probably not become pregnant, because implantation cannot occur in such a short period of time. In this study aiming at evaluating the efficacy of the tested combinations on ovulation inhibition, it

was necessary to only include women with the highest fertility potential. Women who did not fulfill the ovulation criteria did not continue their participation in the trial.
As said above, the primary aim was to study the effect of the different E4 containing contraceptive combinations tested on the HPO axis and ovarian activity. The degree of residual ovarian activity during the intake of a COC is related to the type and the dose of both the progestin and the estrogen included in the combination, and is best appreciated using a scoring method called the *Hoogland Score* (Hoogland and Skouby 1993). To calculate the Hoogland Score of a subject in a given cycle, three parameters are combined, namely the maximal ovarian follicular size obtained by transvaginal ultrasonography, along with the maximal levels of serum E2 and serum progesterone recorded in that cycle. The results are reported in the Hoogland scoring system (Table 18). A Hoogland score of 1 is indicative of low ovarian activity while a score of 6 indicates the presence of ovulation. Naturally, the goal of a COC is to have a maximum of treated women presenting a low Hoogland score (ideally close to a score of 1).

Table 18. Hoogland score used to evaluate the ovarian activity (Hoogland and Skouby 1993)

Hoogland Score	Corresponding ovarian activity	Size of follicle-like structure (mm)	Serum progeterone (mmol/l)	Serum Estradiol (mmol/l)
1	***No activity***	<10	-	-
2	***Potential activity***	10-13	-	-
3	***Non active follicle-like structure***	>13	-	≤0.1
4	***Active follicle-like structure***	>13	≤5	>0.1
5	***Luteinized unruptured follicle***	>13, persisting	>5	>0.1
6	***Ovulation***	>13, ruptured	>5	>0.1

The Hoogland score has been used for more than two decades in clinical research to evaluate the efficacy of anti-ovulatory methods and is nowadays considered the gold standard test for such kind of study.
The objective nature of the primary endpoint evaluated in this trial and the need to compare the new combinations with an already approved therapy explains the use of an active comparator, rather than a placebo. The marketed COC YAZ®, which is a combination of 20 mcg EE and 3 mg DRSP, was chosen as active comparator for three reasons: first, it is a widely marketed COC with a well-established good acceptability and efficacy in terms of ovulation inhibition; secondly, it was at the time of the trial, the only COC administered in a 24/4 day regimen allowing a reliable comparison of the Hoogland scores without the confounding aspect that

would automatically arise from different hormone-free interval lengths; finally, using a DRSP combination permitted a direct comparison between E4 and EE when comparing the results obtained with EE/DRSP to those obtained with the E4/DRSP combinations tested.

1.2. Article

The European Journal of Contraception and Reproductive Health Care, 2015; 20: 476–489

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

Ingrid J.M. Duijkers*, Christine Klipping*, Yvette Zimmerman†, Nicole Appels†, Maud Jost‡, Catherine Maillard‡, Marie Mawet‡, Jean-Michel Foidart‡,§,# and Herjan J. T. Coelingh Bennink†,#

*Dinox BV, Groningen, the Netherlands, †Pantarhei Bioscience BV, Zeist, the Netherlands, ‡Estetra SPRL, Liège, Belgium, and §University of Liège, Liège, Belgium

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

#Jean-Michel Foidart and Herjan J.T. Coelingh Bennink equally contributed to this study.

Correspondence: Marie Mawet, Rue Saint Georges 5–7, 4000 Liège, Belgium. Tel: +32 4 3492822. Fax: +32 4 3492821. E-mail: mmawet@mithra.com

This Original Article accompanies the Article - Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives, also in this issue. DOI:10.3109/13625187.2015.1068934 by Marie Mawet et al.

DOI: 10.3109/13625187.2015.1074675

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

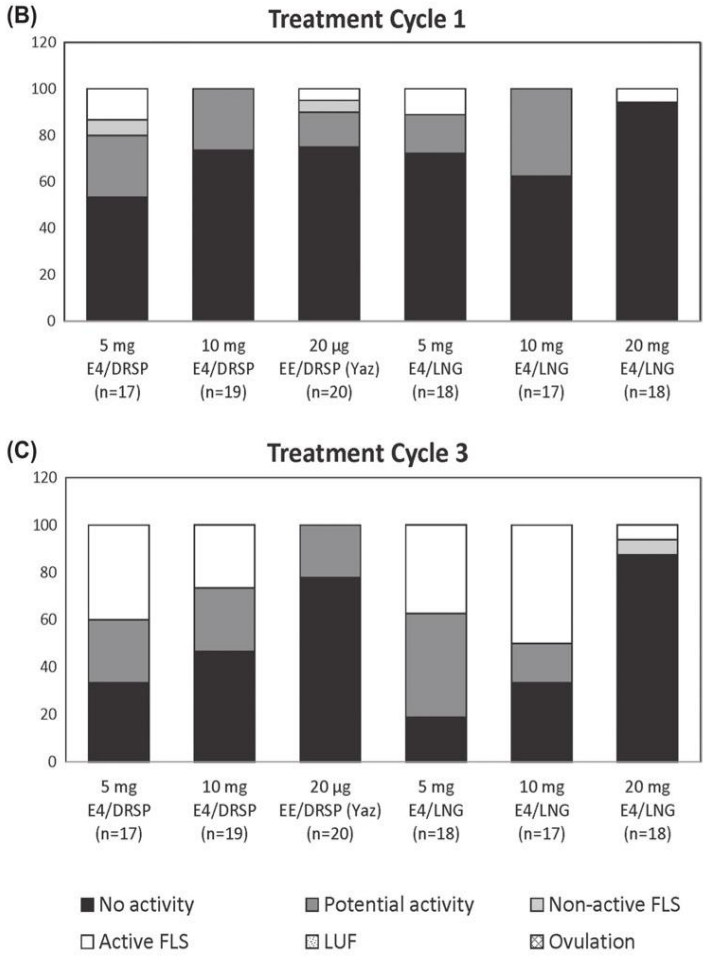

Figure 1 Ovulation inhibition according to the Hoogland score (A). Hoogland scores obtained during cycle 1 (B) and cycle 3 (C) with the different combinations tested during the trial. Results are expressed in percentage of participants.

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 ($p < 0.0001$

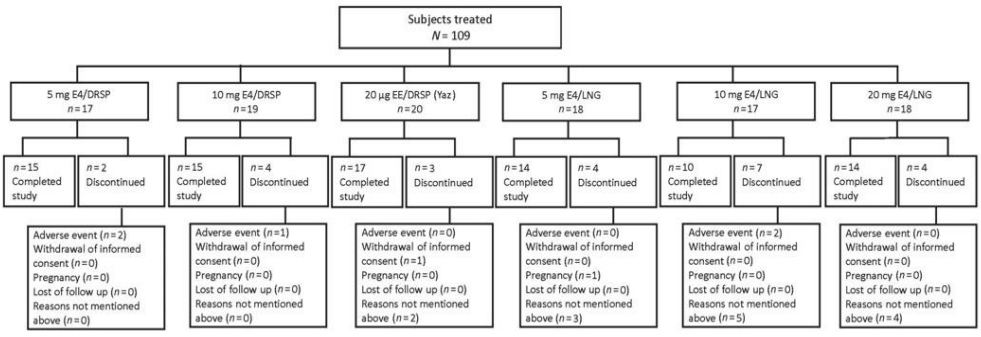

Figure 2 Participant disposition by treatment group.

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

(B)

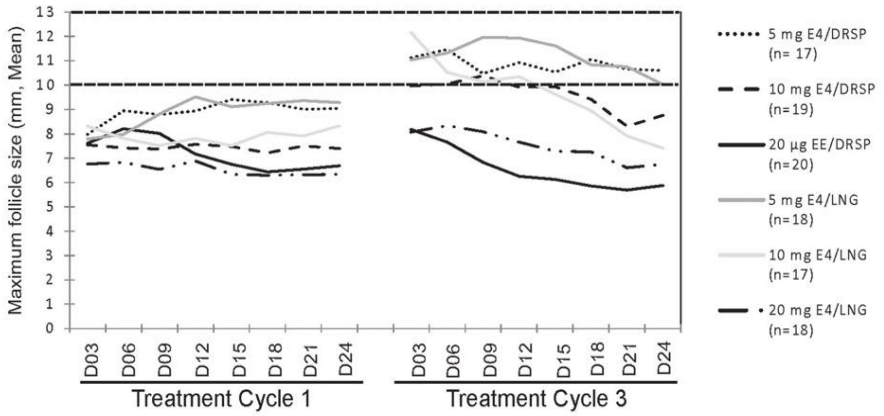

Figure 3 Mean (± SD), minimum and maximum value of the largest follicular diameter per participant in each group over the entire treatment period (A). Mean diameter of the largest follicle (mm) measured in each treatment group every 3 days during cycles 1 and 3 (B).

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)

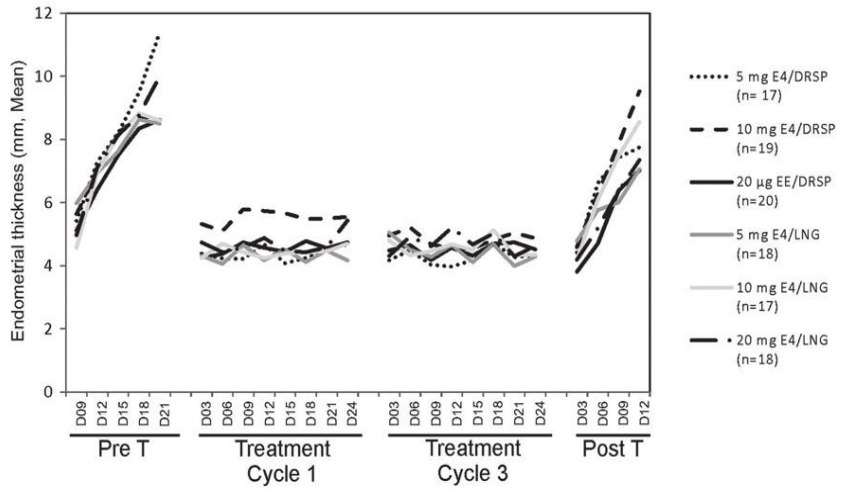

Figure 4 Maximum (mean ± SD) endometrial thickness value observed for each group during the trial (A). Mean endometrial thickness assessed in each treatment group every 3 days across the pretreatment cycle, treatment cycles 1 and 3, and post-treatment cycle (B). D, day; Pre T, pretreatment cycle; Post 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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Declaration of interest: IJMD and CK are employees of the contract organisation Dinox BV which performed the study; YZ is an employee of Pantarhei Bioscience BV; NA was an employee of Pantarhei Bioscience BV. MJ, CM, MM and J-MF are employees of Estetra SPRL; HJTCB is the CEO of Pantarhei Bioscience BV.

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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

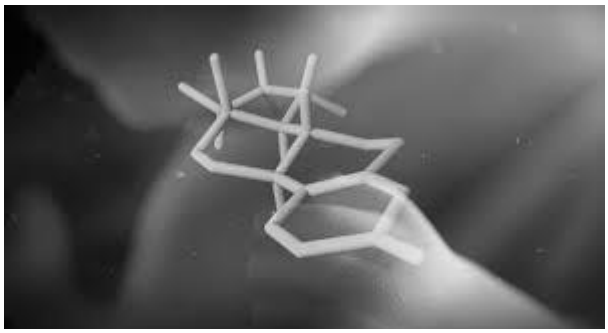
necessary and sufficient for uterine epithelial cell proliferation in vivo. *Endocrinology* 2013;154:2222–33.
28. Adlanmerini M, Solinhac R, Abot A, *et al.* Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. *Proc 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.

1.3. Discussion

At the end of this trial, three important information had been gathered when analyzing the impact of the different E4-containing combinations tested on the HPO axis and on the ovarian activity:

- First, the administration of up to 20 mg E4 associated to a progestin was safe and well-tolerated during 3 treatment cycles.
- Second, ovulation was inhibited in all groups, including that containing the smallest dose (5 mg E4), confirming the suitability of E4 as a new estrogen in the hormonal contraceptive fields.
- Third, the degree of ovarian activity suppression was proportional to the E4 dose. As described in Chapter 1 Section 5, the bleeding pattern associated with a COC is strongly correlated with the degree of ovarian function inhibition of that COC. A better ovarian activity suppression/bleeding pattern were achieved when more than 10 mg E4 was used.

Consequently, we decided to perform the second phase 2 dose-finding trial using the already tested dose of 20 mg E4 and an intermediary dose between 10 and 20 mg, namely 15 mg E4.



2nd Publication

Reduced haemostatic effects with drospirenone-based oral contraceptives containing estetrol versus ethynilestradiol

Contraception, 2017; 95(2):140-147

Kluft C, Zimmerman Y, Mawet M, Klipping C, Duijkers IJ, Neuteboom J, Foidart JM, Coelingh Bennink HC

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2.1. Introduction

During the first phase 2 trial, the impact of the different combinations on hemostasis parameters was also evaluated.

It is widely accepted that COC use enhances the VTE risk mainly (but probably not only) by modifying the expression of several factors of the coagulation/inhibitors of coagulation and of the fibrinolysis pathways. However, as explained in the introduction section of this work, the mechanism by which the estrogen and the progestin compounds negatively impact hemostasis is still not fully understood (see Chapter 1, Section 4 for more details).

Currently, the conduct of large epidemiological studies is the only reliable way to estimate the VTE risk associated with a hormonal contraceptive. These studies encompass at least hundred thousand subjects and are therefore only feasible after commercialization of the COC, in so called "post-marketing surveillance studies". Consequently, the identification of one biomarker able to predict the VTE risk of a hormonal contraceptive would be ideal as it would allow to determine in small clinical settings the VTE risk associated with a hormonal contraceptive before its commercialization. Different studies have been conducted to find out such biomarker, but up to now, no one has appeared to be sufficiently predictive. Therefore, in the absence of one reliable surrogate marker, EMA recommends to evaluate a panel of 10 different biomarkers when developing a CHC (EMA 2006). This evaluation does not replace the large epidemiological studies, however it already gives good informative data on the hemostasis changes induced by a hormonal contraceptive.

In the first phase 2 study, 14 surrogate markers of VTE risk (including those recommended by EMA) were measured at baseline (before starting the study drug) and at the end of the third treatment cycle. The changes from baseline, expressed in percentage, were compared between the different treatment groups.

Very interestingly, having chosen YAZ® (a combination of 20 mcg EE with 3 mg DRSP) as comparator in the trial allows a direct comparison of the hemostatic effects of EE and E4, as two treatment groups of the study were a combination of 5 and 10 mg E4 with 3 mg DRSP. The results of this comparison have been extensively described in the publication below.

2.2. Article

ELSEVIER

Contraception 95 (2017) 140 – 147

Contraception

Original research article

Reduced hemostatic effects with drospirenone-based oral contraceptives containing estetrol vs. ethinyl estradiol☆,☆☆,★

Cornelis Kluft[a,*], Yvette Zimmerman[b], Marie Mawet[c], Christine Klipping[d], Ingrid J.M. Duijkers[d], Jacoline Neuteboom[a], Jean-Michel Foidart[c,1], Herjan Coelingh Bennink[b,1]

[a]*Good Biomarker Sciences, Zernikedreef 8, 2333CL, Leiden, the Netherlands*
[b]*Pantarhei Bioscience, P.O. Box 464, 3700, AL, Zeist, the Netherlands*
[c]*Estetra SPRL, Rue Saint Exupery, 4460 Grace-Hollogne, Belgium*
[d]*Dinox BV, Hanzeplein 1, 9713GZ, Groningen, the Netherlands*

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Summary

Objective: The effects of estetrol (E4), a natural fetal estrogen, combined with drospirenone (DRSP) were evaluated on plasma levels of sex hormone-binding globulin (SHBG), angiotensinogen and 12 hemostasis markers.
Study design: Combinations of 3 mg DRSP with 5 or 10 mg E4 were compared with YAZ® (20 mcg ethinyl estradiol and 3 mg DRSP; EE/DRSP) in parallel groups of 15–18 healthy young women. Main outcome was the relative change from pretreatment to the end (day 24±1) of the third treatment cycle.
Results: All E4 combinations showed low estrogen impact compared to EE/DRSP. Effects on SHBG and angiotensinogen of 10 mg E4 combined with DRSP were 15%–20% that of EE/DRSP.

Both E4/DRSP combinations reduced D-dimer level and the 5 mg E4/DRSP combination also decreased fragment 1+2.
Conclusions: The reduction in coagulation markers suggests an anticoagulant effect from DRSP.

The indications of a low thrombosis risk for E4 preparations should be validated in larger studies.
Implication statement:

- The oral estrogens, 17-β-estradiol and ethinyl estradiol, are known for significant effects on estrogenic and hemostatic variables.
- Effects of oral estetrol (E4) combined with drospirenone (DRSP) are significantly less for these variables.
- This suggests a low procoagulant effect of E4/DRSP that should be clinically verified for low antithrombotic consequences.

Keywords: Estetrol; Ethinyl estradiol; Drospirenone; Hemostasis; SHBG; Estrogenicity

1. Introduction

Current hormonal contraceptive preparations contain ethinyl estradiol (EE) predominantly, and more recently also the natural 17β-estradiol (E2) [1]. Hagen et al. [2] discovered the natural fetal estrogen, estetrol (E4). It is produced by the fetus and detected in the maternal circulation during pregnancy from 9 weeks of gestation until only shortly after birth [3]. Estrogenic potency is lower than that of EE and E2, but at dosages of 5–20 mg, E4 was

☆ Conflict of interest: CKT and JN are employees of Good Biomarker Sciences and received financial support from Estetra for laboratory analysis and interpretation of data and drafting the manuscript; YZ and HCB are employees of Pantarhei Bioscience; MM and JMF are employees of Estetra; CKG and ID are employees of Dinox and received financial support from Estetra for the clinical part of the study. JMF is also a paid consultant for Mithra.

☆☆ Clinical trial registration number: TC2102.

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* Corresponding author.
E-mail addresses: kluft@kluft.in (C. Kluft), yz@pantarheioncology.nl (Y. Zimmerman), mmawet@mithra.com (M. Mawet), c.klipping@dinox.umcg.nl (C. Klipping), i.j.m.duijkers@dinox.umcg.nl (I.J.M. Duijkers), neuteboom@gbsleiden.nl (J. Neuteboom), jfevaconsulting@gmail.com (J.-M. Foidart), hcb@pantarheibio.com (H.C. Bennink).

[1] Contributed equally.

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successfully evaluated for contraception and vaginal bleeding patterns with either levonorgestrel (LNG) or drospirenone (DRSP) as progestin [4–6].

Interestingly, E4 neither binds to sex hormone-binding globulin (SHBG) nor induces its synthesis in vitro by hepatocytes [7]. E4 shows selective estrogen receptor (ER)α and ERβ receptor binding with a preference for ERα [8]. E4 selectively activates the nuclear ER (ERα) but blocks the membrane ERα [9]. Transgenic mice lacking this membrane ERα do not ovulate, demonstrating that this receptor is essential for ovulation and fertility [10]. The selective blockade of the membrane ERα by E4 could therefore contribute to the blockade of ovulation.

Thus, E4 is an option for addition to the repertoire of estrogenic components of oral combined contraceptives (COCs) [6]. Whether oral E4 exerts effects on the liver and endothelium similar to oral EE, and oral E2, resulting in increases in estrogenic markers such as SHBG, angiotensinogen and changes in hemostasis variables is an important question.

These liver estrogenicity markers and hemostasis variables are also sensitive to the progestin used in COC. Markers and variables are notably less modified with LNG compared to desogestrel, gestodene and DRSP [11–17].

Hepatic estrogenicity and hemostasis markers are in the list of the European Medicines Agency (EMA) [18] advised to evaluate potential risk for thrombotic side effects of hormonal contraceptives. Accordingly, we checked the behavior of these safety markers in combinations of E4 dosages with DRSP.

Our aim was to document the effects on hemostasis variables and liver estrogenicity markers of combinations of E4 (5 and 10 mg) with DRSP (3 mg) in comparison with YAZ® (20 mcg EE with 3 mg DRSP; EE/DRSP). We administered all combinations according to a 24- to 4-day regimen.

2. Materials and methods

2.1. Study design

This was an open-label, parallel, dose-finding, single-center (Dinox BV, Groningen, the Netherlands) study with young, healthy female volunteers of reproductive age. The study was performed from November 2009 through November 2010 and was registered as TC2102 (http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=2102).

The local ethics committee approved the study protocol and the protocol was conducted in accordance with the tenets of the Declaration of Helsinki and the International Conference on Harmonization and Good Clinical Practice. From all participants, written informed consent was obtained prior to entry into the study.

2.2. Study population

Healthy women 18–35 years of age with a body mass index (BMI) of 18–30 kg/m^2 were eligible for inclusion. Subjects who were using a hormonal contraceptive at the time of screening had at least one washout cycle prior to the start of the study. Spontaneous ovulation between day 9 (±1) and day 24 (±1) was verified in the pretreatment cycle by a progesterone concentration ≥16 nM (5 ng/mL) and a luteal phase duration of ≥6 days. We applied the following exclusion criteria: failure to ovulate before day 24 in the observational pretreatment cycle, contraindications for the use of contraceptive steroids, clinically relevant abnormal laboratory findings, duration of the washout cycle after stopping hormonal contraceptives >42 days, pregnancy or lactation, prior pregnancy despite accurate hormonal contraceptive use, history of breast cancer, uterine and/or ovarian abnormalities, at least one abnormal cervical smear in the 3 years prior to screening, renal insufficiency, hepatic dysfunction, adrenal insufficiency, use of drugs that affect CYP3A4 activity, use of antihypertensive drugs, use of an injectable hormonal contraceptive within 6 months of screening, delivery or abortion in the past 2 months, use of investigational drugs in the past 2 months, and a recent history (i.e., within 12 months) of alcohol and/or drug abuse. Cigarette smoking (up to 10 per day) was permitted in participants up to 30 years of age; participants >30 years of age were required to be nonsmokers. The use of additional sex steroids was prohibited throughout the study.

The study utilized a parallel design (Fig. 1).

2.3. Study treatment

The study included the following three treatment groups: 20 mcg EE combined with 3 mg DRSP (EE/DRSP), 5 mg E4 combined with 3 mg DRSP (5 mg E4/DRSP), and 10 mg E4 combined with 3 mg DRSP (10 mg E4/DRSP). All subjects were stratified according to the day of ovulation in the pretreatment cycle and then assigned to a treatment group. E4 was supplied as 5 or 10 mg tablets. DRSP was supplied as 3 mg tablets. EE/DRSP was supplied as tablets in their original package. The participants in the two E4/DRSP groups were blinded with respect to the E4 dose; blinding of the EE/DRSP group was not possible.

E4 was synthesized by Cambridge Major Laboratories Europe (Weert, the Netherlands). The study medication was produced, packaged, labeled and released in accordance with Good Manufacturing Practice guidelines (Haupt Pharma, Munster, Germany).

Oral treatment was started on the first day of menstruation following the pretreatment cycle and continued daily for 24 days followed by a 4-day break; treatment compliance was verified by the use of a diary. The percentages of previous users were 40%, 20% and 28% in the 5 mg E4/DRSP, 10 mg/DRSP and EE/DRSP groups, respectively. The treatment period (which included three treatment cycles) was followed by a posttreatment cycle with no hormonal treatment.

2.4. Study measurements

Blood samples for the present analysis were collected at four time points during the study (i.e., Samples 1–4 in Fig. 1).

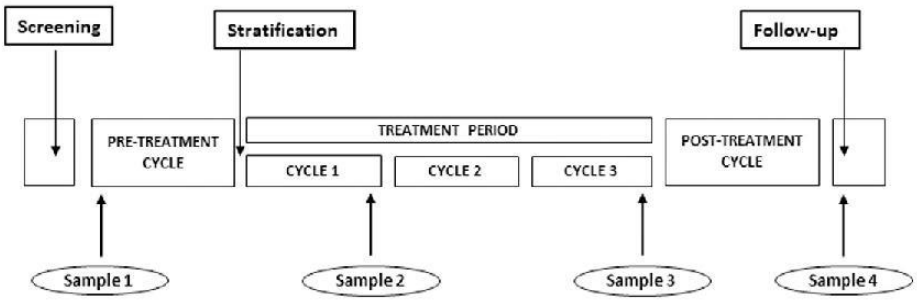

Fig. 1. Design of the parallel study. Blood samples 1–4 are taken at the moments indicated by the arrows.

The blood samples contained either 0.11 mM citrate or CTAD (0.11 mM citrate plus theophylline, adenosine and dipyridamol); ETP-based APCr, APTT-based APC global and prothrombin fragment 1+2 (F1+2) were measured in the CTAD samples.

Sample 1 was taken on day 3 (±1 day) in the pretreatment cycle (Sample 1). Samples 2 and 3 were taken on day 24 (±1 day) in the first and third treatment cycles, respectively. Sample 4 was taken on day 3 (±1 day) in the first cycle following the posttreatment cycle ("Follow-up" in Fig. 1). For primary analysis, the measured values were compared between Sample 1 and Sample 3 for each participant.

Specimens were collected after the participants fasted and abstained from alcohol overnight and at least 1 h after smoking. Subjects were instructed to rest comfortably in a sitting position for at least 15 min prior to blood collection. Venipuncture using minimal stasis (≤15 mmHg pressure) was used for blood collection. In accordance with the study design, the first tube was not used for hemostasis analysis. Plasma samples were stored below −60 °C until analysis at the end of the study.

We measured the following hemostasis and hepatic estrogenicity markers: antithrombin (Coamatic LR Antithrombin, Instrumentation Laboratory, Bedford, MA, USA; CV: <10%, reference range: 75%–130%); ETP-based APC resistance (ratio of thrombin generation with and without APC (Activated Protein C: APE 1660 PAL, ERL); tissue factor (Innovin, Dade Behring, Miami, FL, USA), phospholipid micelles 15 μM, DOPC/DOPE/DOPS 3:1:1 (Avanti Polar Lipids, Alabaster, AL, USA), Substrate S2238 (Instrumentation Laboratory); defibrination of plasma with Reptilase (Pentapharm, Basel, Switzerland; reference range <2.4; CV: <20%); APC-APTT global (Coamatic APC resistance, Chromogenix, Instrumentation Laboratory; CV: <15%; ratio reference range: 2.1–3.7); protein S activity (STA Protein S Clotting, Diagnostica Stago, Gennevilliers, France; CV: <10%; reference range: 70%–140%); protein C activity (Coamatic Protein C, Chromogenix, Instrumentation Laboratory; CV: <10%; reference values: 70%–140%); fibrinogen (STA Fibrinogen, Diagnostica Stago; CV: <10%; reference range: 1.7%–4.5 g/L); prothrombin fragment 1+2 [Enzygnost F1+2 (monoclonal) kit; Siemens Healthcare Diagnostics Products GmbH, The Hague, the Netherlands; CV: <15%; reference range: 69–229 pM]; prothrombin antigen (FII-EIA; Affinity Biologicals Inc., Ancaster, Ontario, Canada; CV: <10%; reference range: 75%–130%); D-dimer (GBS-EIA, GBS monoclonal 14: 7/8/7, Conjugate DD13/PO; CV: <10%; reference range: <310 ng FE/mL); free TFPI (Asserachrom Free TFPI=Tissue Factor Pathway Inhibitor), Diagnostica Stago; CV: <10%, reference range: 0.6–8.9 ng/mL); E-selectin (Quantikine Human sE-Selectin kit; R&D Systems, Minneapolis, MN, USA; CV: <10%, reference range: 23–80 ng/mL); t-PA antigen (Trinilize tPA antigen kit; Trinity Biotech, Jamestown, NY, USA; CV: <10%, reference range: 1.2–12.5 ng/mL); SHBG (Cobas ECLIA assay; Roche Diagnostics, Almere, the Netherlands; CV%: <10%, reference range: 26–130 nM); and angiotensinogen (Human Total Angiotensinogen assay kit; IBL, Gunma, Japan; CV: <10%, reference range: 430–1040 pM).

2.5. Safety

We recorded adverse events throughout the study period. In addition, general physical and gynecological examinations (including vital signs, breast palpation and transvaginal ultrasonography) were performed at screening and within 2 weeks after discontinuation of the study treatment [19].

2.6. Statistics

For each target variable, the percent change relative to Sample 1 was calculated, and the median value and interquartile range (Q1–Q3) was calculated for each treatment group. The relative change was tested using the Wilcoxon signed rank test; differences were considered significant at $p<.05$.

3. Results

3.1. Subjects

The demographics and baseline characteristics were similar between treatment groups. The mean age was 23.8 years (range: 18 to 33 years), and the majority of subjects

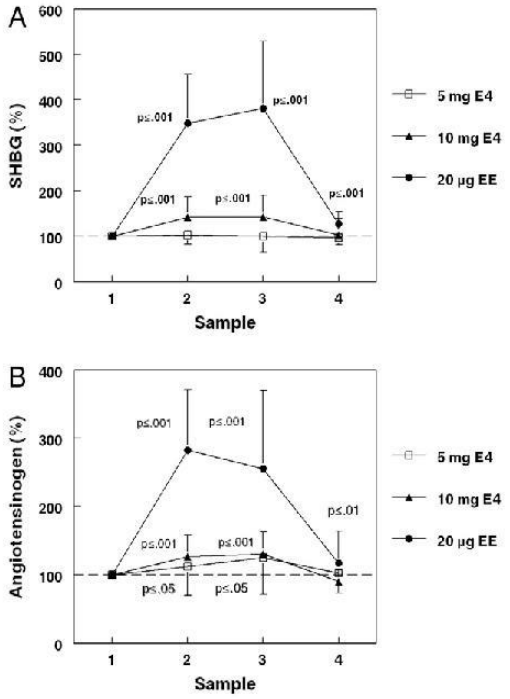

Fig. 2. (A) Median levels (interquartile range up or down) of SHBG relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test. (B) Median levels (interquartile range up or down) of angiotensinogen relative to the pretreatment value (=100%). Data for YAZ® and 10 mg E4 combinations with DRSP and LNG are plotted. Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test.

Table 1
Plasma levels of estrogenic and hemostasis markers at the end of treatment cycle 3 expressed as percentage of the individual pretreatment values (=100%): median and (Q1–Q3 range)

	YAZ®	5 mg E4-DRSP	10 mg E4-DRSP
Number[a]	*n*=17	*n*=15	*n*=15
Estrogenicity markers			
SHBG antigen	381 (313–462)	100 (90–125)	143 (129–176)
	p≤.001	p>.05	p≤.001
Angiotensinogen antigen	256 (229–344)	125 (92–146)	131 (113–145)
	p≤.001	p≤.05	p≤.01
Global assays, markers			
APTT-based APC global	92 (83–97)	103 (90–106)	100 (90–105)
	p≤.001	p>.05	p>.05
ETP-based APCr	275 (196–348)	105 (93–129)	99 (87–154)
	p≤.001	p>.05	p>.05
D-dimer antigen (FbDP)	127 (101–154)	74 (48–92)	74 (57–94)
	p≤.05	p≤.01	p≤.05
F 1+2 antigen	163 (131–193)	77 (68–83)	97 (76–114)
	p≤.001	p≤.001	p>.05
Levels			
Fibrinogen activity	119 (113–126)	107 (97–116)	99 (93–107)
	p≤.001	p>.05	p>.05
Prothrombin antigen	113 (96–134)	110 (88–123)	118 (108–141)
	p≤.05	p>.05	p≤.01
Protein C activity	111 (107–125)	99 (88–102)	99 (91–106)
	p≤.001	p>.05	p>.05
Protein S activity	73 (67–80)	107 (101–116)	103 (96–117)
	p≤.001	p>.05	p>.05
Free TFPI antigen	55 (46–58)	85 (77–101)	83 (80–92)
	p≤.001	p≤.01	p≤.001
Antithrombin activity	95 (90–99)	99 (94–110)	102 (95–107)
	p≤.01	p>.05	p>.05
t-PA antigen	52 (43–68)	92 (75–104)	90 (60–104)
	p≤.001	p>.05	p>.05
sE-Selectin antigen	80 (74–88)	101 (96–114)	92 (82–105)
	p≤.001	p>.05	p>.05

Paired statistics: Wilcoxon signed rank test.
[a] Evaluated number of individuals with data from all sampling points

were of Caucasian descent. The mean height was 170.9 cm (range: 152–188 cm), the mean weight was 67.16 kg (range: 44.3 to 90.3 kg), and the mean BMI was 22.92 kg/m^2 (range: 18.3 to 30.0 kg/m^2).

In total, 48 women provided blood samples through Sample 4. The 5 mg E4/DRSP and 10 mg E4/DRSP groups contained 15 participants each, and the EE/DRSP group contained 18 participants. Based on the subjects' diary records, treatment compliance was generally good (>91%) in all treatment groups. Overall, 14 subject's committed 18 protocol violations, all of which were considered minor. With respect to the hemostasis baseline characteristics, the values measured in Sample 1 (on day 3 of the pretreatment cycle) were all within their respective normal range (see Materials and methods).

3.2. Hepatic estrogenicity markers

In the EE/DRSP group, both SHBG and angiotensinogen increased significantly in the third cycle (to 381% and 256% of baseline, respectively) (Fig. 2). In contrast, SHBG and angiotensinogen were 100% (i.e., no change) and 125% of baseline in the 5 mg E4/DRSP group and 143% and 131% of baseline in the 10 mg E4/DRSP group. Thus, compared to 20 mcg EE, both 5 mg E4 and 10 mg E4 had nearly no effect on SHBG and minor on angiotensinogen.

3.3. Hemostasis variables

The percent change in hemostasis variables measured at the end of treatment cycle 3 (Sample 3) relative to baseline is summarized in Table 1. In addition, the values for the three most relevant coagulation inhibitors (free TFPI, protein S and antithrombin) and for the global coagulation inhibition (ETP-based APCr) test are shown graphically in Fig. 3.

Importantly, both concentrations of E4 had no effect on antithrombin (Fig. 3B), protein S activity (Fig. 3C) or APCr (Fig. 3D), and had only a relatively minor effect on free TFPI (Fig. 3A); in contrast, as expected EE had a significant negative effect on all four markers of coagulation inhibition, thereby promoting coagulation.

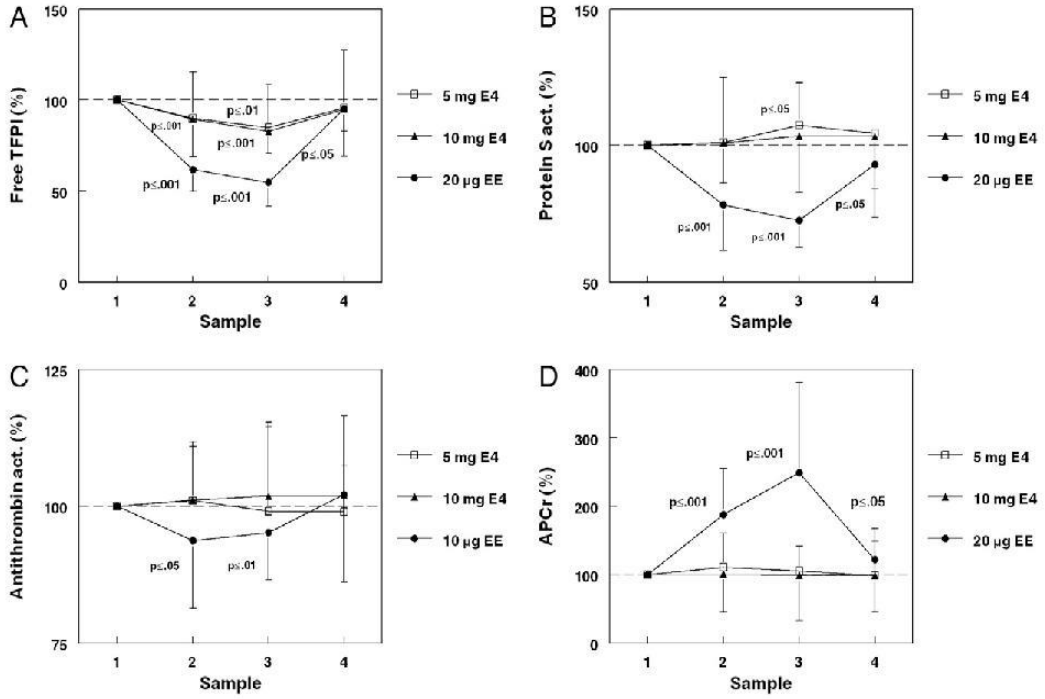

Fig. 3. (A) Median levels (interquartile range up or down) of free TFPI relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test. (B) Median levels (interquartile range up or down) of protein S activity relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test. (C) Median levels (interquartile range up or down) of antithrombin activity relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test. (D) Median levels (interquartile range up or down) of ETP-based APC resistance relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test.

At visit 4 (about 5 weeks after treatment discontinuation), participants who had received the 20 mcg EE/DRSP combination had values of SHBG, angiotensinogen, protein S activity, free TFPI and ETP-APC resistance that remained elevated compared to their pretreatment values (Figs. 2A and B, and 3A, B, and D). The residual change from baseline was compared to the peak value at visit 3 and calculated to be 10%–15% of that peak value.

The effects of E4 and EE on D-dimer and F1+2 (two molecular markers of coagulation activity) are illustrated in Fig. 4A and B, respectively. As reported previously, EE-containing COCs increased both markers, indicating activation of coagulation. Strikingly, however, E4-containing COCs did not increase either marker, but rather decreased both markers.

4. Discussion

Here, we report that E4-containing COCs have considerably reduced effects with respect to hemostasis. None of the studied levels except free TFPI showed a significant change (Table 1). Specifically, with the exception of the modest decrease in free TFPI, E4/DRSP had no effect on coagulation inhibition which is in striking contrast to the well-documented significant negative effects of EE on multiple factors involved in coagulation inhibition [11,12,14,15].

E4 has estrogenic effects on reproductive tissues and organs. The estrogenic effect of 10 and 20 mg E4 alone, administered during 28 days, was previously shown in a study in premenopausal women. Ovulation in the 10 and 20 mg E4 groups was inhibited in one-third and two-thirds of the cycles, respectively [6]. In postmenopausal women, E4 alone in a dose range of 2–40 mg per day dose-dependently showed estrogenic effects on vaginal cytology and hot flushes [20].

The current study provides evidence that COCs containing 5 or 10 mg E4 have some hepatic and endothelial estrogenicity, although the estrogenic effects are much less than the effects of an EE-containing COC. The estrogenicity as indicated by the liver variables SHBG and angiotensinogen is 15%–20% compared to EE/DRSP (Table 1), and the free TFPI reduction is 33%–38% of that of EE/DRSP (Table 1).

Previously, the pharmacodynamic effects of E4/DRSP and EE/DRSP on a broad range of biochemical liver parameters, including carrier proteins, lipids, liver function

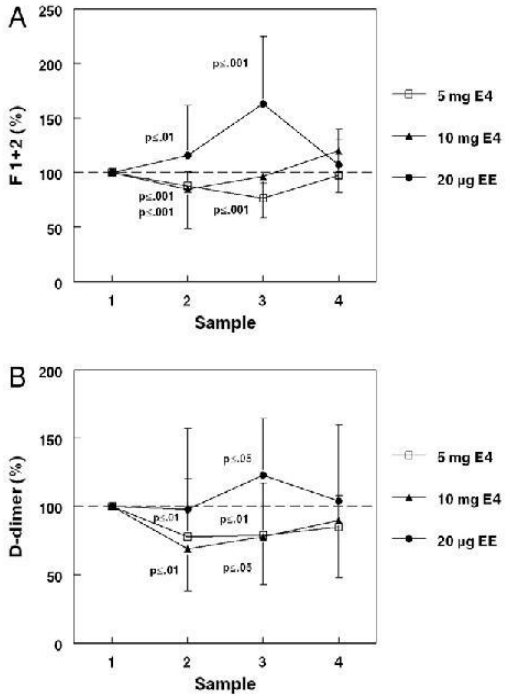

Fig. 4. (A) Median levels (interquartile range up or down) of prothrombin fragment F 1+2 relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test. (B) Median levels (interquartile range up or down) of D-dimer (FbDP) relative to the pretreatment value (=100%). Data for 5 mg E4, 10 mg E4 and 20 mcg EE combinations with 3 mg DRSP. For sample identification, see Fig. 1. Paired statistics: Wilcoxon signed rank test.

parameters and markers of bone metabolism, have been reported and are consistent with the current data [19].

Estrogen in COCs serves three primary functions. First, estrogen regulates vaginal bleeding. Second, estrogen inhibits follicle development by inhibiting the secretion of follicle-stimulating hormone, thereby reducing the amount of progestin needed to effectively inhibit ovulation. Third, the estrogen in COCs serves to replace estradiol that is lost due to the suppression of ovarian function. EE satisfies all three estrogenic functions; however, EE also has a strong estrogenic effect on liver function and vascular endothelium. These hepatic and vascular effects are believed to underlie the increased risk of venous thromboembolism (VTE) in women who use EE-containing COCs. Although this risk can be reduced by some progestins such as LNG, other progestins such as DRSP do not affect the hepatic and vascular estrogenicity of EE.

Interestingly, we observed a *reduction* in D-dimer and F1+2 in women taking E4-DRSP.

Very recently, Regidor et al. [21] reported the first data on DRSP-only treatment (4 mg) and observed a 19% significant reduction in D-dimer. Our data on reduction of both F1+2 and D-dimer suggest an anticoagulant mechanism for the combination E4 and 3 mg DRSP.

We propose that the effect we observed is due to DRSP.

The study of Regidor et al. [21] showed no appreciable effect of DRSP-only on APC resistance, antithrombin and factor VIII, while protein C was shown to be reduced and factor VII increased by 5%. It indicates low impact of DRSP on these liver hemostatic factors.

Previous mechanistic studies suggest a possible mechanism through which DRSP reduces coagulation activation. DRSP is an antagonist of aldosterone, which induces endothelial inflammation, dysfunction and stiffness [22–25] via mineralocorticoid receptors expressed on the endothelium [26,27]. Both in vivo and in vitro studies have shown that aldosterone activates NF-κB [28], decreases t-PA [29], and increases PAI-1 [29–31], Von Willebrand factor [32], ICAM-1 [33], tissue factor [29], VCAM-1 [34], MCP-1 [34], E-selectin [35], TAT, membrane-bound EPCR [36] and microparticles.

That levels of strong reacting variables for the 20 mcg EE/DRSP combination did not return to pretreatment values in the posttreatment sample being around 5 weeks after discontinuation of treatment, is an observation that may provide on retrospect a limitation to our study only employing a 1-month wash-out. It is not clear whether this is relevant for all COCs used before the study, which concerned 20%–40% of the participants. The residual 10%–15% of the peak value observed in the present study indicates a long-lasting effect that cannot be explained by merely plasma clearance of the factors.

A major weakness of our study is the absence of a treatment arm in which only DRSP was administered. The further limitations concern the small sample sizes, the limited range of BMI and the limited age range (only women of a certain BMI and age were included). Further, we did not identify factor V Leiden in our patient group preventing separate analysis and possibly underlying the largest variation (CV%=69, for sample 3) in effect in ETP-based APC resistance. The selection of biomarkers analysis is based on regulatory guidance as provided by EMA. It is based on expert opinions and includes hemostasis variables known to provide a risk in patients with genetic deviations in these factors and risk factors from epidemiological evaluations. The changes induced by the COCs are generally smaller than those of genetic abnormalities and may indicate an increased risk, when in combination with other risk factors and in relation to the fact that COC induce multiple changes that may cooperate in risk during COC use. The data about the listed factors thus do not necessarily reflect clinical outcome, the more so since unknown factors may play a role.

In conclusion, because E4/DRSP has considerably lower hepatic and vascular estrogenicity than EE/DRSP, we expect that women who take E4-containing COCs may ultimately prove to have a lower risk of VTE compared to women who take EE-containing COCs. Our results using COCs with a given dose of DRSP support this hypothesis, at least with

respect to intermediate endpoints. Large studies on DRSP only and the E4/DRSP combination are not yet available and will be required in order to document whether or not unexpected problems arise and to document the putative reduced incidence of VTE among women using COCs containing E4 and DRSP.

Acknowledgment

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References

[1] Fruzzetti F, Tremollieres F, Bitzer J. An overview of the development of combined oral contraceptives containing estradiol: focus on estradiol valerate/dienogest. Gynecol Endocrinol 2012;28:400–8.
[2] Hagen AA, Barr M, Diczfalusy E. Metabolism of 17-beta-oestradiol-4-14-C in early infancy. Acta Endocrinol 1965;49:207–20.
[3] Coelingh Bennink F, Holinka CF, Visser M, Coelingh Bennink HJ. Maternal and fetal estetrol levels during pregnancy. Climacteric 2008;11(Suppl 1):69–72.
[4] Coelingh Bennink HJ, Holinka CF, Diczfalusy E. Estetrol review: profile and potential clinical applications. Climacteric 2008;11(Suppl 1):47–58.
[5] Coelingh Bennink HJ, Skouby S, Bouchard P, Holinka CF. Ovulation inhibition by estetrol in an in vivo model. Contraception 2008;77:186–90.
[6] Visser M, Coelingh Bennink HJ. Clinical applications for estetrol. J Steroid Biochem Mol Biol 2009;114:85–9.
[7] Hammond GL, Hogeveen KN, Visser M, Coelingh Bennink HJ. Estetrol does not bind sex hormone binding globulin or increase its production by human HepG2 cells. Climacteric 2008;11(Suppl 1):41–6.
[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] Abot A, Fontaine C, Buscato M, Solinhac R, Flouriot G, Fabre A, et al. The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation. EMBO Mol Med 2014;6:1328–46.
[10] Adlanmerini M, Solinhac R, Abot A, Fabre A, Raymond-Letron I, Guihot AL, et al. Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. S A 2014;111:E283–90.
[11] Winkler UH. Hemostatic effects of third- and second-generation oral contraceptives: absence of a causal mechanism for a difference in risk of venous thromboembolism. Contraception 2000;62:11S–20S [discussion 37S–8S].
[12] Kluft C. Effects on haemostasis variables by second and third generation combined oral contraceptives: a review of directly comparative studies. Curr Med Chem 2000;7:585–91.
[13] Klipping C, Marr J. Effects of two combined oral contraceptives containing ethinyl estradiol 20 microg combined with either drospirenone or desogestrel on lipids, hemostatic parameters and carbohydrate metabolism. Contraception 2005;71:409–16.
[14] Kemmeren JM, Algra A, Meijers JC, Bouma BN, Grobbee DE. Effects of second and third generation oral contraceptives and their respective progestagens on the coagulation system in the absence or presence of the factor V Leiden mutation. Thromb Haemost 2002;87:199–205.
[15] Middeldorp S, Meijers JC, van den Ende AE, van Enk A, Bouma BN, Tans G, et al. Effects on coagulation of levonorgestrel- and desogestrel-containing low dose oral contraceptives: a cross-over study. Thromb Haemost 2000;84:4–8.
[16] Odlind V, Milsom I, Persson I, Victor A. Can changes in sex hormone binding globulin predict the risk of venous thromboembolism with combined oral contraceptive pills? Acta Obstet Gynecol Scand 2002;81:482–90.
[17] van der Vange N, Blankenstein MA, Kloosterboer HJ, Haspels AA, Thijssen JH. Effects of seven low-dose combined oral contraceptives on sex hormone binding globulin, corticosteroid binding globulin, total and free testosterone. Contraception 1990;41:345–52.
[18] EMA. European Medicines Agency, Committee For Medicinal Products For Human Use. Guideline on clinical investigation of steroid contraceptives in women. EMEA/CPMP/EWP/519/98 rev 1; 2005.
[19] Mawet M, Maillard C, Klipping C, Zimmerman Y, Foidart JM, Coelingh Bennink HJ. Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives. Contracept Reprod Health Care 2015;20:463–75.
[20] Coelingh Bennink HJ, Verhoeven C, Zimmerman Y, Visser M, Foidart JM. Clinical effects of the fetal estrogen estetrol in a multiple-rising-dose study in postmenopausal women. Maturitas 2016;91:93–00.
[21] Regidor PA, Colli E, Schindler AE. Drospirenone as estrogen-free pill and hemostasis: coagulatory study results comparing a novel 4 mg formulation in a 24+4 cycle with desogestrel 75 μg per day. Gynecol Endocrinol 2016:1–3, http://dx.doi.org/10.3109/09513590.2016.1161743.
[22] Funder JW. Aldosterone, mineralocorticoid receptors and vascular inflammation. Mol Cell Endocrinol 2004;217:263–9.
[23] Hashikabe Y, Suzuki K, Jojima T, Uchida K, Hattori Y. Aldosterone impairs vascular endothelial cell function. J Cardiovasc Pharmacol 2006;47:609–13.
[24] Oberleithner H. Aldosterone makes human endothelium stiff and vulnerable. Kidney Int 2005;67:1680–2.
[25] Brown NJ. Aldosterone and vascular inflammation. Hypertension 2008;51:161–7.
[26] Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. Circ Res 1992;71:503–10.
[27] Golestaneh N, Klein C, Valamanesh F, Suarez G, Agarwal MK, Mirshahi M. Mineralocorticoid receptor-mediated signaling regulates the ion gated sodium channel in vascular endothelial cells and requires an intact cytoskeleton. Biochem Biophys Res Commun 2001;280:1300–6.
[28] Fiebeler A, Schmidt F, Muller DN, Park JK, Dechend R, Bieringer M, et al. Mineralocorticoid receptor affects AP-1 and nuclear factor-kappab activation in angiotensin II-induced cardiac injury. Hypertension 2001;37:787–93.
[29] Stankiewicz A, Gromotowicz A, Szemraj J, Wojewodzka-Zelezniakowicz M, Skrzypkowski P, Chabielska E. Acute aldosterone infusion enhances thrombosis development in normotensive rats. Thromb Haemost 2007;98:697–9.
[30] Yuan J, Jia R, Bao Y. Aldosterone up-regulates production of plasminogen activator inhibitor-1 by renal mesangial cells. J Biochem Mol Biol 2007;40:180–8.
[31] Chun TY, Pratt JH. Aldosterone increases plasminogen activator inhibitor-1 synthesis in rat cardiomyocytes. Mol Cell Endocrinol 2005;239:55–61.
[32] Jeong Y, Chaupin DF, Matsushita K, Yamakuchi M, Cameron SJ, Morrell CN, et al. Aldosterone activates endothelial exocytosis. S A 2009;106:3782–7.
[33] Terada Y, Ueda S, Hamada K, Shimamura Y, Ogata K, Inoue K, et al. Aldosterone stimulates nuclear factor-kappa B activity and transcription of intercellular adhesion molecule-1 and connective tissue growth factor in rat mesangial cells via serum- and glucocorticoid-inducible protein kinase-1. Clin Exp Nephrol 2012;16:81–8.
[34] Chander PN, Rocha R, Ranaudo J, Singh G, Zuckerman A, Stier Jr CT. Aldosterone plays a pivotal role in the pathogenesis of thrombotic microangiopathy in SHRSP. J Am Soc Nephrol 2003;14:1990–7.

[35] Seeger H, Wallwiener D, Mueck AO. Effects of drospirenone on cardiovascular markers in human aortic endothelial cells. Climacteric 2009;12:80–7.

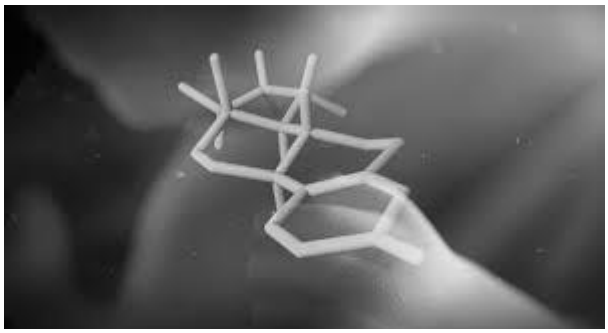
[36] Ducros E, Berthaut A, Mirshahi SS, Faussat AM, Soria J, Agarwal MK, et al. Aldosterone modifies hemostasis via upregulation of the protein-C receptor in human vascular endothelium. Biochem Biophys Res Commun 2008;373:192–6.

2.3. Discussion

Drospirenone often refers as the fourth generation progestin. With its anti-mineralocorticoid activity and anti-androgenic properties, DRSP in combination with EE, was shown to improve the metabolic profile, blood pressure, acne and seborrhea, and PMDD (see Chapter 1, Section 6.2 for more details). These features are most probably the reason of the worldwide success of the EE/DRSP COCs. However, large epidemiological studies have indicated that the VTE risk is two times higher with the EE/DRSP COCs in comparison to the second-generation COCs. This increased VTE risk appears difficult to accept, particularly as it concerns young healthy women and also because it exists safer alternatives. Consequently, several guidelines and recommendations on contraception, including those from United Kingdom and Belgium, have been modified in order to recommend the use of second-generation COCs containing LNG as progestin, in first intention (FSRH 2019b).

Drospirenone was falsely accused of being responsible for the increased VTE risk. However, Regidor and co-workers have recently shown that the newly developed DRSP only-pill did not display any meaningful modifications in APCr, antithrombin III, factor VII, factor VIII, protein C and D-Dimer (Regidor, Colli, and Schindler 2016). The authors concluded that DRSP administered alone does not induce any change in the hemostasis. In our publication, we demonstrated that the changes in the surrogate markers of VTE risk elicited by E4/DRSP are small and much lower than the changes seen with the combination EE/DRSP. Women receiving YAZ® (EE/DRSP) exhibited higher levels of D-Dimers and of the proteolytic fragments 1, 2 of Prothrombin, while these markers of activated coagulation were decreased in women receiving E4/DRSP. Both publications prove that the estrogen compound of the pill is the main responsible of the imbalance in the hemostasis factors favoring the hypercoagulability. These data were completed and confirmed in a second study. The publication of these results is currently under submission and the manuscript is reported in Section 6 below.

In contrast to what is seen with EE/DRSP combinations, in our study, E4/DRSP did not significantly increase the angiotensinogen plasma level. Angiotensinogen being synthetized by the liver under the influence of estrogens, this low impact of E4 on its production is an additional demonstration of the low hepatic impact of E4 in contrast to EE. Being neutral on angiotensinogen level is an additional benefit for an E4/DRSP combination given the well-known deleterious effect of high blood pressure on cardiovascular health.



3rd Publication

Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives

The European Journal of Contraception and Reproductive Health Care,
2015; 20: 463 – 475

Mawet M, Maillard C, Klipping C, Zimmerman Y, Foidart JM, Coelingh Bennink HJ

3. Third publication

Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives

The European Journal of Contraception and Reproductive Health Care, 2015; 20: 463 – 475

Mawet M, Maillard C, Klipping C, Zimmerman Y, Foidart JM, Coelingh Bennink HJ

3.1. Introduction

The first phase 2 trial was also the occasion to evaluate the impact of the different combinations tested on several pharmacodynamic parameters, known to be affected by the use of COC.

First, an analysis on the impact on the lipid profile was carried on as COC use is known to modify the metabolism of lipids. As far as the cholesterol is concerned, literature tends to show that the changes are mostly linked to the progestin compound of the COC: androgenic progestins are associated with a worsening of the cholesterol profile (increased total cholesterol and LDL cholesterol levels; decreased HDL cholesterol level) while less androgenic, and particularly anti-androgenic progestins, do not impact the lipid profile or tend to improve it, like it is the case with EE/DRSP COCs which decrease total cholesterol and LDL cholesterol levels and increase the HDL cholesterol level. Both the strong synthetic EE and the natural E2 are associated with a significant increase in plasma triglycerides.

Secondly, the "estrogenicity on liver" of the combinations was also reported in this article through the measurement of carrier proteins synthetized by the liver under the influence of estrogen: ceruloplasmin, CBG, and SHBG. The synthesis of these carrier proteins by the liver has been shown to be a relevant marker of estrogenicity (see Chapter 1, Section 4.7 for more details).

We also evaluated the impact on bone turnover through the measurement of osteocalcin and C-terminal telopeptide (CTX1). Osteocalcin is the most abundant non-collagenous protein in bone and is produced almost exclusively by osteoblasts. In consequence, osteocalcin concentration is a sensitive biomarker of bone synthesis. Osteocalcin levels correlate with histomorphometric measurements of bone formation in bone biopsy specimens. CTX1 reflects the rate of bone resorption (Nappi, et al. 2005). Changes in bone turnover assessed by biomarkers are more rapid and range on a larger scale compared with the changes in BMD, therefore the use of biomarkers is particularly useful when investigating dose-dependent responses in shorter-term studies. COC use is associated with a reduction in bone turnover biomarkers. The impact of this on fracture risk is still matter of debate (Herrmann and Seibel 2010).

Impact of the different combinations on growth endocrinology was also evaluated during this study, through the measurement of growth hormone (GH), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), insulin-like growth factor-binding protein 1 (IGFBP-1), and insulin-like growth factor-binding protein 3 (IGFBP-3). GH is secreted by the pituitary gland under the stimulating effect of growth hormone releasing hormone and stimulates the peripheral production of IGF-1, IGF-2, IGFBP-1 and IGFBP-3, mostly by the liver. In

premenopausal women, during spontaneous menstrual cycle, endogenous levels of E2 influence the GH/IGF-1 axis: increasing levels of E2 during the follicular phase of the menstrual cycle is associated with an increased in GH and IGF-1 levels. Accordingly, exogenous oral administration of estrogen (notably during COC administration) also increases GH levels but, in the opposite of what is seen with endogenous estrogen, decreases IGF-1 synthesis. This dichotomous activity of exogenous estrogen on the GH/IGF-1 axis is currently explained by the liver first-pass effect: administered orally, exogenous estrogens directly reach the liver where their metabolism impairs the hepatic synthesis of IGF-1, resulting in a lower production of IGF-1 (Southmayd and De Souza 2017). Up to now, the studies aiming at evaluating the impact of COC use on the GH/IGF-1 axis were conducted with EE-containing COCs. Consequently, our study was the occasion to evaluate if another estrogen like E4 could have a different impact on the GH axis and to compare it with an EE-containing COC.

All the above-mentioned parameters were measured before at baseline (before starting the treatment), at the end of the third treatment cycle, and in the menstrual cycle following the end of the treatment.

Finally, serum concentrations of E4 at steady state were also reported. A first measurement was performed during treatment cycle 1 and a second during treatment cycle 3.

3.2. Article

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Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives

Marie Mawet*, Catherine Maillard*, Christine Klipping†, Yvette Zimmerman‡, Jean-Michel Foidart*,§,# and Herjan J.T. Coelingh Bennink‡,#

*Estetra SPRL, Liège, Belgium, †Dinox BV, Groningen, the Netherlands, ‡Pantarhei Bioscience BV, Zeist, the Netherlands, and §University of Liège, Liège, Belgium

ABSTRACT

Objectives Estetrol (E_4) is a natural estrogen produced by the human fetal liver. In combination with drospirenone (DRSP) or levonorgestrel (LNG), E_4 blocks ovulation and has less effect on haemostatic biomarkers in comparison with ethinylestradiol (EE) combined with DRSP. This study evaluates the impact of several doses of E_4/DRSP and E_4/LNG on safety parameters such as liver function, lipid metabolism, bone markers and growth endocrine parameters.

Methods This was a dose-finding, single-centre, controlled study performed in healthy women aged 18 to 35 years with a documented pretreatment ovulatory cycle. Participants received 5 mg or 10 mg E_4/3 mg DRSP; 5 mg, 10 mg or 20 mg E_4/150 µg LNG; or 20 µg EE/3 mg DRSP as a comparator for three consecutive cycles in a 24/4-day regimen. Changes from baseline to end of treatment in liver parameters, lipid metabolism, bone markers and growth endocrinology were evaluated.

Results A total of 109 women were included in the study. Carrier proteins were minimally affected in the E_4/DRSP and E_4/LNG groups, in comparison with the EE/DRSP group, where a significant increase in sex hormone-binding globulin was observed. Similarly, minor effects on lipoproteins were observed in the E_4 groups, and the effects on triglycerides elicited by the E_4 groups were significantly lower than those in the EE/DRSP group. No imbalances in bone markers were observed in any groups. No alterations in insulin-like growth factor were observed in the E_4 groups.

Conclusions E_4-containing combinations have a limited effect on liver function, lipid metabolism, and bone and growth endocrine parameters.

KEYWORDS Bone markers; Drospirenone; Endocrinology; Estetrol; Levonorgestrel; Lipid metabolism; Liver function

#Jean-Michel Foidart and Herjan J.T. Coelingh Bennink contributed equally to this study.
Correspondence: Marie Mawet, Estetra SPRL, Rue Saint Georges 5-7, 4000 Liège, Belgium. Tel: +32 4 349 2822. Fax: +32 4 349 2821. E-mail: mmawet@mithra.com

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INTRODUCTION

Combined oral contraceptives (COCs) are a well-known and reliable method of reversible contraception used by women worldwide[1]. An optimal COC would completely block ovulation and suppress endogenous ovarian activity, while causing minimal changes in haemostasis, lipid metabolism, liver function and growth hormone (GH) endocrinology. In addition, it would provide an adequate spotting and bleeding pattern, with no deterioration in quality of life. The majority of the currently available COCs contain ethinylestradiol (EE), as the estrogen component, and a variety of progestogens including drospirenone (DRSP) and levonorgestrel (LNG).

The estrogens estradiol (E_2) and E_2 valerate have recently been introduced; however, EE remains the most widely used estrogen in COCs. Although EE has been shown to be safe, it causes subjective side effects (breast tension and tenderness, weight gain, oedema, nausea, vomiting, bloating, headache and mood changes)[2]. In addition, EE increases the synthesis of various liver proteins, such as lipoproteins, angiotensinogen, sex hormone-binding globulin (SHBG), corticosteroid-binding globulin (CBG) and ceruloplasmin[3]. Furthermore, the use of EE and its enterohepatic recirculation is related to a doubling of all types of gallbladder diseases[4]. The most serious adverse effects of EE and other exogenous estrogens are cardiovascular complications, both arterial (hypertension, myocardial and cerebral infarction) and venous (deep vein thrombosis and pulmonary embolism). These cardiovascular complications are rare but serious, especially in young, healthy women[5,6]. Strategies to reduce the risk of deep vein thrombosis are: (i) lowering the dose of EE; and (ii) substituting EE with E_2 or another estrogen[7,8].

The development of new estrogens such as estetrol (E_4) holds promise for the safety and tolerability of future COCs[3]. E_4 is only synthesised by human fetal liver and is therefore present only during human pregnancy[9]. Late pregnancy maternal plasma levels are around 1 ng/ml, while fetal plasma levels are 12 to 19 times higher at term[9,10]. E_4 does not bind to SHBG, has a low impact on SHBG production by human hepatocytes, and is mainly excreted in the urine rather than through the biliary route[9,11]. In postmenopausal women, E_4 was found to cause dose-dependent decreases in both the marker of bone resorption (C-telopeptide) and the marker of bone formation (osteocalcin)[12]. The inhibitory effect was found to be more prominent on bone resorption, suggesting the possibility of positive bone formation[12].

Recent studies of uterine and vascular actions of E_4 delineate a distinctive profile of estrogen receptor alpha (ERα) modulation. E_4 activates the nuclear ERα, but antagonises the membrane-bound ERα. Transgenic mice lacking this membrane receptor fail to ovulate and are infertile. E_4 could therefore be particularly suitable for a contraceptive indication[13,14].

Preclinical and phase I clinical research has suggested that E_4 may be a suitable replacement for EE in COCs[12,15]. There is preclinical proof that E_4 effectively inhibits ovulation in a dose-dependent manner similar to the action of EE[16]. In a recent open-label phase II dose-finding study (NTR2102/EudraCT 2009-011858-17), two E_4/DRSP and three E_4/LNG combinations vs. 20 µg EE/3 mg DRSP were investigated for their effects on ovulation inhibition and haemostatic biomarkers (I.J.M. Duijkers, unpublished data; C. Kluft, unpublished data). All doses of 5 mg, 10 mg or 20 mg E_4, in combination with DRSP or LNG, blocked ovulation and dose-dependently decreased ovarian activity. In addition, minor effects were observed on haemostatic biomarkers.

In order to more precisely characterise the safety of E_4-containing COCs, we evaluated in the present study the pharmacodynamic effects of E_4/DRSP and E_4/LNG vs. EE/DRSP on liver function, lipid metabolism, bone markers and growth endocrine parameters. This study provides clinically relevant information on E_4 pharmacokinetics.

METHODS

This was an open-label, dose-finding phase II study conducted at a single centre in the Netherlands (Dinox BV, Groningen, the Netherlands) and registered as NTR2102/EudraCT 2009-011858-17. The study was approved by an independent ethics committee and conducted in accordance with the ethical principles established by the Declaration of Helsinki and the International Conference on Harmonization—Good Clinical Practice Guidelines. Written informed consent was obtained from all participants before enrolment in the study.

Participants

Healthy women, aged 18 to 35 years with a BMI between 18 and 30 kg/m^2, were eligible for inclusion in the study. The exclusion criteria were in line with the World Health Organization's medical eligibility criteria for COC use[17]. Women unwilling to use a non-hormonal method of contraception during the study were also excluded. At screening, all women underwent thorough medical and gynaecological examinations, and blood and urine were sampled for routine laboratory analyses. Eligible women had to use a barrier method of contraception during a washout cycle, the pretreatment cycle and the subsequent cycles in the study up to 7 days after the follow-up visit. Moreover, all women had to have at least two spontaneous cycles before starting the study medication.

Treatment and study design

The study consisted of a washout cycle (when using COCs), followed by one pretreatment observational cycle to verify ovulation, three 28-day treatment cycles (cycles 1–3) and one post-treatment cycle. The pretreatment study visit was held on day 3 (± 1) after the start of spontaneous menses. Eligible women were assigned to one of six treatment groups: 5 mg or 10 mg E_4 combined with 3 mg DRSP; 5 mg, 10 mg or 20 mg E_4 combined with 150 μg LNG; or the comparator 20 μg EE combined with 3 mg DRSP (Yaz; Bayer HealthCare Pharmaceuticals, Berlin, Germany). All subjects were stratified according to the day of ovulation in the pretreatment cycle, except those in the 20 mg E_4/LNG group, as this group was added later during the course of the study. The sample size was primarily determined to allow conclusions on ovulation inhibition (I.J.M. Duijkers, unpublished data), which was expected to vary between 1% and 14% in a worst case scenario for three consecutive cycles. As a result, approximately 18 participants had to be included in each treatment group.

The first study treatment was taken on the first day of the next menses and continued over three cycles of 24 days, each followed by 4 days of no treatment (E_4 groups) or placebo (comparator) intake. Compliance was assessed by recording study treatment (pill intake) on diary cards. Blood samples for laboratory measurements (liver function, bone biomarkers, growth endocrine parameters) were taken on day 3 (± 1) of the pretreatment cycle, on days 3 (± 1) and 24 (± 1) of cycles 1 and 3, and at the follow-up visit on day 3 (± 1) of the cycle following the post-treatment cycle. Blood samples for pharmacokinetic assessments in subjects allocated to one of the E_4/DRSP or E_4/LNG groups were taken on day 24 (± 1) of cycles 1 and 3 before the pill intake scheduled for that day. Excretion of E_4 and E_4 conjugates was investigated by collection of 24 h urine twice between day 21 and 24 of cycle 3 in the 10 mg E_4/LNG group only.

Laboratory measurements

Laboratory analyses were performed under the responsibility of the Clinical-Chemical Laboratory, Canisius Wilhelmina Hospital, Nijmegen, the Netherlands. Pharmacokinetic analyses were performed by Xendo Drug Development BV, Groningen, the Netherlands.

Liver function and lipid metabolism

The carrier proteins SHBG, CBG and ceruloplasmin were measured by the respective immunoassays: COBAS ECLIA (Roche Diagnostics, Mannheim, Germany), CBG RIA (BioSource, San Diego, California, USA) and COBAS INTEGRA (Roche Diagnostics). Serum levels of lipids and lipoproteins (HDL-, LDL- and total cholesterol, and triglycerides) were measured by enzymatic (colorimetric) tests (Roche Diagnostics). The liver enzymes aspartate aminotransferase/glutamic-oxalocetic transaminase (ASAT/SGOT), alkaline phosphatase and γ-glutamyl transferase (γGT) were measured by enzymatic (colorimetric) tests (Roche Diagnostics).

Bone parameters

The biomarkers of bone turnover C-telopeptide and osteocalcin were measured by the immunoassays β-CrossLaps/serum and N-MID Osteocalcin (Roche Diagnostics), respectively.

Growth endocrinology

Insulin-like growth factor (IGF)-I and GH were measured using immunometric techniques (Siemens Medical Solutions Diagnostics, Los Angeles, USA), IGF-II by radioimmunoassay (RIA) in Sep-Pak C18 extracts of plasma[18], and IGF-binding protein (IGFBP)-1 and -3 by specific RIAs.[19,20]

Pharmacokinetics

Plasma E_4 trough levels were investigated by collection of steady-state samples in the E_4 treatment groups 24 h after study medication intake.

The excretion of E_4 and E_4 conjugates was investigated by collection of 24 h urine at steady state from 10 subjects in the 10 mg E_4 treatment groups between day 21 and day 24 of cycle 3. Urinary recovery was expressed as total excretion of E_4 in urine compared with daily intake of 10 mg E_4.

E_4 plasma and urine levels were measured by liquid chromatography, followed by tandem mass spectrometry detection (LC-MS/MS; lower limit of quantification 25 pg/ml). For the E_4 conjugates in urine samples, hydrolysis was performed by β-glucuronidase and sulfatase before the measurement of the released E_4 by LC-MS/MS. The E_4 glucuronide and E_4 sulfate levels were then calculated semi-quantitatively, based on the assumption that hydrolysis is complete. Total E_4 excretion was calculated by summing the excreted quantities of unconjugated E_4, E_4 glucuronide and E_4 sulfate after the E_4 conjugate quantities were corrected using the E_4/E_4 glucuronide and E_4/E_4 sulfate molecular weight ratio.

Statistical analysis

This was an exploratory study. The intention-to-treat (ITT) population (subjects who received at least one dose of study treatment and had at least one post-baseline assessment) served as a basis for the statistical analysis. The ITT population was identical to the all-subjects-treated (AST) population.

Primary analyses included the investigation of pharmacodynamic effects on liver function, lipid metabolism and bone biomarkers. Quantitative summary statistics and a summary of change from baseline to treatment cycle 3 day 24 were performed on these parameters, as well as for SHBG at cycle 1 day 24. Box whisker plots for relative changes from baseline to treatment cycle 3 day 24 were prepared for lipid and lipoprotein parameters. Values assessed on pretreatment cycle day 3 served as baseline.

Secondary analyses included the investigation of pharmacodynamic effects on growth endocrine parameters.

In addition, the differences in the changes from baseline to the end of treatment in carrier proteins, lipids parameters, bone biomarkers and growth endocrine parameters were compared using ANOVA between the different groups after having pooled the two E_4/DRSP groups and the three E_4/LNG groups. In the statistical analyses, $p < 0.01$ was used as a criterion for statistical significance.

Finally, quantitative summary statistics were performed for pharmacokinetic analyses of E_4 steady-state levels and excretion data.

RESULTS

The study was performed from November 2009 until November 2010. In total, 111 subjects were included and assigned to one of the six treatment groups. Two women dropped out before starting study medication, one withdrew her consent and the other became pregnant in the pretreatment cycle. Of the 109 subjects treated, the majority (85/109; 78.0%) completed the study. The proportion of subjects completing the study was similar across the treatment groups (77.8–88.2%), with the exception of the 10 mg E_4/LNG group, in which 58.8% (10/17) completed the study (Figure 1). Fifteen subjects discontinued the study during the treatment phase and nine did not complete the post-treatment cycle. Reasons for discontinuation in these cases were adverse events

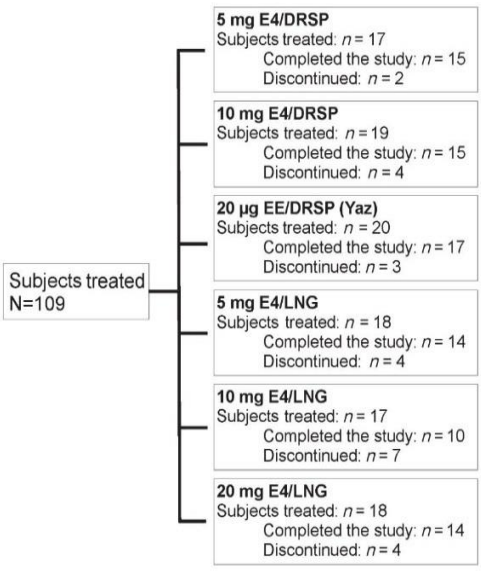

Figure 1 Allocation of subjects by treatment group (ITT and AST populations).

(five subjects: emotional lability and acute bronchitis, tiredness, increased frequency of headache, mood swings, decreased libido and headache, respectively), intracyclic bleeding (one subject), personal reasons (one subject), use of prohibited concomitant medication to treat acute bronchitis (one subject), incorrect study medication intake (one subject), withdrawal of consent (one subject), pregnancy during the post-treatment cycle (one subject) and inability to adhere to the visit schedule (13 subjects).

Overall demographic and baseline characteristics of the 109 subjects treated were generally similar across the treatment groups (Table 1). The mean age was 22.9 years (range 18–33 years). Mean BMI was slightly different between groups and ranged from 21.5 kg/m^2 in the 5 mg E_4/LNG group to 24.3 kg/m^2 in the 20 mg E_4/LNG group. The distribution of races was similar among groups and was predominantly white (82.4–100%). The safety and tolerability profile associated with the different combinations is reported in the publication on ovulation inhibition in the same subjects (I.J.M. Duijkers, unpublished data).

LIVER FUNCTION AND LIPID METABOLISM

A dose-dependent response was observed in the E_4/DRSP and E_4/LNG groups for the carrier protein parameters (Table 2; C. Kluft, unpublished data). A decrease in SHBG was observed in the E_4/LNG groups (mean change up to − 69.0%). By contrast, increases in SHBG were observed in the 5 mg (7.9%) and 10 mg (44.5%) E_4/DRSP groups, but these were still considerably less than the 306.3% observed in the EE/DRSP group. All effects on SHBG were already apparent at treatment cycle 1 (Figure 2).

In the E_4/DRSP and E_4/LNG groups, CBG was marginally affected (mean changes between − 6.9% and 28.1%), whereas a substantial increase was observed in the EE/DRSP group (mean change 170.3%). Ceruloplasmin, the major copper-carrying protein in the blood, is synthesised by hepatocytes under the influence of estrogens[21]. By increasing copper availability, increased ceruloplasmin levels contribute to the enhanced oxidative stress observed in COC users[22]. The effect on ceruloplasmin was minimal in the E_4/DRSP (8.2% and 16.1%) and E_4/LNG groups (between − 5.4% and 16.2%), whereas for the EE/DRSP group a more pronounced increase of 69.0% was noted (Table 2). After pooling the E_4/DRSP and E_4/LNG groups, SHBG, CBG and ceruloplasmin levels were significantly lower in the E_4 groups compared with the EE/DRSP group. The differences in SHBG level between the E_4/DRSP and the E_4/LNG groups also reached statistical significance but the changes in CBG and ceruloplasmin did not.

In the DRSP-treated groups, HDL-cholesterol increased in the E_4/DRSP and EE/DRSP groups (up to 8.1% and 15.2%, respectively), but decreased in the E_4/LNG groups (up to − 19.0%). LDL-cholesterol slightly increased in the E_4/DRSP and 20 mg E_4/LNG groups (up to 6.7% and 8.9%, respectively), but decreased in the other E_4/LNG groups (up to − 13.2%), as well as in the EE/DRSP

Table 1 Main demographics and baseline characteristics (ITT and AST populations).

Characteristic	*5 mg E_4/DRSP n = 17*	*10 mg E_4/DRSP n = 19*	*20 µg EE/DRSP n5 20*	*5 mg E_4/LNG n = 18*	*10 mg E_4/LNG n = 17*	*20 mg E_4/LNG n = 18*
Age, years						
Mean (SD)	24.5 (3.2)	23.7 (3.7)	23.4 (3.9)	22.3 (2.6)	22.4 (2.4)	21.1 (2.3)
Range	20–33	20–32	18–33	18–28	19–27	18–26
BMI, kg/m^2						
Mean (SD)	22.7 (2.4)	23.2 (3.2)	23.0 (2.9)	21.5 (1.7)	21.8 (2.5)	24.3 (3.4)
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
Race, *n* (%)						
White	14 (82.4)	18 (94.7)	19 (95.0)	16 (88.9)	17 (100)	16 (88.9)
Black	1 (5.9)	0	0	0	0	2 (11.1)
Asian	2 (11.8)	0	1 (5.0)	0	0	0
Other	0	1 (5.3)	0	2 (11.1)	0	0

Table 2 Percentage change from baseline at treatment cycle 3 day 24 for carrier proteins, lipoproteins, bone biomarkers, and growth and steroid endocrine parameters (ITT population).

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*
SHBG	7.9 (26.2)	44.5 (34.1)	306.3 (117.7)	−69.0 (11.8)	−64.8 (11.9)	−44.2 (18.0)
CBG	17.1 (16.6)	28.1 (19.6)	170.3 (75.6)	−6.9 (17.2)	5.9 (13.3)	25.2 (25.0)
Ceruloplasmin	8.2 (12.2)	16.1 (11.1)	69.0 (22.9)	−5.4 (14.6)	0.7 (9.9)	16.2 (6.1)
HDL-cholesterol	8.1 (14.0)	5.6 (11.5)	15.2 (11.3)	−16.9 (20.7)	−11.9 (14.1)	−19.0 (10.9)
LDL-cholesterol	6.7 (20.7)	6.3 (18.3)	−9.2 (22.1)	−5.9 (16.1)	−13.8 (20.2)	8.9 (17.9)
Total cholesterol	5.2 (9.8)	5.0 (9.6)	4.9 (10.3)	−12.8 (9.1)	−15.5 (14.4)	−7.6 (9.1)
Triglycerides	6.4 (36.7)	10.0 (48.5)	61.2 (51.2)	−24.6 (33.7)	−29.7 (26.5)	−27.4 (16.5)
ASAT/SGOT	−4.0 (11.9)	2.0 (22.1)	−9.6 (25.6)	−11.6 (24.9)	−12.4 (21.9)	−13.3 (18.6)
Alkaline phosphatase	−11.3 (6.6)	−17.6 (8.6)	−20.6 (11.8)	−7.5 (12.5)	−5.8 (14.9)	−4.7 (12.5)
γGT	−4.8 (18.5)	−8.2 (14.6)	−11.0 (20.9)	−0.6 (19.8)	3.6 (16.0)	2.7 (17.7)
C-telopeptide	−8.6 (16.8)	−13.4 (20.2)	−34.9 (17.8)	−6.4 (22.5)	−12.4 (23.0)	−22.4 (18.8)
Osteocalcin	−10.4 (11.1)	−16.3 (11.9)	−22.3 (11.7)	−4.1 (16.6)	0.8 (19.7)	−13.0 (16.1)
IGF-I	−5.9 (10.8)	−11.5 (17.7)	−41.9 (14.0)	1.4 (7.4)	3.4 (20.0)	−8.8 (12.0)
IGF-II	−0.7 (8.4)	−2.3 (13.1)	4.7 (7.7)	−0.7 (9.4)	4.8 (18.6)	−2.2 (6.9)
IGFBP-1	21.1 (52.3)	0.0 (30.6)	190.9 (245.0)	−7.5 (46.7)	56.5 (251.5)	41.9 (83.8)
IGFBP-3	7.4 (8.0)	1.4 (11.0)	3.9 (12.6)	1.3 (12.4)	2.0 (12.9)	16.3 (11.0)
GH	100.0 (191.6)	314.1 (722.4)	238.4 (508.4)	173.9 (755.1)	357.5 (750.3)	467.7 (1191.3)

Values are mean (SD) percentage change.

group (−9.2%). Consequently, total cholesterol increased slightly in the DRSP-treated groups (from 4.9% to 5.2%), but decreased in the E_4/LNG groups (up to −15.5%). Triglyceride levels decreased by up to −29.7% in the E_4/LNG treatment groups, but increased in the E_4/DRSP and EE/DRSP groups (mean change up to 10.0% and 61.2%, respectively) (Table 2, Figure 3).

When comparing the pooled results of the E_4/LNG groups with those of the E_4/DRSP groups, the changes in HDL-cholesterol, total cholesterol and triglycerides were all statistically different, while the changes in LDL-cholesterol did not reach statistical significance.

The changes in lipid parameters elicited by the comparator (EE/DRSP) were statistically significantly different from those in the pooled E_4/LNG groups, except the decrease in LDL-cholesterol. The E_4/DRSP combinations did not elicit significant differences in lipid parameters in comparison with EE/DRSP.

Small decreases in the liver enzymes ASAT/SGOT and alkaline phosphatase (mean changes up

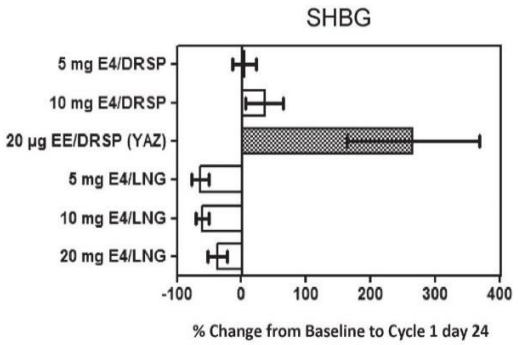

Figure 2 Mean (SD) percentage change from baseline to cycle 1 day 24 in SHBG (ITT population).

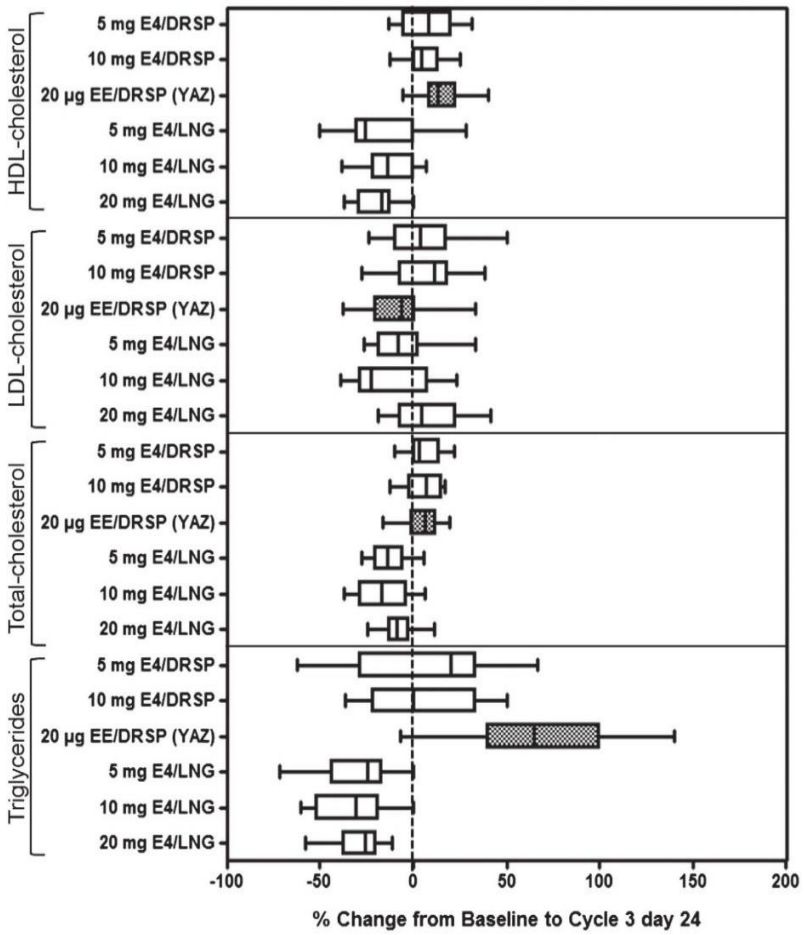

Figure 3 Box whisker plots for E_4/DRSP, E_4/LNG (all treatment groups) and EE/DRSP of relative changes from baseline to cycle 3 day 24 for lipid and lipoprotein parameters (ITT population). The edges of the boxes represent the 25th and 75th sample percentiles (quartiles); the vertical line in the boxes shows the median; and whiskers are drawn up to the smallest and largest value within 1.5 times the interquartile range.

to − 13.3% and − 7.5%, respectively) were observed in the E_4/LNG groups and in the 5 mg E_4/DRSP (− 4.0% and − 11.3%, respectively) and EE/DRSP groups (− 9.6% and − 20.6%, respectively). A small increase in ASAT/SGOT was observed in the 10 mg E_4/DRSP group (2.0 %) and a decrease in alkaline phosphatase (− 17.6%). γGT remained stable in all E_4/LNG groups, whereas in both E_4/DRSP groups and in the EE/DRSP group small decreases were observed (− 4.8%, − 8.2% and − 11.0%, respectively). Due to the large interindividual variations, no statistically significant differences were found between the pooled E_4/DRSP and E_4/LNG groups or between the E_4 groups and the EE/DRSP group in the liver enzymology parameters, except for the alkaline phosphatase level, which was significantly lower in the EE/DRSP group compared with the E_4/LNG group.

Bone parameters

In the E_4 groups, a dose-related decrease was observed for the biomarkers of bone resorption (C-teloppetide) and bone formation (osteocalcin) (mean changes up to − 22.4% and − 16.3%, respectively). A

non-significant greater suppression of bone turnover (− 34.9% and − 22.3%, respectively) was observed in the EE/DRSP groups (Table 2).

The pooled E_4/DRSP combinations had significantly less impact on C-telopeptide levels than either of the other combinations. Osteocalcin levels were significantly lower in the EE/DRSP group than in the pooled E_4/LNG groups, while no statistically significant differences were seen between the E_4-containing combinations and the DRSP-containing combinations.

Growth endocrinology

No clear effects on the growth endocrine parameters IGF-I, IGF-II and IGFBP-3 were observed in any of the E_4/DRSP and E_4/LNG groups. In the EE/DRSP group, IGF-I was statistically significantly decreased (mean change − 41.9%) in comparison with the pooled E_4/DRSP and E_4/LNG groups, while no effect was observed on IGF-II and IGFBP-3 (Table 2). An effect on IGFBP-1 was observed in the E_4/LNG groups (mean change up to 56.5%), and a limited effect (21.1% and 0%, respectively) in the 5 mg and 10 mg E_4/DRSP groups. By contrast, a large increase was observed in the EE/DRSP group (190.9%) (Table 2). This was statistically significantly different from the pooled E_4/DRSP and E_4/LNG groups. Although there were large inter-individual differences, GH levels increased in all groups. For the E_4/DRSP, E_4/LNG and EE/DRSP groups, the mean changes were up to 314.1%, 467.7% and 238.4%, respectively, with no statistical differences (Table 2). In the follow-up cycle, a return to baseline levels was observed for all growth endocrine parameters in all groups, except for GH, which remained increased in the E_4/DRSP (up to 174.1%), E_4/LNG (up to 216.9%) and EE/DRSP (129.9) groups (data not shown).

Pharmacokinetics

A dose-dependent increase was observed in E_4 plasma levels. Administration of 20 mg E_4/LNG resulted in mean E_4 trough levels of 2268 pg/ml and 2005 pg/ml in treatment cycles 1 and 3, respectively (Table 3).

In 10 subjects from the 10 mg groups (E_4/DRSP or E_4/LNG), E_4 was primarily excreted in the urine as E_4 glucuronide (median 60.7%, range 47.6–77.2%) and, to a lesser extent, as E_4 sulfate (median 17.6%, range 13.2–22.1%). Urinary excretion of unconjugated E_4 was negligible (range 0.2–0.7%). The median total E_4 excretion in urine was 79.7% (range 61.1–99.0%).

DISCUSSION

Findings and interpretation

A clinical programme has been initiated to develop fetal E_4 as a replacement for EE in COCs. Ovulation inhibition by E_4 has been assessed preclinically,[16] and in young healthy women (I.J.M. Duijkers, unpublished data). E_4, in combination with DRSP or LNG, has been shown to be effective in suppressing ovulation (I.J.M. Duijkers, unpublished data). In addition, it was observed that E_4 had a minor effect on haemostatic biomarkers, both on coagulation and on fibrinolysis (C. Kluft, unpublished data).

Pharmacokinetic studies in early postmenopausal women revealed an elimination half-life ($t_{1/2}$) of E_4 of approximately 28 h, allowing once-daily oral administration[15]. This was recently confirmed in a study in healthy women, aged 18 to 45 years and receiving combined 20 mg E_4/LNG tablets, in which a $t_{1/2}$ of 26.9 h was observed (data not published). For this formulation the maximum plasma concentration of E_4 was 3490 pg/ml, reached at a t_{max} of 1.3 h. The present

Table 3 E_4 plasma trough levels (pg/ml).

Treatment cycle	*5 mg E_4/DRSP n = 17*	*10 mg E_4/DRSP n = 19*	*5 mg E_4/LNG n = 18*	*10 mg E_4/LNG n = 17*	*20 mg E_4/LNG n = 18*
Cycle 1	638 (445)	1361 (455)	510 (216)	1006 (395)	2268 (851)
Cycle 3	568 (331)	1366 (387)	527 (239)	880 (354)	2005 (793)

Values are mean (SD).

observations reveal an E_4 trough level of 2005 pg/ml in the third cycle for the same combination.

The present study compared the effects of 5 mg and 10 mg E_4 plus DRSP, and 5 mg, 10 mg and 20 mg E_4 plus 150 µg LNG, vs. 20 µg EE plus 3 mg DRSP, administered in a 24-day regimen during three cycles, on a series of liver function and endocrine parameters.

Compared with EE/DRSP, the E_4/DRSP and E_4/LNG combinations were associated with a significantly lower effect on SHBG. EE/DRSP raised SHBG levels over 300% compared with baseline. Similarly, E_4/DRSP and E_4/LNG had a limited effect on the other carrier proteins CBG and ceruloplasmin, in contrast to increases observed with EE/DRSP. This limited effect on SHBG in healthy young women is a confirmation of previous findings[11]. These findings suggest that E_4 practically does not stimulate the production of SHBG in human hepatocytes, and *in vivo* E_4 has limited influence on the SHBG plasma concentration or E_4 availability to target tissues. Moreover, it is considered to be of relevance, since a change in SHBG with a COC could be interpreted as a measure of total estrogenicity and used as a predictor of the risk of venous thromboembolism[23–25]. Although there is some debate regarding SHBG as a thrombotic marker[26,27], the European Medicines Agency recommends SHBG measurements for the estimation of thrombotic safety of a COC[28]. It was previously demonstrated that EE/DRSP use increases lipid peroxidation. The elevated levels of oxidised LDLs and lipid peroxides were correlated with the increase in plasma levels of copper induced by EE. The increase in plasma copper levels related to COC use is well known and has been attributed to the induction by estrogen of hepatic synthesis of the acute-phase protein ceruloplasmin, the main copper carrier protein[29–31]. It may, therefore, be hypothesised that the demonstrated low impact of E_4 on ceruloplasmin levels may also result in a lower impact of an E_4-containing COC on oxidative stress.

Both the E_4/DRSP and E_4/LNG combinations showed minor effects on lipid levels (HDL- and LDL-cholesterol). In comparison with EE/DRSP, the pooled E_4/DRSP group was associated with a non-significant increase in HDL- and LDL-cholesterol levels and, consequently, in total cholesterol. In accordance with data from the literature, the EE/DRSP combination increased the level of HDL-cholesterol and decreased the level of LDL-cholesterol, leading to an increased level of total cholesterol[32–37]. All E_4/LNG regimens reduced plasma triglyceride levels by approximately 30% (statistically significantly different from EE/DRSP), whereas the E_4/DRSP combinations non-significantly raised triglyceride levels by 10%. The impact of EE/DRSP on the rise in triglyceride levels was more pronounced (approximately 60%). Increased triglyceride levels are considered a marker for cardiometabolic diseases, and it has been suggested that long-term use of COCs might increase the risk of acute metabolic syndrome[38]. Although a retrospective cohort study failed to confirm this association, the lack of increase in triglyceride levels in women exposed to E_4-containing COCs might be beneficial[39].

A balance between bone resorption and bone formation maintains the regulation of bone mineral density. E_4 acts like a weak estrogen on several body systems, including liver function, but displays a comparable potency to that of EE on others such as bone turnover. This finding has been demonstrated in an osteoporosis rat model[12,40]. The present study did not detect any imbalances after treatment with E_4/DRSP, E_4/LNG or the comparator EE/DRSP in serum osteocalcin and C-telopeptide. Serum osteocalcin is produced almost exclusively by osteoblasts and is a sensitive marker of bone formation that correlates with histomorphometric measurements of bone formation in bone biopsy specimens[41,42]. C-telopeptide is a sensitive biomarker of bone degradation and turnover[43]. Previous short-term studies confirm the usefulness of these markers to document the effect of COC on bone metabolism. For example, serum osteocalcin levels were somewhat, but not significantly, lower during short-term (3 months) E_2 valerate/dienogest COC pill use in comparison with basal values[44]. Serum osteocalcin was unchanged in women receiving a contraceptive vaginal ring or dermal patch for 6 or 12 months[45]. The decreased bone turnover observed in the present study with the E_4/DRSP, E_4/LNG and EE/DRSP COCs is indicative of a similar positive influence on bone turnover in young post-adolescent women.

No clear effects on IGF growth parameters were observed in any of the E_4/DRSP or E_4/LNG groups, but a decrease in IGF-I was noted in the women who received EE/DRSP, similar to that reported for other COCs containing EE/dienogest or EE/LNG[46]. No relevant differences were observed in plasma concentrations of IGFBP-1 and IGFBP-3 following

E_4/DRSP or E_4/LNG. IGF-I is produced by the liver and excreted in the circulation, where it binds to IGFBP-1 and IGFBP-3. Because circulating IGF-I is mainly of hepatic derivation, its suppression by estrogen is probably a hepatocellular effect of the estrogen, whereas GH increase seems to be a consequence of IGF-I reduction[47]. As a result, hormonal contraceptives can modulate the GH/IGF-I axis during the reproductive years. The currently observed changes in levels of IGF-I and IGF-II, as well as in GH and IGFBP-1 and IGFBP-3 levels, with the EE/DRSP combination, are probably the consequence of the potent estrogenic action of EE on the hepatocytes. Hence, the negligible impact of E_4 on liver function does not result in significant changes in IGF-I plasma concentrations.

Strengths and weaknesses of the study

This study is part of an exploratory open-label, dose-finding phase II study (NTR2102/EudraCT 2009-011858-17) investigating the efficacy and safety of COCs containing the new estrogen E_4. In this initial report, the effects of two E_4/DRSP and three E_4/LNG combinations vs. 20 μg EE/3 mg DRSP were evaluated on ovulation inhibition, biomarkers of haemostasis and on liver function (present paper). The information gained from this initial programme is that E_4, in combination with DRSP or LNG, at all doses tested, blocked ovulation and dose-dependently reduced ovarian activity (I.J.M. Duijkers, unpublished data). In addition, E_4 had minor effects on haemostatic biomarkers (C. Kluft, unpublished data). Together with the findings in the present study (showing a limited effect on hepatic, lipid, bone and growth endocrine parameters), relevant original information has been obtained on contraceptive efficacy and safety of COCs containing E_4 as the estrogen component. Finally, the pharmacokinetic data demonstrate that most of the E_4 is bioavailable and is excreted in the urine as sulfate and glucuronide conjugates, in contrast to other estrogens, which are mainly excreted through the bile. This urinary excretion pattern may convey a significant advantage, since COCs containing EE significantly increase the incidence of gallbladder diseases[48].

These preliminary safety data require confirmation and further evaluation in a larger population of healthy women exposed for longer periods of time to various combinations of E_4/DRSP or E_4/LNG. Longer term and larger studies are necessary to select a definitive regimen that not only efficiently blocks ovulation and adequately inhibits ovarian function but also delivers excellent quality of life and a satisfactory vaginal spotting and bleeding pattern. More detailed and focused studies should then confirm the minimal impact of the selected regimen on carbohydrate, lipid and lipoprotein metabolism, on oxidative stress markers, coagulation and fibrinolysis markers, and on bone metabolism.

Differences in results and conclusions in relation to other studies

The present study confirms earlier findings of E_4 on liver cell metabolism and bone-sparing effects[12,15]. The effects of the EE/DRSP combination on bone turnover and bone mineral density have recently been investigated[49,50]. The positive influence of short-term EE/DRSP on bone turnover in young fertile women[49] was in line with the findings in the present study, which did not detect any imbalances. However, a decrease in bone formation was observed after EE/DRSP administration during six consecutive cycles[50].

Relevance of the findings: Implications for clinicians

According to preclinical and phase I clinical research, E_4 seems suitable to replace EE in COCs[12,15]. This has been confirmed in healthy young women, where suppression of ovarian activity and inhibition of ovulation have been demonstrated (I.J.M. Duijkers, unpublished data). Also, based on the results of the present study, E_4-containing COCs appear to interfere less with lipid metabolism and coagulation parameters while maintaining adequate bone protection. The available data will assist in selecting the optimal doses of E_4 and progestogen for further evaluation.

Unanswered questions and future research

So far, there is substantial information on the pharmacological, pharmacokinetic and ovulation inhibition activities of E_4[12]. However, current knowledge on the bleeding pattern is minimal due to the limited number of subjects treated. Future trials are needed to provide information on the bleeding pattern of the E_4 COC to be selected.

In a recent review of the pharmacological profile of estrogens in COCs, it was stated that new estrogens

such as E_4 differ from EE with regard to their pharmacological properties and target organs[3]. These differences hold promise for the safety and tolerability of future COCs.

CONCLUSION

The present study shows that, compared with the EE/DRSP combination, both E_4/DRSP and E_4/LNG have a limited effect on liver function, lipid metabolism, and bone and growth endocrine parameters.

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MM, CM and J-MF are employees of Estetra SPRL; CK is an employee of the Contract Organisation, Dinox BV, which performed the study; YZ and HCB are employees of Pantarhei Bioscience BV.

REFERENCES

1. United Nations. World contraceptive use 2011. Available from: www.unorg/esa/population/publications/contraceptiv$E_2$011/wallchart_frontpdf.
2. Westhoff CL, Heartwell S, Edwards S, *et al.* Oral contraceptive discontinuation: Do side effects matter? *Am J Obstet Gynecol* 2007;196:412.
3. Bitzer J. Pharmacological profile of estrogens in oral contraception. *Minerva Ginecol* 2011;63:299–304.
4. Cirillo DJ, Wallace RB, Rodabough RJ, *et al.* Effect of estrogen therapy on gallbladder disease. *JAMA* 2005;293:330–9.
5. Kiley J, Hammond C. Combined oral contraceptives: A comprehensive review. *Clin Obstet Gynecol* 2007;50: 868–77.
6. Stegeman BH, de Bastos M, Rosendaal FR, *et al.* Different combined oral contraceptives and the risk of venous thrombosis: Systematic review and network meta-analysis. *BMJ* 2013;347:f5298.
7. Rosenberg MJ, Meyers A, Roy V. Efficacy, cycle control, and side effects of low- and lower-dose oral contraceptives: A randomized trial of 20 micrograms and 35 micrograms estrogen preparations. *Contraception* 1999; 60:321–9.
8. Hoffmann H, Moore C, Zimmermann H, *et al.* Approaches to the replacement of ethinylestradiol by natural 17beta-estradiol in combined oral contraceptives. *Exp Toxicol Pathol* 1998;50:458–64.
9. Holinka CF, Diczfalusy E, Coelingh Bennink HJ. Estetrol: A unique steroid in human pregnancy. *J Steroid Biochem Mol Biol* 2008;110:138–43.
10. Coelingh Bennink F, Holinka CF, Visser M, *et al.* Maternal and fetal estetrol levels during pregnancy. *Climacteric.* 2008;11(Suppl.1):69–72.
11. Hammond GL, Hogeveen KN, Visser M, *et al.* Estetrol does not bind sex hormone binding globulin or increase its production by human HepG2 cells. *Climacteric* 2008;11(Suppl.1):41–6.
12. Visser M, Coelingh Bennink HJ. Clinical applications for estetrol. *J Steroid Biochem Mol Biol* 2009;114:85–9.
13. Abot A, Fontaine C, Buscato M, *et al.* The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation. *EMBO Mol Med* 2014;6:1328–46.
14. 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 U S A* 2014;111:$E_2$83–90.
15. Coelingh Bennink HJ, Holinka CF, Diczfalusy E. Estetrol review: profile and potential clinical applications. *Climacteric* 2008;11(Suppl.1):47–58.
16. Coelingh Bennink HJ, Skouby S, Bouchard P, *et al.* Ovulation inhibition by estetrol in an in vivo model. *Contraception* 2008;77:186–90.
17. World Health Organization. Combined hormonal contraceptives (CHCs). In: *Medical eligibility criteria for contraceptive use*, 4th edn. Geneva: WHO 2010:15–44.
18. Rikken B, van Doorn J, Ringeling A, et al. Plasma levels of insulin-like growth factor (IGF)-I, IGF-II and IGF-binding protein-3 in the evaluation of childhood growth hormone deficiency. *Horm Res* 1998;50:166–76.
19. de Boer L, Hoogerbrugge CM, van Doorn J, *et al.* Plasma insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), acid-labile subunit (ALS) and IGFBP-3 proteolysis in individuals with clinical characteristics of Sotos syndrome. *J Pediatr Endocrinol Metab* 2004;17:615–27.

20. de Vries BB, Robinson H, Stolte-Dijkstra I, *et al.* General overgrowth in the fragile X syndrome: Variability in the phenotypic expression of the FMR1 gene mutation. *J Med Genet* 1995;32:764–9.
21. Gyulikhandanova NE, Tsymbalenko NV, Platonova NA, *et al.* Regulation of ceruloplasmin gene in mammals. *Bull Exp Biol Med* 2004;137:485–9.
22. De Groote D, Perrier d'Hauterive S, Pintiaux A, *et al.* Effects of oral contraception with ethinylestradiol and drospirenone on oxidative stress in women 18–35 years old. *Contraception* 2009;80:187–93.
23. Odlind V, Milsom I, Persson I, *et al.* Can changes in sex hormone binding globulin predict the risk of venous thromboembolism with combined oral contraceptive pills? *Acta Obstet Gynecol Scand* 2002;81:482–90.
24. Raps M, Helmerhorst F, Fleischer K, *et al.* Sex hormone-binding globulin as a marker for the thrombotic risk of hormonal contraceptives. *J Thromb Haemost* 2012;10:992–7.
25. Stegeman BH, Helmerhorst FM, Vos HL, *et al.* Sex hormone-binding globulin levels are not causally related to venous thrombosis risk in women not using hormonal contraceptives. *J Thromb Haemost* 2012;10: 2061–7.
26. Kluft C, Skouby SO, Jespersen J, *et al.* Sex hormone-binding globulin as a marker for the thrombotic risk of hormonal contraceptives: A rebuttal. *J Thromb Haemost* 2013;11:394–5.
27. Raps M, Helmerhorst FM, Fleischer K, *et al.* Sex hormone-binding globulin as a marker for the thrombotic risk of hormonal contraceptives: reply to a rebuttal. *J Thromb Haemost* 2013;11:396–7.
28. Committee for Medical Products for Human Use (CPMP). *Guideline on clinical investigation of steroid contraceptives in women.* London: European Medicines Agency 2005.
29. Akhter S, Shamsuzzaman AK, Banarjee M, *et al.* Serum copper in rural women taking combined oral contraceptive. *Mymensingh Med J* 2006;15:25–9.
30. Berg G, Kohlmeier L, Brenner H. Effect of oral contraceptive progestins on serum copper concentration. *Eur J Clin Nutr* 1998;52:711–5.
31. Benes B, Spevackova V, Smid J, *et al.* Effects of age, BMI, smoking and contraception on levels of Cu, Se and Zn in the blood of the population in the Czech Republic. *Cent Eur J Public Health* 2005;13:202–7.
32. Agren UM, Anttila M, Maenpaa-Liukko K, *et al.* Effects of a monophasic combined oral contraceptive containing nomegestrol acetate and 17beta-oestradiol compared with one containing levonorgestrel and ethinylestradiol on haemostasis, lipids and carbohydrate metabolism. *Eur J Contracept Reprod Health Care* 2011;16:444–57.
33. Sitruk-Ware R. Pharmacology of different progestogens: The special case of drospirenone. *Climacteric* 2005;8(Suppl. 3):4–12.
34. Endrikat J, Klipping C, Cronin M, *et al.* An open label, comparative study of the effects of a dose-reduced oral contraceptive containing 20 microg ethinyl estradiol and 100 microg levonorgestrel on hemostatic, lipids, and carbohydrate metabolism variables. *Contraception* 2002;65: 215–21.
35. Scharnagl H, Petersen G, Nauck M, *et al.* Double-blind, randomized study comparing the effects of two monophasic oral contraceptives containing ethinylestradiol (20 microg or 30 microg) and levonorgestrel (100 microg or 150 microg) on lipoprotein metabolism. *Contraception* 2004;69:105–13.
36. Skouby SO, Endrikat J, Dusterberg B, *et al.* A 1-year randomized study to evaluate the effects of a dose reduction in oral contraceptives on lipids and carbohydrate metabolism: 20 microg ethinyl estradiol combined with 100 microg levonorgestrel. *Contraception* 2005;71:111–7.
37. Tuppurainen M, Klimscheffskij R, Venhola M, *et al.* The combined contraceptive vaginal ring (NuvaRing) and lipid metabolism: A comparative study. *Contraception.* 2004;69:389–94.
38. Josse AR, Garcia-Bailo B, Fischer K, *et al.* Novel effects of hormonal contraceptive use on the plasma proteome. *PLoS One.* 2012;7:e45162.
39. Hurwitz BE, Henry N, Goldberg RB. Long-term oral contraceptive treatment, metabolic syndrome and measures of cardiovascular risk in pre-menopausal women: National Health and Nutrition Examination Survey 1999–2004. *Gynecol Endocrinol* 2009;25:441–9.
40. Coelingh Bennink HJ, Heegaard AM, Visser M, *et al.* Oral bioavailability and bone-sparing effects of estetrol in an osteoporosis model. *Climacteric* 2008;11(Suppl. 1): 2–14.
41. Delmas PD. Biochemical markers of bone turnover for the clinical assessment of metabolic bone disease. *Endocrinol Metab Clin North Am* 1990;19:1–18.
42. Robins SP. Biochemical markers of bone metabolism. *CPD Bull Clin Biochem* 1999;1:116–21.
43. Rosen HN, Moses AC, Garber J, *et al.* Serum CTX: A new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. *Calcif Tissue Int* 2000;66:100–3.
44. Di Carlo C, Gargano V, Sparice S, *et al.* Short-term effects of an oral contraceptive containing oestradiol valerate and dienogest on bone metabolism and bone mineral density: An observational, preliminary study. *Eur J Contracept Reprod Health Care* 2013;18: 388–93.

45. Massaro M, Di Carlo C, Gargano V, et al. Effects of the contraceptive patch and the vaginal ring on bone metabolism and bone mineral density: A prospective, controlled, randomized study. *Contraception* 2010; 81:209–14.
46. Balogh A, Kauf E, Vollanth R, *et al.* Effects of two oral contraceptives on plasma levels of insulin-like growth factor I (IGF-I) and growth hormone (hGH). *Contraception* 2000;62:259–69.
47. Weissberger AJ, Ho KK, Lazarus L. Contrasting effects of oral and transdermal routes of estrogen replacement therapy on 24 h growth hormone (GH) secretion, insulin-like growth factor I, and GH-binding protein in postmenopausal women. *J Clin Endocrinol Metab* 1991;72:374–81.
48. Thijs C, Knipschild P. Oral contraceptives and the risk of gallbladder disease: A meta-analysis. *Am J Public Health* 1993;83:1113–20.
49. Gargano V, Massaro M, Morra I, *et al.* Effects of two low-dose combined oral contraceptives containing drospirenone on bone turnover and bone mineral density in young fertile women: A prospective controlled randomized study. *Contraception* 2008;78:10–5.
50. Paoletti AM, Orru M, Lello S, *et al.* Short-term variations in bone remodeling markers of an oral contraception formulation containing 3 mg of drospirenone plus 30 microg of ethinyl estradiol: Observational study in young postadolescent women. *Contraception* 2004;70:293–8.

3.3. Discussion

3.3.1. Carrier proteins

The results demonstrate the much lower estrogenicity of the E4-containing COCs in comparison to the marketed EE/DRSP COC (YAZ®). When comparing the E4/DRSP groups to the EE/DRSP COC, we observed that the E4 combinations displayed a much lower impact on the carrier proteins SHBG, CBG and Ceruloplasmin. In accordance with the anti-androgenic properties of LNG, resulting in a further decrease in the total estrogenicity, the E4/LNG combinations were even associated with a decrease in carrier proteins levels. It is to our knowledge the first time that a decrease in carrier proteins levels was reported with a combined hormonal preparation. If we accept the widespread hypothesis that links total estrogenicity to VTE risk, we may conclude that E4 will allow for a safer profile in terms of VTE risk, even when it is combined with DRSP, an anti-androgenic progestin.
In this regard, the E4/LNG combination would appear superior to that of E4/DRSP. However, comparison of the E4/LNG versus E4/DRSP on hemostasis parameters failed to demonstrate significant differences of one combination versus the other (data not shown).

3.3.2. Lipid profile

This estrogenicity analysis may also explain the differences in the lipids seen in our study. The high estrogenicity of the comparator EE/DRSP led to assumed protective changes in the lipid profile (higher HDL/LDL ratio) while the low estrogenicity of the E4/LNG combinations were associated with a worsening of the lipid parameters (lower HDL/LDL ratio). The E4/DRSP combinations seemed intermediate and neutral, since it kept the HDL/LDL ratio rather unchanged. Impact on triglycerides plasma levels was drastically lower with E4 than EE, confirming the data generated in phase 1 studies (reported in Section 7.11.7).

3.3.3. Bone turnover

Bone mineral density assessment suggests that COC use during adolescence may impair bone peak acquisition, although, the data are conflicting regarding the relationship between BMD and the real fracture risk (Herrmann and Seibel 2010). Long epidemiological studies are necessary to accurately evaluate the exact fracture risk associated with COC use because, first, changes in BMD are slow and secondly, the main fracture risk appears after onset of menopause: only a study evaluating the incidence of fracture in post-menopausal women who used COC during their adolescence could answer this question. Currently the analysis of bone-turnover biomarkers is considered a useful tool at short notice in small clinical settings. However, the link between bone turnover biomarkers change and facture risk is still questioned. Almost all studies suggest that the use of COC induces a significant reduction in the markers of bone turnover in comparison to non-users. Our results are in line with this. However, although the sample size of our study is certainly too limited to draw definitive conclusion, it

seems that the decrease in bone turnover biomarkers was less pronounced with E4 than with EE.

3.3.4. Growth endocrinology

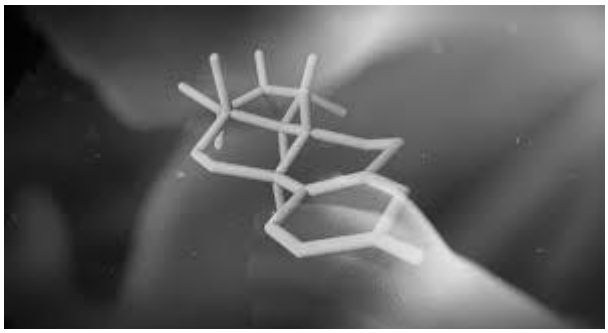
Regarding the impact on growth endocrinology, in accordance with previously published articles, the EE-containing COC increased the GH level while it decreased the IGF-1 level. IGFBP-1 was also increased probably in line with the increase in GH level. The E4-containing combinations all increased the GH production and all but one, decreased IGF-1 level but to a lower extent than what is seen with the potent EE. This lower impact of E4 on the production of IGF-1 is probably linked to the lower hepatic impact of this estrogen. This could have positive clinical consequence since some studies have demonstrated a significant correlation between IGF-1 and bone density (Elkazaz and Salama 2015).

3.3.5. Pharmacokinetics

In accordance with the data generated during the phase 1 studies (see Sections 7.10.2 and 7.11.2), the serum E4 concentrations were dose-proportional. They appear to be influenced by the progestin compound with, for the same E4 dose administered, a lower E4 concentration when it was associated to LNG than to DRSP. However, due to the quite large inter-subject variability, no absolute conclusion may be drawn on a possible progestin-related difference in E4 concentrations. In any case, the PK data gathered during our study are reassuring and suggest that the use of E4 in a COC is safe since the E4 concentration with a daily dose of 20 mg E4 is more than 4 times lower than the mean E4 concentration to which fetuses at term are exposed to (F. Coelingh Bennink, et al. 2008). In the course of the Estelle development program, additional PK studies were performed including mass balance studies and single dose and multiple dose PK data were obtained (data not shown).

3.4. Conclusion

In conclusion, we analyzed during this first phase 2 study additional parameters known to affect the safety of COCs. The results suggest that the low estrogenicity of E4-containing COC allows for a probably safer use of DRSP than what is currently available with EE/DRSP. These results encouraged us not to exclude DRSP from the next step of the dose-finding program, namely, a second larger phase 2 trial of which the results have been described in two publications reported below.



4th Publication

Bleeding pattern and cycle control with estetrol-containing combined oral contraceptives: results from a phase II, randomized, dose-finding study (FIESTA)

Contraception, 2016; 94: 366 – 373

Apter D, Zimmerman Y, Beekman L, Mawet M, Maillard C, Foidart JM, Coelingh Bennink HJ

4. Fourth publication

Bleeding pattern and cycle control with estetrol-containing combined oral contraceptives: results from a phase II, randomized, dose-finding study (FIESTA)

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4.1. Introduction

The second phase 2 study was entitled: *"A randomized, open-label, multicenter, dose-finding study to evaluate cycle control of 15 mg or 20 mg estetrol combined with either 150 mcg levonorgestrel or 3 mg drospirenone, compared to a combined oral contraceptive containing estradiol valerate and dienogest"***.** The study was conducted in 10 centers located in Finland. The very good safety profile of E4 observed during the first trial allowed to treat a larger population (311 subjects treated with an E4-containing COC) for a longer period (6 cycles) than what was done in the first trial.

To participate, the subjects had to be between 18 and 35 years of age, be healthy with no contra-indications for COC use and give their written informed consent. The same 24/4 day therapeutic regimen was used for the E4-containing COCs.

The choice of the E4-containing combinations to be tested was based on the data generated in the first study: the same progestins as previously evaluated, namely 3 mg DRSP and 150 mcg LNG, were studied in this trial but they were this time combined with doses of 15 mg and 20 mg E4 as it was shown that a dose above 10 mg E4 achieves a higher ovarian function inhibition.

The primary objective of this second phase 2 trial was to evaluate the bleeding profile associated with the different combinations tested. The results were compared to a commercialized COC containing E2V, a prodrug of the naturally occurring estrogen E2, in association with DNG as progestin. This commercialized COC is called Qlaira® in Europe and Natazia® in the USA. It is administered following a 26/2 day regimen (26 active pill intake followed by 2 days of placebo intake). It is a quadriphasic COC, meaning that the doses of E2V and DNG vary 4 times during the treatment cycle. This comparator was chosen as it was, at the time of the conduct of our study, the only COC on the market incorporating a "natural" estrogen, like E4. Therefore, it was relevant to compare both natural estrogenic compounds, since the primary endpoint of this trial, *i.e* the bleeding pattern, is primarily influenced by the estrogenic component. While low incidence of irregular spotting/bleeding is reported in users of EE containing COCs, in clinical practice, more frequent episodes of irregular spotting/bleeding are reported in E2 containing COC users.

Evaluation of the bleeding pattern was selected as primary objective since it is a main topic of concern for many COC users and physicians. The advent of the pill has radically changed women's quality of life, not only because it offers a reliable and easy contraceptive method, but also because it improves several menstruating issues like irregular menstruation, frequent menstruation, and heavy or prolonged bleeding (De Leo, et al. 2016). In the opposite,

occurrence of bleeding issues with a contraceptive method is one of the main reasons why women are switched to another method: for example, LNG-intra uterine systems are regularly removed in the first months of use due to the frequent intermenstrual spotting episodes in the beginning of the treatment. Another example is the need to remove a copper intrauterine device for inacceptable increased menstrual bleeding. The importance of the bleeding pattern associated with a contraceptive method is well-known from the health authorities and, consequently, data on the bleeding pattern are requested by EMA when submitting a dossier for a new contraceptive method (EMA 2006).

The goal of the study was to find out an E4-combination associated with adequate compliance (at most 20 % of intermenstrual breakthrough bleeding and at most 20 % of absence of withdrawal bleeding).

To evaluate the bleeding pattern during our study, every day a bleeding was experienced, the participants were asked to complete a diary to characterize this bleeding in either “spotting” (evidence of minimal vaginal blood loss that did not require new use of any type of sanitary protection, including panty liners) or “bleeding” (evidence of vaginal blood loss that required the use of sanitary protection with a tampon, pad, or panty liner). The definitions of spotting and bleeding used in this study are those recommended by Mishell et al. in an article defining the way the bleeding pattern of a hormonal contraceptive should be evaluated and reported (Mishell, et al. 2007). This article aims at standardizing the reporting of bleeding pattern across the different hormonal contraceptives in order to facilitate the comparison between methods or molecules. These recommendations, even if they are not yet included in the official guidelines from EMA, were regularly cited or requested by the health authorities during the scientific advices conducted for the Estelle project.

In this publication, we also report an overview of the adverse events recorded during the study.

Finally, the absence of ovulation detected during the preceding study allowed for a safe use of the E4-combinations as unique contraceptive method. This study was consequently also the occasion to confirm the contraceptive efficacy of the E4-containing COCs in conditions close to real life.

4.2. Article

ELSEVIER

Contraception 94 (2016) 366 – 373

Contraception

Original research article

Bleeding pattern and cycle control with estetrol-containing combined oral contraceptives: results from a phase II, randomised, dose-finding study (FIESTA)☆

Dan Apter[a], Yvette Zimmerman[b], Louise Beekman[b], Marie Mawet[c,*], Catherine Maillard[c], Jean-Michel Foidart[c,d,1], Herjan J.T. Coelingh Bennink[b,1]

[a]*Sexual Health Clinic (Väestöliitto), 00100 Helsinki, Finland*
[b]*Pantarhei Bioscience BV, 3700 AL Zeist, the Netherlands*
[c]*Estetra SPRL, 4000 Liège, Belgium*
[d]*University of Liège, 4000 Liège, Belgium*

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Abstract

Objectives: This study aims to assess vaginal bleeding patterns and cycle control of oral contraceptives containing estetrol (E4) combined with either drospirenone (DRSP) or levonorgestrel (LNG).
Study design: An open-label, multicentre, randomised, dose-finding study lasting six cycles in healthy women aged 18–35 years was used. Four treatments (15 mg or 20 mg E4, combined with either 3 mg DRSP or 150 mcg LNG) were administered in a 24/4-day regimen. A marketed dosing regimen of estradiol valerate with dienogest (E2V/DNG) served as reference since it contains (like E4) a natural oestrogen.
Results: A total of 396 women were randomised, of whom 389 received study medication, and 316 completed the study. By cycle 6, the frequencies of unscheduled bleeding and/or spotting and absence of withdrawal bleeding were the lowest in the 15 mg E4/DRSP group (33.8% and 3.5%, respectively). In the E2V/DNG reference group, these frequencies were 47.8% and 27.1%, respectively. By cycle 6, the frequency of women with absence of withdrawal bleeding was <20% for all E4 treatment groups: 3.5–3.8% combined with DRSP and 14.0–18.5% combined with LNG. By cycle 6, unscheduled intracyclic bleeding was reported by <20% of women in the 20 mg E4/LNG group (18.9%) and in the 15 mg E4/DRSP group (16.9%).
Conclusion: This study showed that, of the four treatment modalities investigated, the 15 mg E4/DRSP combination has the most favourable bleeding pattern and cycle control.
Implications: Due to its favourable bleeding pattern and cycle control, the 15 mg E4/DRSP combination is the preferred combination for further phase III clinical development.

Keywords: Estetrol; Estradiol valerate; Dienogest; Bleeding pattern; Cycle control

1. Introduction

The bleeding pattern of combined oral contraceptives (COCs) containing ethinyl estradiol (EE) is perceived as satisfactory by women, which may be due to the effective stabilisation of the endometrium, induced by its potent oestrogenic activity. However, EE causes subjective side effects [1], and it affects the synthesis of various liver proteins leading to an increased risk of cardiovascular complications [2–4]. Strategies have been developed to lower the EE dose or substitute EE with another oestrogen

☆ Declaration of interest: The authors alone are responsible for the content and writing of the paper. MM and CM are employees of Estetra SPRL (a subsidiary of Mithra Pharmaceuticals); J-MF and HCB are Strategic Scientific Advisors at Mithra Pharmaceuticals. YZ and HCB are employees, and LB is a former employee, of Pantarhei Bioscience BV. DA does not report any conflicts of interest.

* Corresponding author. Tel.: +32-4-349-2822; fax: +32-4-349-2821.
E-mail address: mmawet@mithra.com (M. Mawet).

[1] Jean-Michel Foidart and Herjan J.T. Coelingh Bennink contributed equally to this study.

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[5,6]. Original attempts at replacing EE by estradiol (E2) were associated with poor bleeding patterns, and therefore, the development was stopped [7]. Combinations of E2 with nomegestrol acetate (NOMAC/E2) or estradiol valerate with dienogest (E2V/DNG) were subsequently developed and led to acceptable cycle control [7–10] but were still suboptimal since absence of withdrawal bleeding was reported to be 31% and 20%, respectively [7,9].

The development of new oestrogens such as estetrol (E4) holds promise for the safety and tolerability of future COCs [2]. E4 is synthesised by the human foetal liver and is present only during human pregnancy [11]. In contrast to other oestrogens, E4 is an antagonist of the membrane oestrogen receptor alpha, it does not bind to the carrier protein sex-hormone binding globulin SHBG and it does not change the activity of relevant cytochrome P450 related liver enzymes [12,13]. The terminal half-life of E4 is 28 h versus 14 h for E2 [14], an important prerequisite for its development as a once daily oral drug [15].

Preclinical and phase I clinical research suggests that E4 inhibits ovulation and may be a suitable replacement for EE in COCs [16–18]. In a phase II, dose-finding study, 5 mg, 10 mg or 20 mg E4 in combination with drospirenone (DRSP) or levonorgestrel (LNG) completely inhibited ovulation and decreased ovarian activity dose-dependently [19]. These combinations also had a limited effect on liver function, lipid metabolism and bone and growth endocrine parameters [20].

The present phase II, randomised, dose-finding study was conducted with the aim of selecting an E4/progestin combination for phase III development. The primary objective was to investigate the effect of two dosages of E4 combined with either DRSP or LNG on vaginal bleeding patterns and cycle control, using a COC containing E2V/DNG as a reference.

2. Methods

2.1. Design and ethical approval

This was an open-label, multicentre, randomised, dose-finding study in healthy female volunteers of reproductive age. The study was conducted between September 2010 and 2011 in 10 centres in Finland (ClinicalTrials.gov identifier NCT01221831). Approval was obtained by the regional independent ethics committee of the Hospital District of Helsinki and Uusimaa and by the Finnish Medicines Agency. The study was conducted in accordance with the ethical principles established by the Declaration of Helsinki and the International Conference on Harmonisation — Good Clinical Practise Guidelines. Written informed consent was obtained from all participants before enrolment.

2.2. Participants

Healthy women, aged 18–35 years with a body mass index between 18 and 30 kg/m^2 and a regular menstrual cycle (24–35 days), were eligible for inclusion. Women who were already using hormonal contraceptives (switchers) and hormonal contraceptive-naïve women (starters) were able to participate. Switchers changed from a COC, vaginal ring or transdermal patch. Women were defined as starters when they had not used a hormonal contraceptive in the 3 months prior to randomisation. Women who used any hormonal contraceptive method within 3 months prior to randomisation, but not at screening (for starters), or women using a depot progestogen within 6 months prior to randomisation were excluded. Untreated chlamydia infection also led to exclusion. The other exclusion criteria were in line with the World Health Organisation's medical eligibility criteria for COC use [21] and included contraindications for contraceptive steroids (e.g., a history of, or existing thromboembolic, cardiovascular or cerebrovascular disorder or hypertension, defined as systolic and diastolic blood pressure >140 and >90 mmHg, respectively).

2.3. Treatment

There were five treatment groups: (1) 15 mg E4 plus 3 mg DRSP (15E4/DRSP), (2) 20 mg E4 plus 3 mg DRSP (20E4/DRSP), (3) 15 mg E4 plus 150 mcg LNG (15E4/LNG), (4) 20 mg E4 plus 150 mcg LNG (20E4/LNG) and (5) E2V plus DNG (E2V/DNG) (commercial packaging of 4-phasic Qlaira®, Bayer HealthCare, Germany). E2V/DNG was chosen as a reference because it is the only global COC containing a natural oestrogen (E2V), like E4.

To achieve equal distribution across the groups, randomisation was stratified by switchers, starters and sites. After randomisation, switchers completed their last pill blister (or completed the vaginal ring or patch treatment cycle) before starting study treatment. Starters took their first study medication on the first day of the first menstruation occurring after randomisation. Participants completed six treatment cycles of 28 days. For the E4 groups, one cycle comprised of 24-study medication days, followed by 4 placebo days. Women assigned to E2V/DNG took active study treatment for 26 days followed by 2 placebo days, according to the labelling of Qlaira®.

2.4. Assessments

Study visits were scheduled at randomisation, on days 1–14 of treatment cycles 2, 3, 4 and 5 and between days 25 and 28 of cycle 6 (final study visit). Women completed a daily diary to monitor vaginal bleeding. The intensity of vaginal bleeding was evaluated based on the number of sanitary protections needed (0, 1 or ≥2).

Safety was evaluated by recording treatment-emergent adverse events (TE-AEs), standard laboratory safety results, physical and gynaecological examination and vital signs. Of drug-related TE-AEs, headache/migraine and anxiety/depression were considered of special interest.

A pregnancy test (urinary β-hCG) was performed at randomisation and at monthly visits. Ovulation inhibition was assessed during cycles 1–4 by urinary pregnanediol glucuronide measurements [22].

2.5. Outcome variables

The primary aim of the study was to assess vaginal bleeding patterns and cycle control of 15 and 20 mg E4 combined with either DRSP or LNG, administered during six treatment cycles in a 24/4-day regimen.

The purpose of the study was to find a dosing combination with not more than 20% absence of withdrawal bleeding and not more than 20% unscheduled intracyclic bleeding in cycle 6. The optimal E4/progestin combination will be selected for further phase III clinical development.

Cycle control was evaluated on the basis of daily vaginal bleeding patterns. The primary bleeding parameters were unscheduled bleeding and spotting combined (referred to as bleeding/spotting) and absence of withdrawal bleeding. Secondary bleeding parameters comprised a bleeding/spotting cycle pattern by cycle day and early withdrawal bleeding (i.e., occurring between days 21 and 24). Bleeding or spotting reported during cycle days 5–24 was considered unscheduled. If bleeding or spotting did not occur on cycle days 1 and 2, but occurred on cycle day 3 or 4, it was also considered unscheduled (except for days 1–7 of cycle 1 [23]).

2.6. Statistical analyses

Data analyses were descriptive; 2-sided 95% confidence intervals (CIs) were calculated per treatment group and cycle for the primary and secondary bleeding endpoints versus the E2V/DNG reference group. The analyses were performed for both the intent-to-treat (ITT) and the per-protocol (PP) populations for cycles 2, 3 and 6. A *t* test was performed to determine whether there are significant differences in the mean number of unscheduled bleeding/spotting days between E4 treatment groups and the E2V/DNG group. The ITT population comprised all-subjects-treated (AST) with at least one evaluable cycle, and PP population comprised all ITT subjects without any major protocol violation. Since the aim of the present study was to select an E4/progestin combination for phase III development, the present paper focussed on the outcome of primary and secondary bleeding parameters in the PP population. The safety analysis was performed for the AST population, and only tabulations and frequencies are presented.

Since this was an exploratory study, the sample size was based on the precision of the estimates in the treatment groups. When the frequencies of absence of withdrawal bleeding and of unscheduled intracyclic bleeding are 15–20% in a group, a size of 80 evaluable subjects per group leads to standard errors of 4–4.5%.

3. Results

3.1. Study population

A total of 396 women were randomised (Fig. 1), of whom 389 (98.2%) received study medication, and 316 completed the study (79.8%). The number of completers was highest for 15E4/DRSP (72/79; 91.1%) and E2V/DNG (70/78; 89.7%) and was lowest for 20E4/LNG (54/77; 70.1%) (Fig. 1). TE-AEs were the most common reason for discontinuing the study (41/80; 51.3%). Seven women who had been randomised withdrew from the study before receiving treatment.

The proportion of switchers (66.8% overall) and starters (33.2% overall) was generally similar across treatment groups (Table 1). Of the 41 women who discontinued due to AEs, 27 (65.9%) were switchers and 14 (34.1%) were starters. The completion rate was similar among starters, but among switchers, it was highest for E2V/DNG and 15E4/DRSP (97.4% and 96.2%, respectively) (data not shown).

Demographic and baseline characteristics were similar across the treatment groups, with the exception of the proportion of smokers, which varied between 10.7% for 20E4/DRSP and 24.7% for 20E4/LNG (Table 1).

3.2. Primary bleeding parameters

The frequency of unscheduled bleeding/spotting was lower in the E4/DRSP groups across treatment cycles 2, 3 and 6, compared to the other treatment groups. By cycle 6, the frequency varied between 33.8% in the 15E4/DRSP group and 47.8% in the E2V/DNG group (Table 2 and Fig. 2). The maximum intensity of unscheduled bleeding ($\geq$2 sanitary protections needed) per cycle increased over time in the E2V/DNG group (up to 60%) and stayed the same with minor fluctuations in the 15 mg E4/LNG and 15 mg E4/DRSP groups (25–35%) (data not shown).

For both switchers and starters, the incidence of unscheduled bleeding/spotting generally decreased with time in all groups. By cycle 6, the frequency of unscheduled bleeding/spotting varied in switchers between 29.3% for 15E4/DRSP and 48.7% for 20E4/DRSP, and in starters, it varied between 38.5% for 20E4/LNG and 66.7% for E2V/DNG (Fig. 2).

The frequency of women with absence of withdrawal bleeding was <20% for all E4 treatment groups throughout the study. For E4/DRSP, it was absent for 1.3–1.5% in cycle 2, 1.5–2.8% in cycle 3 and 3.5–3.8% in cycle 6. In the E4/LNG groups, these frequencies were 2.9–9.9%, 10.8–13.6% and 14.0–18.5%, respectively, and for E2V/DNG, these were 12.1%, 16.4% and 27.1%, respectively. By cycle 6, this resulted in a difference of 23.6% fewer subjects with an absence of withdrawal bleeding in the 15E4/DRSP group than in the E2V/DNG group (95% CI: −35.9, −11.3) (Table 2 and Fig. 3).

In starters, none or only one single subject in any treatment group had an absence of withdrawal bleeding in cycle 2 or 3. By cycle 6, this remained the same in the E4 treatment groups but increased to 22.2% for E2V/DNG. For switchers, the pattern was similar to the overall population, showing that the lowest incidence of absence of withdrawal bleeding was observed in the E4/DRSP groups in any cycle (data not shown).

3.3. Secondary bleeding parameters

Throughout the study, the frequency of unscheduled intracyclic bleeding was highest for E2V/DNG (35.8–45.5%)

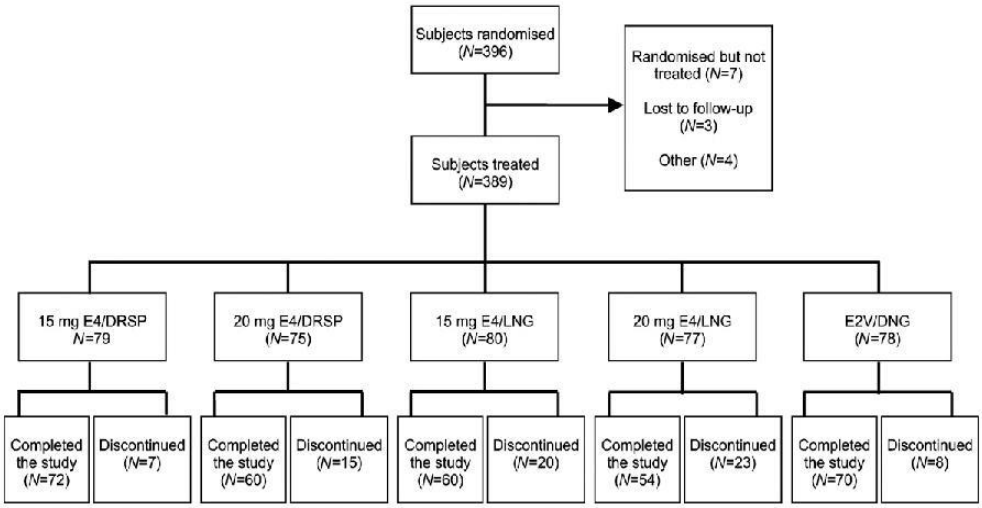

Fig. 1. Subject disposition by treatment group (all-subjects-randomised population). Subject disposition in a study evaluating the bleeding pattern and cycle control of four contraceptive combinations containing E4 (15 and 20 mg E4 combined with either 3 mg DRSP or 150 mcg LNG) and a marketed quadriphasic combination containing E2V/DNG. The E4 combinations were administrated in a 24/4-day regimen while the E2V/DNG combination was administered in a 26/2-day regimen. More women receiving the 15 mg E4/DRSP combination or the E2V/DNG combination completed the study in comparison to the other groups. DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel.

and lowest in the 15E4/DRSP group (16.9–30.6%). By cycle 6, the reported frequency of unscheduled bleeding was <20% for 20E4/LNG (18.9%) and 15E4/DRSP (16.9%) (Table 2 and Fig. 3).

Unscheduled bleeding/spotting on individual days during cycle 1 was reported by 12–34% of women in the E4/DRSP groups and by 16–46% of women in the other groups. The daily frequency of unscheduled bleeding/spotting decreased notably by cycle 2 and remained lower in subsequent cycles in all groups. By cycle 6, the occurrence of unscheduled bleeding/spotting on any cycle day was 3–8% for 15E4/DRSP, 9–19% for the other E4 groups and 10–27% for E2V/DNG (Fig. 4). As shown in Fig. 5, the mean number of days with unscheduled bleeding/spotting was statistically significantly lower in the 15 mg E4/DRSP group in comparison to the E2V/DNG group at cycles 2, 3 and 6 (at cycle 1, there was no statistical difference between the five groups — data not shown). By cycle 6, the mean number of days with unscheduled bleeding/spotting varied between 1.3 in the 15E4/DRSP group and 2.9 in the E2V/DNG group ($p = .008$) (Fig. 5).

Early withdrawal bleeding (between cycle days 21 and 24) was variable across the cycles. The incidence decreased or remained stable over time and, by cycle 6, it was lowest for 15E4/DRSP (20.0%) and 20E4/LNG (17.0%). The incidence in the E2V/DNG reference group was 23.9% by cycle 6 (data not shown).

3.4. Safety parameters and vital signs

The frequency of TE-AEs was 64.6% for 15E4/DRSP and varied between 71.8% and 80.0% in the other groups (Table 3). The majority of women had mild or moderate TE-AEs. Between 23.1% and 45.5% of women reported a drug-related TE-AE in the various groups. The incidence of headache/migraine was 2.5–9.3%, and of anxiety/depression, it was 0%–6.5% across groups. One SAE was reported: thyroid neoplasm in the E2V/DNG group, considered by the investigator as not related to study treatment. No apparent dose- or drug-related trends were observed in standard safety laboratory parameters. There were no in-treatment pregnancies, and ovulation was inhibited for all treatments during the first four cycles tested.

Mean changes from baseline in systolic and diastolic blood pressure and heart rate were generally small throughout the study in all groups, without any obvious trends.

Table 1
Main demographics and baseline characteristics (AST population)

	15E4/DRSP *N*=79	20E4/DRSP *N*=75	15E4/LNG *N*=80	20E4/LNG *N*=77	E2V/DNG *N*=78	Overall *N*=389
Mean age, years (SD)	24.3 (4.6)	24.0 (4.5)	24.8 (4.8)	24.0 (3.6)	23.4 (3.5)	24.1 (4.2)
Mean BMI, kg/m^2 (SD)	22.9 (3.0)	23.1 (2.8)	22.6 (3.0)	22.6 (2.8)	22.4 (2.8)	22.7 (2.9)
Current smoking, *n* (%)	18 (22.8)	8 (10.7)	18 (22.5)	19 (24.7)	10 (12.8)	73 (18.8)
Switchers, *n* (%)	51 (64.6)	50 (66.7)	50 (62.5)	55 (71.4)	54 (69.2)	260 (66.8)
Starters, *n* (%)	28 (35.4)	25 (33.3)	30 (37.5)	22 (28.6)	24 (30.8)	129 (33.2)

BMI: body mass index; DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel; *n*: number of subjects with data; *N*: number of subjects in the AST population; SD: standard deviation.

Table 2
Primary and secondary bleeding outcome parameters (PP population)

	15E4/DRSP *N*=77	20E4/DRSP *N*=73	15E4/LNG *N*=77	20E4/LNG *N*=76	E2V/DNG *N*=75
Occurrence of unscheduled bleeding and/or spotting					
Treatment cycle 2					
• Bleeding/spotting, *n*/*N* (%)	34/75 (45.3)	28/68 (41.2)	43/71 (60.6)	38/68 (55.9)	40/66 (60.6)
• Difference from E2V/DNG, rate (95% CI)	−15.3 (−31.6, 1.0)	−19.4 (−36.0, −2.8)	0.0 (−16.4, 16.3)	−4.7 (−21.4, 12.0)	
• Treatment cycle 3					
• Bleeding/spotting, *n*/*N* (%)	39/72 (54.2)	29/67 (43.3)	35/65 (53.8)	39/66 (59.1)	39/68 (57.4)
• Difference from E2V/DNG, rate (95% CI)	−3.2 (−19.6, 13.3)	−14.1 (−30.8, 2.6)	−3.5 (−20.4, 13.4)	1.7 (−15.0, 18.4)	
Treatment cycle 6					
• Bleeding/spotting, *n*/*N* (%)	22/65 (33.8)	29/57 (50.9)	28/58 (48.3)	22/53 (41.5)	32/67 (47.8)
• Difference from E2V/DNG, rate (95% CI)	−13.9 (−30.5, 2.7)	3.1 (−14.5, 20.8)	0.5 (−17.0, 18.1)	−6.2 (−24.1, 11.6)	
Occurrence of unscheduled bleeding					
Treatment cycle 2					
• Bleeding, *n*/*N* (%)	20/75 (26.7)	16/68 (23.5)	29/71 (40.8)	20/68 (29.4)	30/66 (45.5)
• Difference from E2V/DNG, rate (95% CI)	−18.8 (−34.4, −3.2)	−21.9 (−37.6, −6.2)	−4.6 (−21.2, 12.0)	−16.0 (−32.2, 0.1)	
• Treatment cycle 3					
• Bleeding, *n*/*N* (%)	22/72 (30.6)	15/67 (22.4)	24/65 (36.9)	22/66 (33.3)	29/68 (42.6)
• Difference from E2V/DNG, rate (95% CI)	−12.1 (−28.0, 3.8)	−20.6 (−35.7, −4.8)	−5.7 (−22.3, 10.9)	−9.3 (−25.7, 7.0)	
Treatment cycle 6					
• Bleeding, *n*/*N* (%)	11/65 (16.9)	13/57 (22.8)	21/58 (36.2)	10/53 (18.9)	24/67 (35.8)
• Difference from E2V/DNG, rate (95% CI)	−18.9 (−33.6, −4.2)	−13.1 (−28.8, 2.8)	0.4 (−16.5, 17.3)	−17.0 (−32.5, −1.4)	
Absence of withdrawal bleeding					
Treatment cycle 2					
• No withdrawal, *n*/*N* (%)	1/75 (1.3)	1/68 (1.5)	7/71 (9.9)	2/68 (2.9)	8/66 (12.1)
• Difference from E2V/DNG, rate (95% CI)	−10.8 (−19.1, −2.5)	−10.6 (−19.0, −2.3)	−2.3 (−12.8, 8.2)	−9.2 (−18.0, −0.3)	
Treatment cycle 3					
• No withdrawal,. *n*/*N* (%)	2/72 (2.8)	1/67 (1.5)	7/65 (10.8)	9/66 (13.6)	11/67 (16.4)
• Difference from E2V/DNG, rate (95% CI)	−13.6 (−23.3, −4.0)	−14.9 (−24.3, −5.6)	−5.6 (−17.3, 6.0)	−2.8 (−14.9, 9.4)	
Treatment cycle 6					
• No withdrawal, *n*/*N* (%)	2/57 (3.5)	2/53 (3.8)	10/54 (18.5)	7/50 (14.0)	16/59 (27.1)
• Difference from E2V/DNG, rate (95% CI)	−23.6 (−35.9, −11.3)	−23.4 (−35.8, −10.9)	−8.6 (−24.0, 6.8)	−3.1 (−28.0, 1.8)	

CI: confidence interval; DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel; *n*: number of subjects with data; *N*: number of subjects in the PP population.

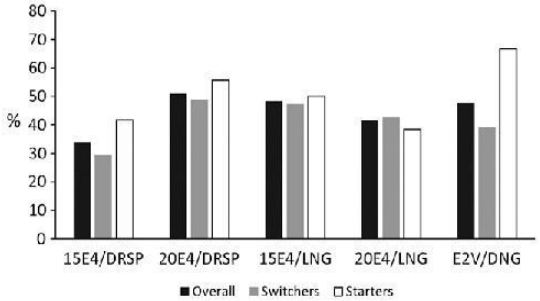

Fig. 2. Frequency (%) of women with unscheduled bleeding/spotting in cycle 6. Percentage of women with unscheduled bleeding/spotting in each treatment group in cycle 6. The data are presented overall and in the subsets of switchers and starters (PP population). DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel.

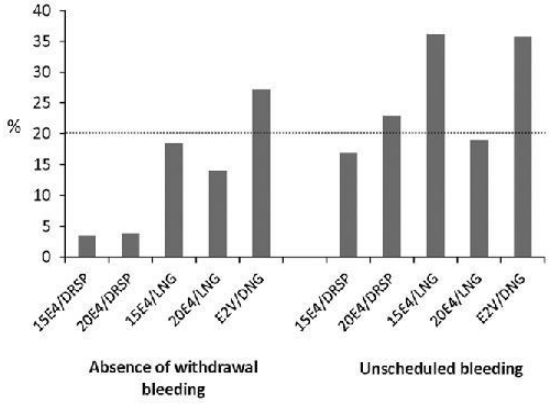

Fig. 3. Frequency (%) of women with absence of withdrawal bleeding/spotting in cycle 6. Percentage of subjects with absence of withdrawal bleeding and unscheduled bleeding in each treatment group in cycle 6 (PP population). Absence of withdrawal bleeding ≤20%, and/or ≤20% unscheduled intracyclic bleeding after six treatment cycles, was set as a limit (dotted bar). DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel.

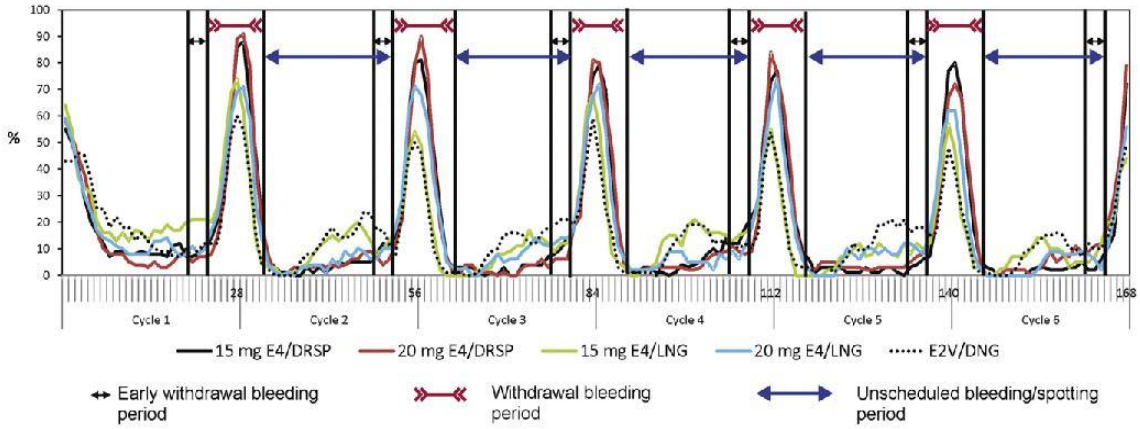

Fig. 4. Incidence of scheduled and unscheduled bleeding/spotting on a daily basis during the six treatment cycles (PP population). Bleeding or spotting reported during cycle days 5–24 was considered unscheduled. Early withdrawal bleeding/spotting occurs between cycle days 21 and 24. DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel.

4. Discussion

Firstly, there were no safety concerns for any of the treatments in this study.

The aim of the present study was to select the E4/progestin dosing regimen for phase III development, based on an optimal bleeding pattern and cycle control. For this reason, absence of withdrawal bleeding for at most 20% of the women and unscheduled intracyclic bleeding without spotting for at most 20% of the women were set as limits. After six cycles, the 15 mg E4/DRSP and the 20 mg E4/LNG combinations were the only ones meeting both criteria: absence of withdrawal bleeding was observed for 3.5% and 14.0%, respectively, and unscheduled intracyclic bleeding was observed for 16.9% and 18.9%, respectively.

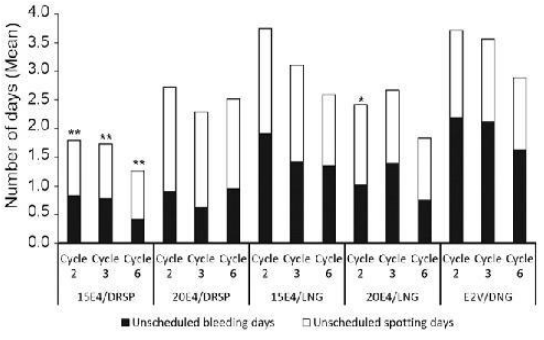

Fig. 5. Mean number of unscheduled bleeding/spotting days in cycles 2, 3 and 6. Bleeding or spotting reported during cycle days 5–24 was considered unscheduled. The mean number of unscheduled bleeding (black bars)/spotting (white bars) days was calculated for each treatment group in the PP population. *t* Test showed a significant difference at cycles 2, 3 and 6 between 15E4/DRSP and E2V/DNG treatment groups (*p<.05, **p<.005). DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel.

Regular monthly withdrawal bleeding is desirable as it reassures the user that she is not pregnant [24]. Therefore, the 3.5% absence of withdrawal bleeding in the 15E4/DRSP group is considered a very positive feature. By cycle 6, the lowest frequency of unscheduled bleeding and spotting was also observed in the 15E4/DRSP group (33.8%), which was considerably lower than the 47.8% observed in the E2V/DNG reference group. Only 8.9% of subjects in the 15E4/DRSP group discontinued prematurely (compared with a discontinuation rate of between 10.3% and 29.9% in the other groups), which may be related to the favourable cycle control with this treatment regimen.

Since E4 is a natural oestrogen, Qlaira® was chosen as reference COC in the present study because it also contains a natural oestrogen (E2V). It is noteworthy that the incidence of unscheduled bleeding in the E2V/DNG group (35.8%) is in contrast with the 14% reported by Ahrendt et al. [9]. An explanation may be the different definitions used for the term 'unscheduled': days 5–24 in the present study and days 3–21 in the E2V/DNG study [9]. This is also supported by the fact that, during E2V/DNG treatment, substantial bleeding was reported between days 21 and 24 (Fig. 4). A satisfactory cycle control has been described for combinations of DRSP or LNG with 20 mcg EE as the oestrogen [25,26]. The results observed with 15 mg E4/DRSP are in line with these findings.

In conclusion, the 15 mg E4/DRSP combination has been shown to be the most efficacious in terms of bleeding pattern and cycle control, compared with the other combinations investigated. Therefore, this COC seems to be the preferred combination for further phase III clinical development.

Acknowledgements

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Table 3
Incidence of TE-AEs, *n* (%) (AST population)

	15E4/DRSP *N*=79	20E4/DRSP *N*=75	15E4/LNG *N*=80	20E4/LNG *N*=77	E2V/DNG *N*=78
TE-AEs	51 (64.6)	60 (80.0)	60 (75.0)	57 (74.0)	56 (71.8)
Deaths	0	0	0	0	0
SAE	0	0	0	0	1 (1.3)
TE-AEs leading to withdrawal	5 (6.3)	8 (10.7)	10 (12.5)	12 (15.6)	4 (5.1)
TE-AEs of known severe intensity	3 (3.8)	2 (2.7)	1 (1.3)	2 (2.6)	3 (3.9)
Drug-related TE-AEs					
• Overall	20 (25.3)	31 (41.3)	28 (35.0)	35 (45.5)	18 (23.1)
• Headache/migraine	3 (3.8)	7 (9.3)	2 (2.5)	6 (7.8)	5 (6.4)
• Anxiety/depression	0	0	1 (1.3)	5 (6.5)	1 (1.3)

AST: all-subjects treated; DNG: dienogest; DRSP: drospirenone; E4: estetrol; E2V: estradiol valerate; LNG: levonorgestrel; *n*: number of subjects with data; *N*: number of subjects in the AST population; SAE: serious adverse event; TE-AEs: treatment-emergent adverse events.

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References

[1] Westhoff CL, Heartwell S, Edwards S, Zieman M, Stuart G, Cwiak C, et al. Oral contraceptive discontinuation: do side effects matter? Am J Obstet Gynecol 2007;196:412.e1–6 [discussion e6-7].
[2] Bitzer J. Pharmacological profile of estrogens in oral contraception. Minerva Ginecol 2011;63:299–304.
[3] Kiley J, Hammond C. Combined oral contraceptives: a comprehensive review. Clin Obstet Gynecol 2007;50:868–77.
[4] Stegeman BH, de Bastos M, Rosendaal FR, van Hylckama Vlieg A, Helmerhorst FM, Stijnen T, et al. Different combined oral contraceptives and the risk of venous thrombosis: systematic review and network meta-analysis. BMJ 2013;347:f5298.
[5] Rosenberg MJ, Meyers A, Roy V. Efficacy, cycle control, and side effects of low- and lower-dose oral contraceptives: a randomized trial of 20 micrograms and 35 micrograms estrogen preparations. Contraception 1999;60:321–9.
[6] Hoffmann H, Moore C, Zimmermann H, Elger W, Schwarz S, Graser T, et al. Approaches to the replacement of ethinylestradiol by natural 17beta-estradiol in combined oral contraceptives. Exp Toxicol Pathol 1998;50:458–64.
[7] Mansour D, Verhoeven C, Sommer W, Weisberg E, Taneepanichskul S, Melis GB, et al. Efficacy and tolerability of a monophasic combined oral contraceptive containing nomegestrol acetate and 17beta-oestradiol in a 24/4 regimen, in comparison to an oral contraceptive containing ethinylestradiol and drospirenone in a 21/7 regimen. Eur J Contracept Reprod Health Care 2011;16:430–43.
[8] Westhoff C, Kaunitz AM, Korver T, Sommer W, Bahamondes L, Darney P, et al. Efficacy, safety, and tolerability of a monophasic oral contraceptive containing nomegestrol acetate and 17beta-estradiol: a randomized controlled trial. Obstet Gynecol 2012;119:989–99.
[9] Ahrendt HJ, Makalova D, Parke S, Mellinger U, Mansour D. Bleeding pattern and cycle control with an estradiol-based oral contraceptive: a seven-cycle, randomized comparative trial of estradiol valerate/dienogest and ethinyl estradiol/levonorgestrel. Contraception 2009;80:436–44.
[10] Fraser IS, Jensen J, Schaefers M, Mellinger U, Parke S, Serrani M. Normalization of blood loss in women with heavy menstrual bleeding treated with an oral contraceptive containing estradiol valerate/dienogest. Contraception 2012;86:96–01.
[11] Holinka CF, Diczfalusy E, Coelingh Bennink HJ. Estetrol: a unique steroid in human pregnancy. J Steroid Biochem Mol Biol 2008;110:138–43.
[12] Hammond GL, Hogeveen KN, Visser M, Coelingh Bennink HJ. Estetrol does not bind sex hormone binding globulin or increase its production by human HepG2 cells. Climacteric 2008;11(Suppl 1):41–6.
[13] 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.
[14] FDA label E2V/DNG; 2012.
[15] 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.
[16] Coelingh Bennink HJ, Skouby S, Bouchard P, Holinka CF. Ovulation inhibition by estetrol in an in vivo model. Contraception 2008;77:186–90.
[17] Visser M, Coelingh Bennink HJ. Clinical applications for estetrol. J Steroid Biochem Mol Biol 2009;114:85–9.
[18] Coelingh Bennink HJ, Holinka CF, Diczfalusy E. Estetrol review: profile and potential clinical applications. Climacteric 2008;11(Suppl 1):47–58.
[19] Duijkers IJ, Klipping C, Zimmerman Y, Appels N, Jost M, Maillard C, et al. Inhibition of ovulation by administration of estetrol in combination with drospirenone or levonorgestrel: Results of a phase II dose-finding pilot study. Eur J Contracept Reprod Health Care 2015;20:476–89.
[20] Mawet M, Maillard C, Klipping C, Zimmerman Y, Foidart JM, Coelingh Bennink HJ. Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives. Eur J Contracept Reprod Health Care 2015;20:463–75.
[21] World Health Organization. Medical eligibility criteria for contraceptive use. 4th ed. WHO Press; 200915–44.
[22] Roos J, Johnson S, Weddell S, Godehardt E, Schiffner J, Freundl G, et al. Monitoring the menstrual cycle: Comparison of urinary and serum reproductive hormones referenced to true ovulation. Eur J Contracept Reprod Health Care 2015;20:438–50.
[23] Mishell Jr DR, Guillebaud J, Westhoff C, Nelson AL, Kaunitz AM, Trussel J, et al. Recommendations for standardization of data collection and analysis of bleeding in combined hormone contraceptive trials. Contraception 2007;75:11–5.

[24] Oddsson K, Leifels-Fischer B, Wiel-Masson D, de Melo NR, Benedetto C, Verhoeven CH, et al. Superior cycle control with a contraceptive vaginal ring compared with an oral contraceptive containing 30 microg ethinylestradiol and 150 microg levonorgestrel: a randomized trial. Hum Reprod 2005;20:557–62.

[25] Gruber DM, Huber JC, Melis GB, Stagg C, Parke S, Marr J. A comparison of the cycle control, safety, and efficacy profile of a 21-day regimen of ethinylestradiol 20mug and drospirenone 3mg with a 21-day regimen of ethinylestradiol 20mug and desogestrel 150mug. Treat Endocrinol 2006;5:115–21.

[26] DelConte A, Loffer F, Grubb GS. Cycle control with oral contraceptives containing 20 micrograms of ethinyl estradiol. A multicenter, randomized comparison of levonorgestrel/ethinyl estradiol (100 micrograms/20 micrograms) and norethindrone/ethinyl estradiol (1000 micrograms/20 micrograms). Contraception 1999;59:187–93.

4.3. Discussion

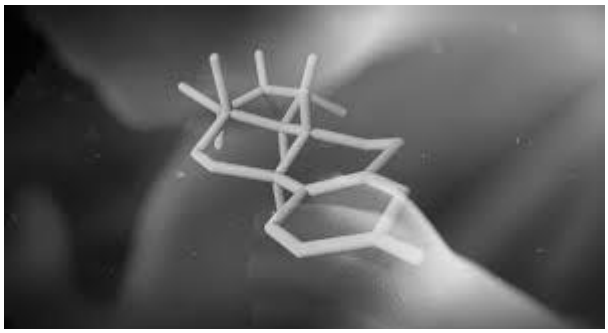
Unwanted intermenstrual bleeding was the lowest (below 20%) with two E4 combinations, namely 15 mg E4/DRSP and 20 mg E4/LNG (at treatment cycle 6, a mean of 16.9% and 18.9% of the women experienced intermenstrual bleeding in each group, respectively). However, the incidence of amenorrhea was high in the 20 mg E4/LNG group in comparison to the 15 mg E4/DRSP groups (18.5% versus 3.5%). Amenorrhea may be appreciated by women and is an additional benefit of certain contraceptive methods (e.g. LNG-releasing intrauterine system), but only when amenorrhea is constant and predictable. Indeed, if amenorrhea occurs irregularly, a pregnancy may be suspected and should be ruled out, a source of anxiety for the woman who may not trust her contraceptive method any longer.

The women treated with the active comparator, Qlaira®, reported a high frequency of intermenstrual bleeding episode (35.8% at treatment cycle 6) and a high frequency of amenorrhea (27.1%). These results are not in line with the published data on this commercialized COC (Ahrendt, et al. 2009).

Altogether, we may conclude that the E4-COCs were associated with a better bleeding profile than the E2V-COC Qlaira® and that, taking into account both incidence of intermenstrual bleeding and amenorrhea, the 15 mg E4/LNG combination was the best combination regarding the bleeding pattern. Interestingly, increasing the dose of E4 to 20 mg in association with DRSP had no further benefit in terms of bleeding pattern, probably because a plateau of activity is achieved.

In addition to the good bleeding pattern, the 15 mg E4/DRSP group also surpassed the other groups regarding the safety. First, the number of adverse events was the lowest in that group with 64.6 % of the subjects reporting an adverse event during the study versus 71.8 to 80.0% in the other groups, including the Qlaira® group. Secondly, this group was associated with the lowest discontinuation rate due to adverse reaction amongst the E4-COCs tested.

Finally, no pregnancy occurred during the study demonstrating the contraceptive reliability of the E4-containing combinations.



5th Publication

Estetrol combined with drospirenone: an oral contraceptive with high acceptability, user satisfaction, well-being and favourable body weight control

The European Journal of Contraception and Reproductive Health Care,
2017; 22: 260-267

Dan Apter, Yvette Zimmerman, Louise Beekman, Marie Mawet, Catherine Maillard, Jean-Michel Foidart, Herjan J T Coelingh Bennink

5. Fifth publication

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5.1. Introduction

One of the major goals when developing a new contraceptive method is achieving high user satisfaction. This is indeed one of the major factor influencing compliance and consequently influencing efficacy associated with the contraceptive method. In line with this consideration, this article reports and discusses three additional parameters that were taken into account in the final dose selection.

First, the discontinuation rates in each group along with the reasons for discontinuation, since it was considered as a good reflect of the subject's tolerance to the study treatments.

Secondly, the general well-being and the willingness to continue with the study drug after the end of the trial. Both parameters were measured using a questionnaire.

Finally, the change in body weight because weight increase (or fear of weight increase) is a common reason for early COC discontinuation (Lindh, Ellstrom, and Milsom 2011). This parameter is consequently seen as discriminant in the dose selection.

5.2. Article

THE EUROPEAN JOURNAL OF CONTRACEPTION & REPRODUCTIVE HEALTH CARE, 2017
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ORIGINAL RESEARCH ARTICLE

Estetrol combined with drospirenone: an oral contraceptive with high acceptability, user satisfaction, well-being and favourable body weight control

Dan Apter[a], Yvette Zimmerman[b], Louise Beekman[b], Marie Mawet[c], Catherine Maillard[c], Jean-Michel Foidart[c,d]* and Herjan J. T. Coelingh Bennink[b]*

[a]Sexual Health Clinic (Väestöliitto), Helsinki, Finland; [b]Pantarhei Bioscience BV, Zeist, the Netherlands; [c]Estetra SPRL, Liège, Belgium; [d]University of Liège, Liège, Belgium

ABSTRACT

Objectives: This study evaluated acceptability, user satisfaction, body weight control and general well-being of estetrol (E4) combined with either drospirenone (DRSP) or levonorgestrel (LNG).

Methods: In this open-label, multi-centre, dose-finding, 6-cycle study, 396 healthy women of reproductive age were randomised into five treatment groups in a 24/4-day regimen: 15 mg or 20 mg E4 combined with either 3 mg DRSP or 150 µg LNG, and as reference estradiol valerate (E2V) combined with dienogest (DNG). Data on acceptability, user well-being, satisfaction and body weight were collected.

Results: The number of completers was the highest in the 15 mg E4/DRSP group (91.1%), and the lowest for 20 mg E4/LNG (70.1%). The largest proportion of treatment satisfaction was reported for 15 mg E4/DRSP (73.1%), and the lowest for 15 mg E4/LNG (50.6%). The number of women willing to continue with the assigned study treatment was the highest in the 15 mg E4/DRSP group (82.1%) and the lowest for 20 mg E4/LNG (58.3%). Well-being with E4/DRSP combinations was statistically significantly better than with E4/LNG combinations: OR (95% CI) 2.00 (1.13; 3.53) and 1.93 (1.06; 3.56) for 15 and 20 mg E4, respectively, and comparable to E2V/DNG. Proportion of women with a 2 kg or more weight loss after 3 and 6 cycles was the highest in the 15 mg E4/DRSP group (30.7 and 36.7%, respectively).

Conclusions: The present study shows that 15 mg estetrol combined with 3 mg DRSP is associated with a high-user acceptability and satisfaction, and with a favourable body weight control.

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KEYWORDS
Estetrol; drospirenone; levonorgestrel; user satisfaction; well-being; weight control

Introduction

Combined oral contraceptives (COCs), which contain a synthetic estrogen and progestin, are highly effective; pregnancy rates range from 0.1 to 0.3% among perfect users [1]. However, failure rates up to 8% in the first year have been reported, most importantly because of lack of compliance [2]. Noncompliance and discontinuation of contraception is often a result of side effects, weight gain and sub-optimal cycle control [3,4]. Meanwhile, it has been shown that (sexual) well-being and user satisfaction can improve treatment compliance [5]. Therefore, there is still a medical need to develop a COC with both an optimal cycle control and good user acceptability.

Estetrol (E4) is a natural estrogen, synthesized by the human fetal liver and is present only during human pregnancy. A summary of E4 research data until 2015 is available [6]. The combination of E4 at doses of 5 or 10 mg with 3 mg drospirenone (DRSP) has been shown to suppress ovulation, as well as the combination of E4 at doses of 5, 10 or 20 mg with 150 µg levonorgestrel (LNG) [7]. These combinations also have a limited impact on the synthesis of liver proteins such as sex hormone-binding globulin, and triglyceride levels, which may indicate a potential reduction of both the risk of venous thromboembolism and cardiovascular disease [8]. Compared to COCs containing ethinylestradiol (EE) and DRSP, the E4/DRSP combinations led to reduction in coagulation markers and reduced haemostatic effects [9].

An open-label, multi-centre, randomized, dose-finding study (FIESTA) was performed to assess bleeding pattern and cycle control of E4 combined with either DRSP or LNG. The 15 mg E4/DRSP combination proved to be the most efficacious with respect to bleeding and cycle control, and was subsequently selected for further phase III clinical development [10]. The FIESTA study also evaluated user acceptability, satisfaction, body weight control and general well-being. The results of this evaluation are presented here.

Methods

This was an open-label, multi-centre, randomized, dose-finding study in healthy women of reproductive age. The study was conducted between September 2010 and September 2011 in 10 centres in Finland (ClinicalTrials.gov identifier NCT01221831). Approval was obtained by the regional independent ethics committee of the Hospital District of Helsinki and Uusimaa (HUS), and the Finnish Medicines Agency (FIMEA). The study was conducted in accordance with the ethical principles established by the Declaration of Helsinki and the International Conference on Harmonization – Good Clinical Practice Guidelines. Written

CONTACT Marie Mawet mmawet@mithra.com Estetra SPRL, Rue Saint Georges 5-7, 4000 Liège, Belgium
*These authors contributed equally to this work.

Table 1. Subject Satisfaction and Health-Related Questionnaire.

Domain	Question	Response possibility
1. General feeling	How did you feel during the use of your current contraceptive or during last cycle?	Better No change Worse
2a. Mood	What is the effect of the contraceptive on your mood?	Much better Better No change Worse Much worse
2b. Sexual life	What is the effect of the contraceptive on your sexual life?	
2c. Premenstrual complaints	What is the effect of the contraceptive on your premenstrual complaints?	
2d. Overall effect	What is the effect of the contraceptive overall?	
3. Satisfaction	How satisfied are you with your study medication?	Very satisfied Satisfied Neutral[a] Dissatisfied Very dissatisfied
4. Future use	Would you consider using the study medication that you have been using during this cycle?	Yes Maybe No Don't know

[a]Described as 'Neither satisfied nor dissatisfied'.

informed consent was obtained from all participants prior to any study related procedures, including screening evaluations and an appropriate wash-out period for medication that participants were using prior to study entry.

Participants

Healthy women, aged 18–35 years with a BMI between 18 and 30 kg/m^2 and a regular menstrual cycle (24–35 days), were eligible for inclusion. Women who were already using hormonal contraceptives and hormonal contraceptive-naïve women (switchers and starters, respectively) could participate. Exclusion criteria were in line with the World Health Organization's medical eligibility criteria for COC use [11], and described previously in more detail [10].

Treatment

Participants were randomly allocated to one of the five treatment groups: (i) 15 mg E4 combined with 3 mg DRSP (15E4/DRSP); (ii) 20 mg E4 combined with 3 mg DRSP (20E4/DRSP); (iii) 15 mg E4 combined with 150 μg LNG (15E4/LNG); (iv) 20 mg E4 combined with 150 μg LNG (20E4/LNG); (v) and as a reference estradiol valerate (E2V) combined with dienogest (DNG) (E2V/DNG), the commercial packaging of 4-phasic Qlaira® (Bayer HealthCare, Berlin, Germany). To achieve equal distributions across the groups, randomization was stratified by switchers, starters, and sites.

Participants were to complete six treatment cycles of 28 days. For the E4 groups one cycle comprised of 24 study medication days, followed by four placebo days. For the E2V/DNG group one cycle comprised of 26 days of active pills followed by two placebo days, as indicated in the labelling of Qlaira®.

Assessments and outcome variables

The primary objective of this study was to assess vaginal bleeding patterns and cycle control, and its results are published elsewhere [10].

The secondary study objectives included the evaluation of acceptability of the study medication, user satisfaction and general well-being by completing a Subject Satisfaction and Health-Related Questionnaire, and recording of body weight and return of menstruation. The results of this evaluation are presented in this article.

Acceptability of the study medication was assessed by recording discontinuation rates, reasons for discontinuations and compliance with the study medication. At each visit during the treatment period (scheduled at the end of cycles 1, 2, 3, 4 and 6), women were asked to return the blisters of study medication in order to evaluate treatment compliance. They were also asked to record their daily intake of the study medication in a diary. This information was used as a measure for the extent of exposure and compliance.

User satisfaction and well-being were evaluated at each visit using a self-reported Subject Satisfaction and Health-Related Questionnaire. The questionnaire assesses user satisfaction with the study medication (domain 1) and its effects on mood (domain 2a), sexual life (domain 2b), premenstrual symptoms (domain 2c), and its overall effect (domain 2d) (Table 1). The domains and questions are similar to those used in validated measures like the Treatment Satisfaction Questionnaire for Medication (TSQM), the Spanish society of Contraception Quality-of-Life (SEC-QOL), or the Quality of Life Enjoyment and Satisfaction Questionnaire (Q-LES-Q) [12–14]. Possible responses varied from (much) better, no change, and (much) worse compared to that recorded at the previous visit.

Body weight was measured using standardized equipment at each study visit. The proportions of women with either a weight gain of $\geq$2 kg or a weight loss of $\geq$2 kg were calculated for each treatment group at the end of cycles 3 and 6 (or end of study), according to the method described by Foidart et al. [15]. In addition, the resulting ratio (weight loss $\geq$2 kg/weight gain $\geq$2 kg) was calculated.

After study completion (or premature discontinuation), women were followed for up to one year until return of spontaneous menstruation or pregnancy occurred. Women starting other forms of hormonal contraception were not followed-up.

Statistical analysis

Data analyses for treatment acceptability, user satisfaction and health-related questionnaire were performed for the

intent-to-treat (ITT) population, comprising all-subjects-treated (AST) with data for any non-bleeding parameter and for dosing compliance.

A mixed-effects proportional odds regression model was used for a *post hoc* multifunctional analysis of the domains 1 and 2a–d (Table 1) [16], including the treatment, the question, the subgroup (starter or switcher), the visit, and all double interactions between these factors as fixed effects, and a random subject-specific intercept. For this purpose, the change from baseline for each of the five visits was longitudinally converted to a three-level ordinal outcome: −1 = worse or much worse, 0 = no change; 1 = better or much better. The results were summarized in a mosaic plot and odds ratios (OR) with 95% confidence intervals (CI) were calculated for pairwise treatment differences of interest. The analyses on the domains satisfaction and future use were only descriptive, and presented by means and frequencies.

Results

A total of 396 women were randomized (Figure 1), of whom 389 (98.2%) received study medication. The proportion of switchers (66.8%) and starters (33.2%) was generally similar across treatment groups. Demographic and baseline characteristics were similar across the treatment groups, except for smoking which varied between 10.7% for 20E4/DRSP and 24.7% for 20E4/LNG (Table 2).

Acceptability of study medication

Of the 389 women receiving study medication, 73 (18.8%) discontinued prematurely (Table 3). The number of completers was the highest for 15E4/DRSP (72/79; 91.1%) and E2V/DNG (70/78; 89.7%), and the lowest for 20E4/LNG (54/77; 70.1%) (Figure 1).

Reasons for discontinuation are displayed in the Figure 1. Treatment-emergent adverse events (TE-AEs) were the most common reason for discontinuation ($n = 39$; 10.0%) (Table 3). In addition, two women (one assigned to 15E4/DRSP and one assigned to E2V/DNG) discontinued because of an AE that started before taking study medication. No women in the 15E4/DRSP and E2V/DNG groups discontinued because of vaginal bleeding, in contrast to a total of three to four (3.9–5.0%) in the other groups (Table 3). Compliance was generally good, with 97.6% of tablets taken per cycle based on diary card entries. In line with the discontinuations across treatment groups, the

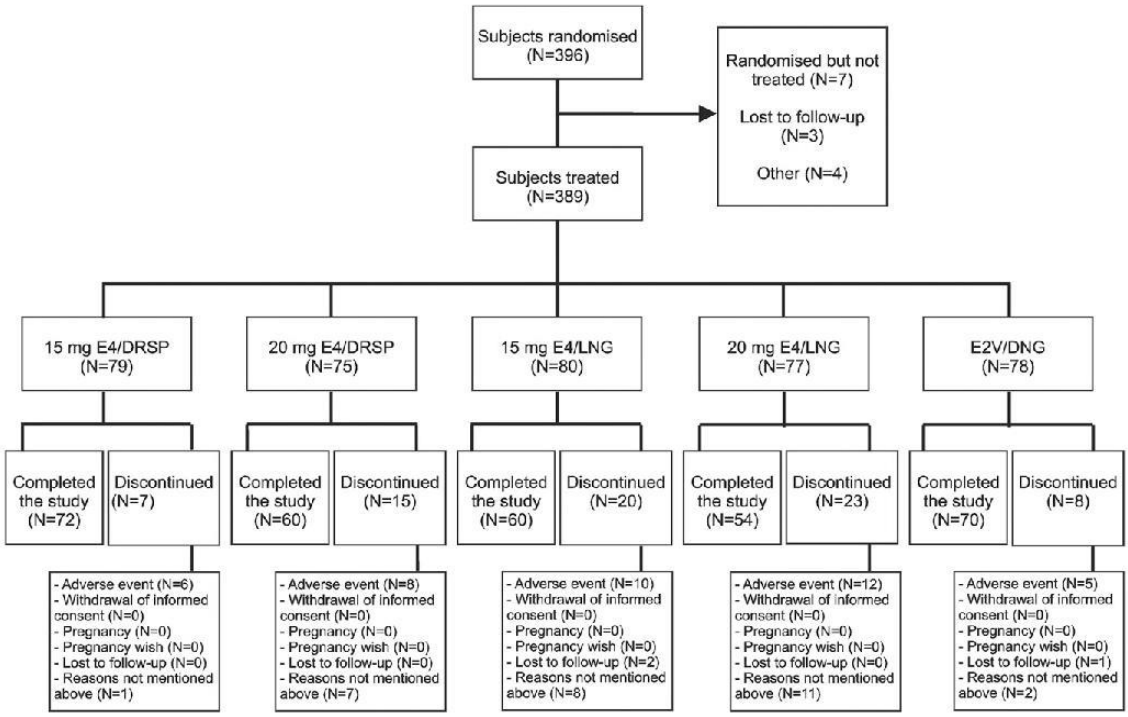

Figure 1. Subject disposition by treatment group (all-subjects-randomised population).

Table 2. Main demographics and baseline characteristics (all-subjects-treated population).

	15E4/DRSP, $N = 79$	20E4/DRSP, $N = 75$	15E4/LNG, $N = 80$	20E4/LNG, $N = 77$	E2V/DNG, $N = 78$	Overall, $N = 389$
Mean age, years (SD)	24.3 (4.6)	24.0 (4.5)	24.8 (4.8)	24.0 (3.6)	23.4 (3.5)	24.1 (4.2)
Mean BMI, kg/m^2 (SD)	22.9 (3.0)	23.1 (2.8)	22.6 (3.0)	22.6 (2.8)	22.4 (2.8)	22.7 (2.9)
Current smoking, *n* (%)	18 (22.8)	8 (10.7)	18 (22.5)	19 (24.7)	10 (12.8)	73 (18.8)
Switchers, *n* (%)	51 (64.6)	50 (66.7)	50 (62.5)	55 (71.4)	54 (69.2)	260 (66.8)
Starters, *n* (%)	28 (35.4)	25 (33.3)	30 (37.5)	22 (28.6)	24 (30.8)	129 (33.2)

SD: standard deviation.

Table 3. Reasons for discontinuation of study treatment, *n* (%) (all-subjects-treated population).

	15E4/DRSP, *N* = 79	20E4/DRSP, *N* = 75	15E4/LNG, *N* = 80	20E4/LNG, *N* = 77	E2V/DNG, *N* = 78	Overall, *N* = 389
TE-AE[a]	5 (6.3)	8 (10.7)	10 (12.5)	12 (15.6)	4 (5.1)	39 (10.0)
Lost to follow- up	0	0	2 (2.5)	0	1 (1.3)	3 (0.8)
Vaginal bleeding	0	3 (4.0)	4 (5.0)	3 (3.9)	0	10 (2.6)
Logistic reasons[b]	1 (1.3)	3 (4.0)	3 (3.8)	3 (3.9)	2 (2.6)	12 (3.1)
Other[c]	0	1 (1.3)	1 (1.3)	5 (6.5)	0	7 (1.8)
Total	7 (8.9)	15 (20.0)	20 (25.0)	23 (29.9)	8 (10.3)	73 (18.8)

[a]Mainly gastrointestinal, nervous system, psychiatric (libido), and skin disorders; two additional women (one assigned to 15E4/DRSDP and one assigned to E2V/DNG) discontinued because of an AE before taking study medication.
[b]e.g. burden to visit the study centre.
[c]e.g. no need for contraception, decision to stop before cycle 6.

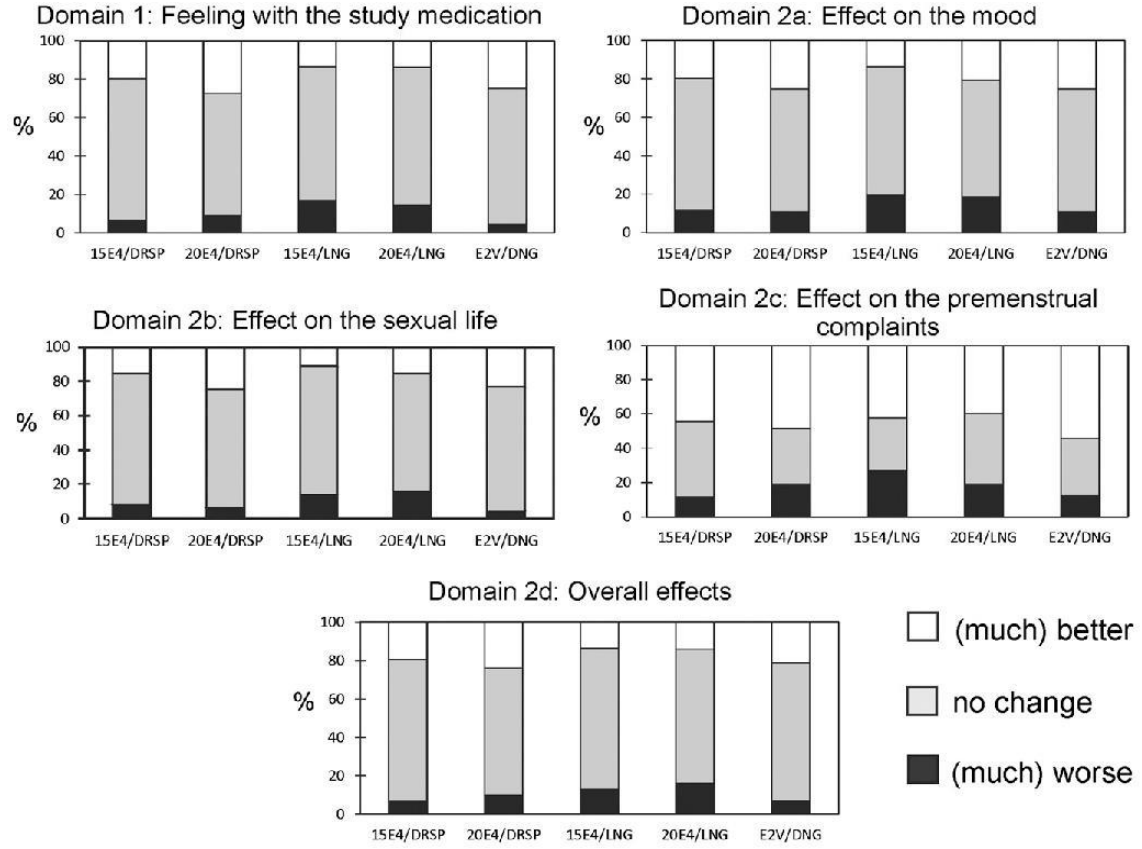

Figure 2. Mosaic plots of the change from baseline score recorded at cycle 6 (or end-of-study) stratified by domains (general feeling, mood, sexual life, premenstrual complaints and overall effect); white: better; grey: no change; black: worse.

mean extent of exposure varied between 144.1 days for 20E4/LNG and 159.5 days for 15E4/DRSP. Accordingly, the mean total number of cycles varied between 5.2 for 20E4/LNG and 5.7 for 15E4/DRSDP and E2V/DNG (data not shown).

Subject Satisfaction and Health-Related Questionnaire

At randomisation, the majority of women (>90%) reported a very good to average general feeling. In contraceptives users the domains 'Mood' and 'Overall effect' were scored high (91–98%), but 'Sexual life' and 'Premenstrual complaints' scored relatively low (58–68%) (data not shown).

The changes at Cycle 6 compared to baseline, expressed as 'Better' (white bar), 'No change' (grey bar) or 'Worse' (black bar) for the domains 1 and 2a–d, are presented in Figure 2. Scoring of better 'General feeling' was 19.9 and 27.6% in the 15E4/DRSP and 20 E4/DRSP groups, respectively, which was comparable for E2V/DNG (25.0%), but higher than observed for 15E4/LNG and 20E4/LNG groups (13.7%). Similar results were observed for the domains 'Mood', 'Sexual life' and 'Overall effect', with better scores in the E4/DRSP groups compared to the E4/LNG groups. Less subjects reported worsening of their premenstrual complaints in the 15E4/DRSP (11.7%) and E2V/DNG (12.2%) groups, compared to 19.2% for 20E4/DRSP. The highest proportion of women reporting a worsening of their premenstrual complaints was observed in the 15E4/LNG (27.1%) and 20E4/LNG groups (19.0%).

Odds ratio's comparing overall treatment differences (Figure 3) showed that well-being scores were significantly

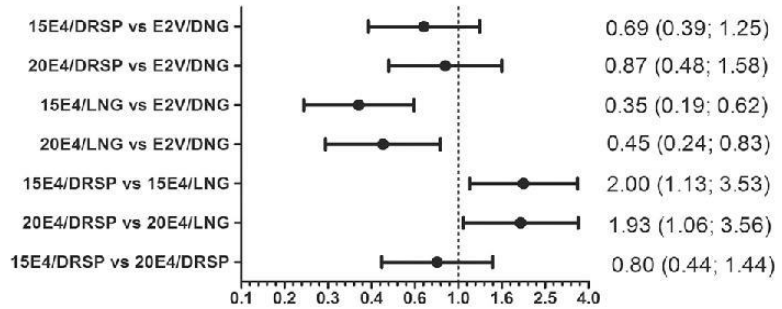

Figure 3. OR (95% CI) comparing the overall outcome* of different treatments (*x*-axis in log10-scale). *Domains: general feeling, mood, sexual life, premenstrual complaints and overall effect; combined recordings at cycles 1, 2, 3, 4, and 6. NB. Values <1 are indicative of a worse well-being outcome, and values >1 are indicative of a better well-being outcome.

Table 4. Subject satisfaction and health-related questionnaire; responses [*n* (%)] at cycle 6 for the items Satisfaction and Future use.

Domain	Response possibility	15E4/DRSP, *N* = 79	20E4/DRSP, *N* = 75	15E4/LNG, *N* = 80	20E4/LNG, *N* = 77	E2V/DNG, *N* = 78
Satisfaction	Very satisfied	11 (14.1)	15 (20.6)	10 (13.3)	9 (12.5)	11 (14.9)
	Satisfied	46 (59.0)	35 (48.0)	28 (37.3)	30 (41.7)	39 (52.7)
	Neutral[a]	10 (12.8)	13 (17.8)	20 (26.7)	20 (27.8)	15 (20.3)
	Dissatisfied	10 (12.8)	10 (13.7)	13 (17.3)	11 (15.3)	9 (12.2)
	Very dissatisfied	1 (1.3)	0	4 (5.3)	2 (2.8)	0
	Missing	1	2	5	5	4
Future use	Yes	28 (35.9)	27 (37.0)	16 (21.3)	15 (20.8)	31 (41.9)
	May be	36 (46.2)	30 (41.1)	32 (42.7)	27 (37.5)	21 (28.4)
	No	14 (18.0)	13 (17.8)	24 (32.0)	27 (37.5)	18 (24.3)
	Don't know	0	3 (4.1)	3 (4.0)	3 (4.2)	4 (5.4)
	Missing	1	2	5	5	4

[a]Described as 'Neither satisfied nor dissatisfied'.

worse for 15E4/LNG and 20E4/LNG compared to E2V/DNG [OR 0.35 (95% CI: 0.19; 0.62) and OR 0.45 (95% CI: 0.24; 0.83), respectively]. Comparison of DRSP groups versus LNG groups showed that DRSP treatment was significantly better with an OR 2.00 (95% CI: 1.13; 3.53) for the 15E4 treatment groups and an OR 1.93 (95% CI: 1.06; 3.56) for the 20E4 treatment groups (Figure 3). The differences between the two E4/DRSP groups and between E4/DRSP groups and E2V/DNG were not statistically significant.

Regarding user satisfaction with study medication, >50% of women in any treatment group reported being satisfied or very satisfied at cycle 6, with the largest proportion in the 15E4/DRSP group (73.1%), and the lowest in the 15E4/LNG group (50.6%). E2V/DNG showed a satisfaction rate of 67.6%. Reporting to be (very) dissatisfied was the highest in the E4/LNG groups (18.1–22.6%) while fewer women were (very) dissatisfied in the other groups (between 12.2 and 14.1%) (Table 4, Figure 4). The response 'yes' or 'may be', to consider future using of the assigned study medication, was the highest for 15E4/DRSP (82.1%) and the lowest for 20E4/LNG (58.3%) (Table 4, Figure 4).

Body weight

The proportion of women with a weight gain of ≥2 kg was the lowest for 20E4/DRSP (8.7 and 13.3% at cycles 3 and 6, respectively), and the highest for 15E4/LNG (21.5 and 29.9%, respectively). The proportion of women with a weight loss of ≥2 kg was the highest for 15E4/DRSP (30.7 and 36.7% at cycles 3 and 6, respectively), and the lowest for 15E4/LNG (7.7 and 13.0%, respectively) (Figure 5). The resulting ratio in the combined E4/DRSP groups to have a weight loss ≥2 kg compared to a weight gain ≥2 kg was 2.8:1 at cycle 3 and 2.0:1 at cycle 6. The corresponding ratios were 1.6:1 and 1.7:1, respectively, in the E2V/DNG group, and 0.7:1 and 0.6:1, respectively, in the combined E4/LNG groups.

Return of menstruation

Of 36 women who did not start with a new hormonal contraceptive method after study completion (or end of treatment), 32 (89%) had a return of menstruation within the first 2 months, and two had (both in the 15E4/LNG group) menstruation returned in 2–4 months. The remaining two women (both in the 15E4/LNG group) became pregnant in the first cycle after stopping study treatment, with an estimated date of conception of more than 14 days after last active tablet.

Discussion

Findings and interpretation

User satisfaction and health-related outcomes (well-being), together with acceptability of the study medication, are the basis of the present evaluation. The responses on the Subject Satisfaction and Health-Related Questionnaire revealed that at treatment cycle 6, using 15 mg E4/DRSP was accompanied by the highest proportion of satisfaction (73.1%) and using 15 mg E4/LNG by the lowest (50.6%). This is in line with the current finding based on ORs for the *post hoc* multifunctional statistical analysis that well-being with E4/DRSP combinations is significantly better than with E4/LNG combinations. In general, the scores in the E4/DRSP

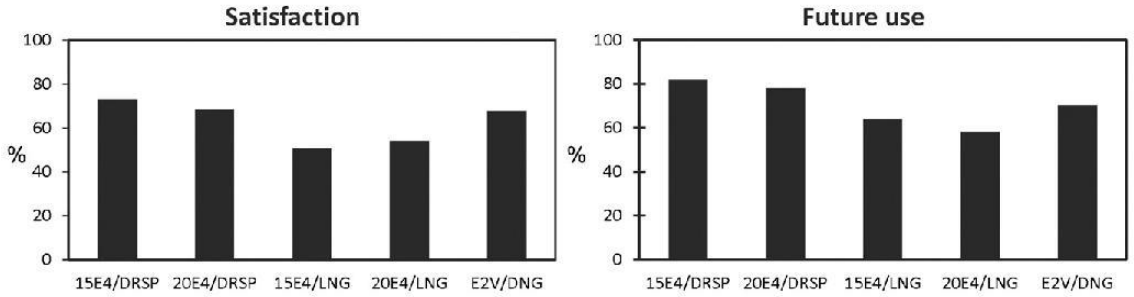

Figure 4. Frequencies (%) of a satisfaction [(very) satisfied] response or willingness of future use (yes and maybe), recorded at cycle 6 (or end-of-study) on the Subject Satisfaction and Health-Related Questionnaire.

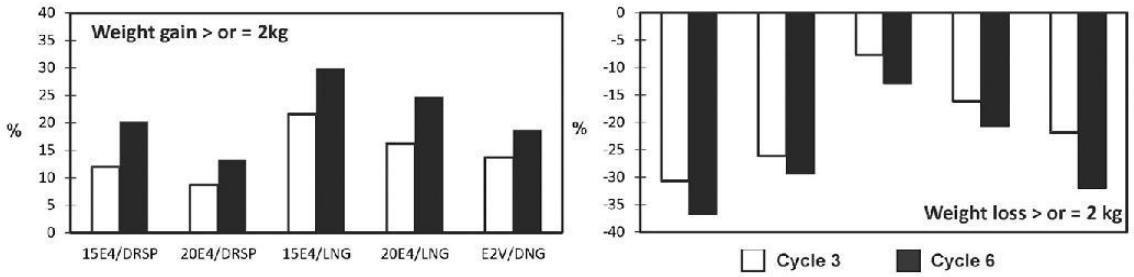

Figure 5. Proportions (%) of women with either a weight gain ≥2 kg or a weight loss ≥2 kg in each treatment at the end of cycle 3 or 6 (or end of treatment).

groups were in the same range as those in the E2V/DNG group.

Since E4 is a natural estrogen, E2V/DNG was chosen as reference COC in the present study, because it was the only marketed COC also containing a natural estrogen. E2V/DNG has shown to improve quality of life (QoL), together with a positive effect on sexuality, although DNG is a progestin with anti-androgenic properties [17–19]. This was recently confirmed in an observational study for women who switched to a COC based on a natural estrogen [20]. For this reason, it was also relevant to use E2V/DNG in the present study as a reference to assess user satisfaction and health-related quality of life with E4-containing COCs.

The EE/DRSP combination has shown to provide added benefits to the user by improving general well-being and subsequent better compliance [5,21,22]. Compared to EE/LNG, the EE/DRSP combination showed a higher self-rated improvement on the Clinical Global Impression Scale (66 versus 59%) [23]. Documented evidence suggests that EE/DRSP has a negative influence on female sexual function [24], a finding however not observed in the current study with E4/DRSP.

In the present study, compliance of study medication was similar across treatment groups. The number of women who completed the six cycles ranged between 91.1% for 15 mg E4/DRSP and 70.1% for 20 mg E4/LNG (Table 3). The high acceptability in the E2V/DNG group (89.7%), despite much heavier and persistent unscheduled bleeding than in the E4-groups [10], may be due to the open-label design of our study. Both because of the small group sizes (77–80 subjects per group) and the open-label design of the study, no *post hoc* statistical analysis was performed on discontinuation rates. Participants allocated to the E4 combinations were aware that they were using a new and experimental COC, whereas those allocated to E2V/DNG knew that they received a commercial product (Qlaira®). Nevertheless, 29.7% of women assigned to the E2V/DNG treatment answered "No or Uncertain" to the question to consider future use of this combination. This negative response was lower in the 15 mg E4/DRSP (18.0%) and 20 mg E4/DRSP (21.9%) groups (Table 4, Figure 4). In line with this finding, a high proportion of women was satisfied or very satisfied at cycle 6 in the 15 mg E4/DRSP group (73.1%) versus 68.6% for 20 mg E4/DRSP and 67.6% for E2V/DNG.

Weight gain is considered as an important and common reason for discontinuation of COCs, despite the continued need for fertility control [5]. Therefore, the higher proportion of women with weight loss in the E4/DRSP treatment groups (Figure 5) is an important advantage and may play a role in treatment compliance and continuation. An explanation for the weight loss with the DRSP COCs may be a decrease of water-retention, associated with the anti-mineralocorticoid activity of DRSP [5]. Of note, is the weight loss seen in this study with E2V/DNG at cycle 6, with a similar magnitude to the weight loss seen with E4/DRSP. This is in contrast to previous studies with E2V/DNG, where weight gain was reported as one of the most common adverse events and reason for treatment discontinuation [25].

Apart from a few women receiving 15 mg E4/LNG, menstruation returned within 2 months in those who did not start with a new hormonal contraceptive method. The absence of persistent ovarian suppression is considered relevant for future fertility of women who are ready to become pregnant [26]. Previous experience has shown that after cessation of COCs, a delay of 3–6 months in conception may occur. The results of the current study are consistent with previous data on return to fertility [26,27].

Moreover, none of the subjects experienced post-pill amenorrhea.

Strengths and weaknesses of the study

Strengths of this study are the favourable effect on body weight and the statistically significant beneficial health-related outcomes of the E4/DRSP combinations.

In this study, a non-validated questionnaire was used; however, since the outcome in the E2V/DNG group was similar to those based on validated questionnaires [17,28], it suggests that the questionnaire used in the present study can be considered reliable for its purpose. Other weaknesses are the absence of blinding of the comparator and the relatively low number of participants in each treatment group, which did not allow to differentiate between starters and switchers of the COCs investigated.

The populations in the treatment groups were balanced for age, BMI, switchers and starters, but not for smoking (Table 2). Current smoking varied between 10.7% for 20 mg E4/DRSP and 24.7% for 20 mg E4/LNG. This difference, probably due to chance, is unlikely to have an effect on the well-being outcome.

Differences in results and conclusions in relation to other studies

The present study was the first investigating the effect of E4 combinations on general well-being and body weight.

Relevance of the findings: implications for clinicians and policymakers

The primary aim of the present study was to assess vaginal bleeding patterns and cycle control of 15 and 20 mg E4 combined with either DRSP or LNG, administered during six treatment cycles in a 24:4 day regimen. It was concluded that the 15 mg E4/DRSP combination had the best properties and was superior to the E2V/DNG contraceptive [10]. The secondary objectives – user satisfaction and health-related outcomes – together with acceptability of the study medication revealed that E4/DRSP combinations have a more favourable user satisfaction outcome than the E4/LNG combinations. In addition and contrary to E4 combined with LNG, the E4/DRSP combinations had a favourable effect on body weight.

In an observational study in 39 subjects, aged <35 years, the effects of E2V/DNG on quality of life and sexual function have been assessed by using the Short Form-36 (SF-36) and Female Sexual Function Index questionnaires [28]. General Health was improved after 6 months ($p < .01$), but overall sexual function remained unchanged. In subjects <48 years, all SF-36 scales improved after 6 months ($p < .01$), and improvement of sexuality was observed based on the Short Personal Experience questionnaire ($p < .05$) [17]. Direct comparison of 15 mg E4/DRSP and E2V/DNG in the present study showed a comparable favourable overall outcome (including sexual function), satisfaction, and weight control (Figures 2–5).

Altogether, the current findings support the recommendation of 15 mg E4/DRSP as a promising new COC for further phase III clinical development [10].

Unanswered questions and future research

The current findings of a favourable effect on well-being and body weight of the E4/DRSP combinations, have to be confirmed in larger numbers of women and over longer treatment periods.

Conclusions

The present study shows that 15 mg estetrol combined with 3 mg DRSP is associated with a high user acceptability and satisfaction, and with a favourable body weight control.

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Disclosure statement

The authors alone are responsible for the content and writing of this article. M.M. and C.M. are employees; J.-M.F. and H.C.B. are Strategic Scientific Advisors at Mithra Pharmaceuticals. Y.Z. and H.C.B. are employees, and L.B. a former employee, of Pantarhei Bioscience BV. D.A. does not report any conflicts of interest.

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References

[1] Kiley J, Hammond C. Combined oral contraceptives: a comprehensive review. Clin Obstet Gynecol. 2007;50:868–877.

[2] Kost K, Singh S, Vaughan B, et al. Estimates of contraceptive failure from the 2002 National Survey of Family Growth. Contraception. 2008;77:10–21.

[3] Huber LR, Hogue CJ, Stein AD, et al. Contraceptive use and discontinuation: findings from the contraceptive history, initiation, and choice study. Am J Obstet Gynecol. 2006;194:1290–1295.

[4] Skouby SO. Contraceptive use and behavior in the 21st century: a comprehensive study across five European countries. Eur J Contracept Reprod Health Care. 2004;9:57–68.

[5] Bitzer J, Paoletti AM. Added benefits and user satisfaction with a low-dose oral contraceptive containing drospirenone: results of three multicentre trials. Clin Drug Investig. 2009;29:73–78.

[6] Coelingh Bennink HJT, Foidart JM. Estetrol, a fetal steroid for the treatment of adults. J Reprod Med Endokrinol Online. 2015;12:399–403.

[7] Duijkers IJ, Klipping C, Zimmerman Y, et al. Inhibition of ovulation by administration of estetrol in combination with drospirenone or levonorgestrel: results of a phase II dose-finding pilot study. Eur J Contracept Reprod Health Care. 2015;20:476–489.

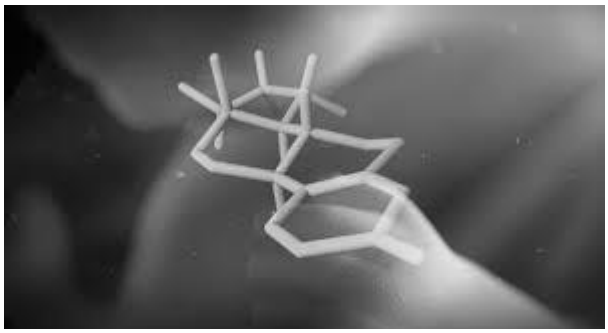
[8] Mawet M, Maillard C, Klipping C, et al. Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives. Eur J Contracept Reprod Health Care. 2015;20:463–475.

[9] Kluft C, Zimmerman Y, Mawet M, et al. Reduced haemostatic effects with drospirenone-based oral contraceptives containing estetrol versus ethinyl estradiol. Contraception. 2017;95: 140–147.
[10] Apter D, Zimmerman Y, Beekman L, et al. Bleeding pattern and cycle control with estetrol-containing combined oral contraceptives: results from a phase II, randomised, dose-finding study (FIESTA). Contraception. 2016;94:366–373.
[11] World Health Organization. Medical eligibility criteria for contraceptive use. 4th ed. Geneva, Switzerland: WHO Press; 2009. p. 15–44.
[12] Atkinson MJ, Sinha A, Hass SL, et al. Validation of a general measure of treatment satisfaction, the Treatment Satisfaction Questionnaire for Medication (TSQM), using a national panel study of chronic disease. Health Qual Life Outcomes. 2004;2:12.
[13] Perez-Campos E, Duenas JL, de la Viuda E, et al. Development and validation of the SEC-QOL questionnaire in women using contraceptive methods. Value Health. 2011;14:892–899.
[14] Egarter C, Topcuoglu MA, Imhof M, et al. Low dose oral contraceptives and quality of life. Contraception. 1999;59:287–291.
[15] Foidart JM, Wuttke W, Bouw GM, et al. A comparative investigation of contraceptive reliability, cycle control and tolerance of two monophasic oral contraceptives containing either drospirenone or desogestrel. Eur J Contracept Reprod Health Care. 2000;5:124–134.
[16] Agresti A. An introduction to categorical data analysis. 2nd ed. Wiley-Interscience; 2007.
[17] Caruso S, Agnello C, Romano M, et al. Preliminary study on the effect of four-phasic estradiol valerate and dienogest (E2V/DNG) oral contraceptive on the quality of sexual life. J Sex Med. 2011;8:2841–2850.
[18] Davis SR, Bitzer J, Giraldi A, et al. Change to either a nonandrogenic or androgenic progestin-containing oral contraceptive preparation is associated with improved sexual function in women with oral contraceptive-associated sexual dysfunction. J Sex Med. 2013;10:3069–3079.
[19] Nappi RE, Serrani M, Jensen JT. Noncontraceptive benefits of the estradiol valerate/dienogest combined oral contraceptive: a review of the literature. Int J Womens Health. 2014;6:711–718.
[20] Lete I, de la Viuda E, Perez-Campos E, et al. Effect on quality of life of switching to combined oral contraception based on natural estrogen: an observational, multicentre, prospective phase IV study (ZOCAL Study). Eur J Contracept Reprod Health Care. 2016;21:276–284.
[21] Apter D, Borsos A, Baumgartner W, et al. Effect of an oral contraceptive containing drospirenone and ethinylestradiol on general well-being and fluid-related symptoms. Eur J Contracept Reprod Health Care. 2003;8:37–51.
[22] Foidart JM. Added benefits of drospirenone for compliance. Climacteric. 2005;8(Suppl. 3):28–34.
[23] Kelly S, Davies E, Fearns S, et al. Effects of oral contraceptives containing ethinylestradiol with either drospirenone or levonorgestrel on various parameters associated with well-being in healthy women: a randomized, single-blind, parallel-group, multicentre study. Clin Drug Investig. 2010;30:325–336.
[24] Ciaplinskiene L, Zilaitiene B, Verkauskiene R, et al. The effect of a drospirenone-containing combined oral contraceptive on female sexual function: a prospective randomised study. Eur J Contracept Reprod Health Care. 2016;21:395–400.
[25] Palacios S, Wildt L, Parke S, et al. Efficacy and safety of a novel oral contraceptive based on oestradiol (oestradiol valerate/dienogest): a phase III trial. Eur J Obstet Gynecol Reprod Biol. 2010;149:57–62.
[26] Bagwell MA, Thompson SJ, Addy CL, et al. Primary infertility and oral contraceptive steroid use. Fertil Steril. 1995;63: 1161–1166.
[27] Chasan-Taber L, Willett WC, Stampfer MJ, et al. Oral contraceptives and ovulatory causes of delayed fertility. Am J Epidemiol. 1997;146:258–265.
[28] Di Carlo C, Gargano V, De Rosa N, et al. Effects of estradiol valerate and dienogest on quality of life and sexual function according to age. Gynecol Endocrinol. 2014;30:925–928.

5.3. Discussion

Altogether, the results exposed in this article point out that the E4/DRSP combinations are superior to the E4/LNG combinations regarding important domains potentially affecting subject's compliance: discontinuation in general, discontinuation specifically due to adverse events, well-being and satisfaction with the drug, impact on premenstrual symptoms, and body weight change. This demonstrates the better tolerance to DRSP than to LNG, a condition often reported by clinicians in everyday practice when these progestins are associated with EE and well-documented in the literature (Kelly, et al. 2010; Sangthawan and Taneepanichskul 2005). Among the two E4/DRSP COCs tested, 15 mg E4/DRSP generally recorded the best results (without statistical difference however in comparison to 20 mg E4/DRSP).

The results obtained with 15 mg E4/DRSP were generally the same as those obtained with the commercialized comparator (Qlaira®). This was an open-label study and consequently, the women knew if they were treated with a marketed COC or not, which can strongly influence their subjective assessments (*i.e.*, they could be more confident in an already approved product than in a product under experimentation). Therefore, having identical results in both 15 mg E4/DRSP and Qlaira® groups is seen as a significant positive sign in favor of this E4-COC over the other combinations tested.



6th Publication

Evaluation of the effect of a new oral contraceptive containing estetrol and drospirenone on hemostasis parameters

Contraception September 19, 2020 (online ahead of printing).

Jonathan Douxfils, Christine Klipping, Ingrid Duijkers, Virginie Kinet, Marie Mawet, Catherine Maillard, Maud Jost, Jan Rosing and Jean-Michel Foidart

6. Sixth publication

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6.1. Introduction

The data gathered during the dose-finding program were sufficiently robust to select the combination intended for the phase 3 program. The COC containing *15 mg E4/3 mg DRSP* was chosen using the following selection process:

- Ovulation inhibition was achieved with all the combinations tested. No selection could be made on this single criterion.
- Ovarian activity was proportional to the E4 dose with doses above 10 mg achieving a deeper ovarian function inhibition. Therefore, doses of 15 and 20 mg E4 were further evaluated.
- Although the bleeding pattern was adequate with two combinations (15 mg E4/3 mg DRSP and 20 mg E4/150 mcg LNG), absence of withdrawal bleeding was considered too high in the 20 mg E4/LNG group.
- User satisfaction, well-being and body weight change were significantly better in the E4/DRSP groups than in the E4/LNG groups. This allows selecting DRSP as the optimal progestin to be combined with E4.
- Number of adverse events and discontinuation rates were the lowest with the 15 mg E4/3 mg DRSP combination.

In line with the EMA guideline, a specific study had to be carried on with this final combination to assess the changes in hemostasis parameters, endocrinology parameters, glucose and lipids metabolism on a 6-cycle basis and to compare them with a second generation COC (EE/LNG). In addition to this first comparator, we decided to add EE/DRSP (YAZ®) as second comparator in this trial. Indeed, comparing 15 mg E4/DRSP with EE/DRSP was the only way to have a direct head-to-head comparison of the hemostatic impact of the two different estrogens.

The full title of this study was: "*A single center, randomized, open-label, controlled, three-arm study to evaluate the effect of a new combined oral contraceptive (COC) containing 15 mg estetrol (E4) and 3 mg drospirenone (DRSP) and of two reference COCs containing either 30 mcg ethinylestradiol (EE) and 150 mcg levonorgestrel (LNG) or 20 mcg EE and 3 mg DRSP on endocrine function, metabolic control and hemostasis during 6 treatment cycles*".

At the time of writing this thesis, the hemostasis and overall safety data gathered through this study were included in one article submitted to Contraception. A first round of

comments were received from the Journal's reviewers. The manuscript was adapted accordingly and the version outlined in the section below was re-submitted to the Journal. The changes in endocrinology parameters, lipid and glucose metabolism will be reported in a separate article.

6.2. Article

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Evaluation of the effect of a new oral contraceptive containing estetrol and drospirenone on hemostasis parameters ☆

Jonathan Douxfils [a,b], Christine Klipping [c], Ingrid Duijkers [c], Virginie Kinet [d], Marie Mawet [d], Catherine Maillard [d], Maud Jost [d,*], Jan Rosing [e], Jean-Michel Foidart [d,f]

[a] Qualiblood SA, Namur, Belgium
[b] Department of Pharmacy, Namur Thrombosis and Hemostasis Center, NAmur Research Institute for LIfe Sciences, University of Namur, Namur, Belgium
[c] Dinox BV, Groningen, the Netherlands
[d] Estetra SPRL, an affiliate's company of Mithra Pharmaceuticals, Liège, Belgium
[e] Department of Biochemistry, Maastricht University, Maastricht, the Netherlands
[f] University of Liège, Liège, Belgium

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ABSTRACT

Objective: To assess the effect on hemostasis parameters of a new combined oral contraceptive (COC).
Study design: In this randomized, single centre, open-label, exploratory study, healthy women received either 15 mg estetrol/3 mg drospirenone (E4/DRSP) ($n = 39$), 30 mcg ethinylestradiol/150 mcg levonorgestrel (EE/LNG) ($n = 30$), or 20 mcg ethinylestradiol/3 mg drospirenone (EE/DRSP) ($n = 32$) for six 28-day cycles. Blood was collected at baseline, cycle 3, and cycle 6. Median change from baseline was evaluated for procoagulant, anticoagulant, and fibrinolytic parameters, and for sex hormone-binding globulin (SHBG).
Results: Median change of endogenous thrombin potential (ETP) based activated protein C sensitivity resistance (APCr) at cycle 6 was +30% for E4/DRSP, +165% for EE/LNG (p-value <0.05 vs E4/DRSP), and +219% for EE/DRSP (p-value <0.05 vs E4/DRSP). Changes to prothrombin fragment 1 + 2 and SHBG for E4/DRSP, EE/LNG, and EE/DRSP were +23%, +71%, and +64% (p-value <0.05 vs E4/DRSP); and +55%, +74% and +251% (p-value <0.05 vs E4/DRSP), respectively. At cycle 6, changes to other hemostasis parameters for E4/DRSP were similar or smaller than for EE/LNG or EE/DRSP.
Conclusions: In this study, changes in hemostasis parameters after treatment with 6 cycles of E4/DRSP were smaller or similar to those observed for EE/LNG. Similar, but more pronounced changes were also observed versus EE/DRSP, which supports the hypothesis that the effect of COCs on hemostasis parameters is mainly mediated by the estrogenic component. Further studies are needed to provide more insight into the venous thromboembolic risk of E4/DRSP.
Implications statement: This study reports that the effects on hemostasis parameters of a COC containing 15 mg E4/3 mg DRSP are less or similar to those for EE/LNG or EE/DRSP. It also demonstrates that the choice of estrogen modulates the effects of COCs on hemostasis parameters.

1. Introduction

Until the mid-1990s, the effects of combined oral contraceptives (COCs) on hemostasis and associated venous thromboembolism (VTE) risk were poorly understood. Discovery of activated protein C resistance (APCr) was an important step forward in understanding the etiology of VTE [1]. Inherited APCr, caused by a mutation in factor V called factor V Leiden (FV Leiden), increases the VTE risk by ~4-fold [2]. In COC users, APCr is also observed, with COCs containing desogestrel (DSG), gestodene (GSD), or drospirenone (DRSP) showing normalized APC sensitivity ratio (nAPCsr) and VTE risk similar to that of heterozygous carriers of

☆ Conflict of interest: J. Douxfils is the director and founder of Qualiblood, a contract research organization that received funding from Mithra for the conduct of the study. He also reports personal fees from Daiichi-Sankyo, Diagnostica Stago, Portola, Roche and Roche Diagnostics. J. Rosing has provided expert witness testimony relating to effects of hormonal contraceptives on blood coagulation and his laboratory executed several industry-sponsored studies on the effects of female sex hormones on coagulation. He reports personal fees from Mithra and from Pantharei Bioscience and Oncology.C. Klipping and I. Duijkers are directors of Dinox BV, a contract research organization that received funding from Mithra for the conduct of the study. V. Kinet, M. Mawet, C. Maillard and M. Jost are employees of Mithra. JM Foidart is a member of the board at Mithra and received financial support for the supervision of this study.

* Corresponding author.
E-mail address: mjost@mithra.com (M. Jost).

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FV Leiden [2–5]. Epidemiologic studies have shown that relatively small increases of coagulation factors (*e.g.* prothrombin and factor VIII), small decreases of anticoagulant proteins (*e.g.* tissue factor pathway inhibitor [TFPI], protein S and antithrombin) and acquired APCr, can explain the increased VTE risk observed in COC users [6]. The use of COCs containing DSG, GSD, or DRSP in combination with ethinylestradiol (EE) results in more pronounced changes of coagulation markers and a higher observed VTE risk in comparison with EE/levonorgestrel (LNG) [7,8][9]. This difference is likely a result of the weaker antagonism of the EE-induced changes in hemostasis variables with DSG, GSD, and DRSP compared to LNG [10]. Combined oral contraceptives containing the natural adult hormone estradiol (E2) appear to have less effect on hemostasis and recent epidemiological data reported the low VTE risk associated with the combination of E2 valerate with dienogest [9–12].

Estetrol (E4) is a native estrogen produced by the human fetal liver. E4, chemically synthesized from estrone, is identical to the natural hormone and has potential for contraceptive use in humans [13,14]. So far, the hemostatic profile of E4 in combination with 3 mg DRSP has been evaluated with doses up to 10 mg [15]. The current study investigated the hemostatic effects of a 15 mg E4/3 mg DRSP combination, as selected for phase 3 development [16,17].

2. Material and methods

2.1. Study design

This single center, randomized, open-label, controlled, three-arm, parallel study in healthy females was conducted from September 2016 through October 2017 at Dinox BV, Groningen, the Netherlands (EudraCT 2016-001316-37, Clinicaltrials.gov NCT02957630). The study, performed in accordance with the Declaration of Helsinki and the ICH E6 (R2) Good Clinical Practice guidelines, was approved by an independent local ethics committee and written informed consent was obtained from all participants before study entry. The study consisted of a pretreatment cycle, followed by six treatment cycles of 28 days. A total of 100 healthy women (40 in the investigational group and 30 per comparator group) was planned to be included in the study. Visits were planned to be at screening, at randomization/baseline, at cycle 3, at cycle 6, and at the end of study.

2.2. Study population

Healthy females aged 18–50 years with a body mass index between 18 and 30 kg/m^2, and a natural menstrual cycle of maximum 35 days were eligible for inclusion. Main exclusion criteria were contraindications for the use of hormonal contraceptives, known coagulopathy or thrombogenic mutation, the use of anticoagulants or other drugs affecting coagulation and platelet aggregation, and an abnormal Papanicolaou smear test. The use of an injectable contraceptive was not allowed within 3–10 months prior to screening, depending on the type of injection. Women with combined hormonal contraceptive use prior to the study had a washout period of one menstrual cycle before the pretreatment cycle. The pretreatment cycle started on the first day of the menstrual cycle following screening or following washout if washout was required.

2.3. Study treatment

Eligible subjects were stratified by previous hormonal contraceptive use (2 cycles or >2 cycles without use before study treatment start) and by age (≤35 years or >35 years of age). Subjects were then assigned, using a computerized random allocation sequence, to one of the following treatments in a 4:3:3 ratio: 15 mg E4 (as monohydrate, equivalent to 14.2 mg anhydrate) combined with 3 mg DRSP (E4/DRSP; 24 day active/4 day placebo regimen), 30 mcg EE combined with 150 mcg LNG (EE/LNG; 21 day active/7 day placebo regimen), or 20 mcg EE combined with 3 mg DRSP (EE/DRSP; 24 day active/4 day placebo regimen). E4/DRSP was manufactured by Haupt Pharma, Münster, Germany and provided by Estetra SPRL, an affiliate's company of Mithra Pharmaceuticals, Liège, Belgium. EE/LNG (Melleva® 150/30, Leon Farma) and EE/DRSP (Yaz®, Bayer Healthcare) were obtained from a local pharmacy. Study treatment started on the first day of the menstrual cycle following the pretreatment cycle. Treatment compliance was verified by the use of a diary and check of returned packages.

2.4. Study assessments and outcome parameters

2.4.1. Evaluation of hemostasis parameters and sex hormone-binding globulin

The primary endpoint included the evaluation of the following coagulation and fibrinolytic parameters: fibrinogen, prothrombin, factor VII, factor VIII, von Willebrand factor, antithrombin, protein S activity, free protein S, protein C, free TFPI, plasminogen, plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen activator (tPA), ETP-based APCr (expressed as nAPCsr), D-dimer and prothrombin fragment 1 + 2. In addition, sex hormone-binding globulin (SHBG) was also measured. Blood samples for measurement of hemostasis parameters and SHBG were collected at baseline and between days 18 and 21 of cycles 3 and 6. Blood was collected in citrate tubes and serum-separating tubes and processed to plasma or serum. Samples were shipped on dry ice to BARC (Gent, Belgium) and QUALIblood laboratories (Namur, Belgium) for analysis. The analytical methods, including reference ranges are presented in the Supplemental Table 1.

2.5. Statistical analysis

Statistical analyses were performed using SAS® software for Windows® (SAS Institute Inc, Cary, NC, USA). Statisticians were unblinded to treatments. All randomized subjects who received at least one dose of the study medication and had at least one hemostasis assessment on treatment, without any major protocol deviation impacting the endpoints, were included in the analysis (per protocol dataset). Hemostasis parameters were summarized using descriptive statistics (*n*, mean, standard deviation [SD], minimum, median, maximum, and coefficient of variation [CV]). No formal sample size calculation was performed, and no formal statistical analysis was planned for this exploratory study. However, additional exploratory nonparametric analysis was performed on the absolute change from baseline. Exploration of the difference between results observed at baseline and cycle 3 and baseline and cycle 6 in each treatment was performed using a Wilcoxon signed-rank test. A Kruskal–Wallis test was used to assess the homogeneity of the change from baseline among the three treatments, separately at cycle 3 and at cycle 6. Where results of additional analyses were significant, pairwise comparison of the treatments was done using the Dwass–Steel–Critchlow–Fligner procedure. Due to the exploratory nature of the study, no correction for multiplicity was made. All statistical tests were evaluated with a level of significance of 0.05.

Table 4
Functional coagulation tests, markers of ongoing coagulation and SHBG: Median (min, max) values at baseline, cycle 3 and cycle 6 and changes from baseline (%) at cycle 3 and cycle 6.

Parameter	Treatment	Baseline	Cycle 3		Cycle 6[1]	
		Value	Value	CFB (%)	Value	CFB (%)
Functional coagulation tests						
APC resistance, ETP-based (nAPCsr)	E4/DRSP	1.67 (0.53, 3.35)	2.31 (0.81, 4.12)	39.5 (−19, 117.0)*	2.09 (0.80, 4.28)	30.0 (−53.0, 233.0)*
	EE/LNG	1.49 (0.00, 5.03)	3.61 (1.74, 6.67)	165.0 (33.0, 496.0)*,#	3.35 (0.73, 7.39)	164.5 (30.0, 424.0)*,#
	EE/DRSP	1.37 (0.46, 3.06)	4.36 (2.64, 6.13)	229.0 (91.0, 781.0)*,#	4.50 (2.54, 5.85)	218.5 (99.0, 763.0)*,#
Markers for ongoing coagulations						
D-dimer (ug/mL FEU)	E4/DRSP	0.27 (0.27, 0.43)	0.27 (0.27, 0.86)	0.0 (−36.0, 219.0)	0.29 (0.27, 0.59)	4.0 (−33.0, 97.0)*
	EE/LNG	0.27 (0.27, 2.28)	0.31 (0.27, 1.82)	0.0 (−65.0, 59.0)	0.33 (0.27, 1.19)	7.0 (−84.0, 93.0)
	EE/DRSP	0.27 (0.27, 1.14)	0.28 (0.27, 1.43)	0.0 (−46.0, 93.0)	0.31 (0.27, 0.93)	0.0 (−33.0, 93.0)
Prothrombin fragment 1 + 2 (nmol/L)	E4/DRSP	0.133 (0.060, 0.261)	0.129 (0.064, 0.284)	7.0 (−39.0, 73.0)	0.151 (0.086, 0.360)	23.0 (−34.0, 108.0)*
	EE/LNG	0.111 (0.060, 0.332)	0.169 (0.098, 0.343)	62.0 (2.0, 125.0)*,#	0.184 (0.118, 0.818)	71.0 (−27.0, 357.0)*,#
	EE/DRSP	0.114 (0.073, 0.0260)	0.169 (0.095, 0.416)	47.5 (−6.0, 187.0)*,#	0.191 (0.094, 0.377)	64.0 (1.0, 127.0)*,#
SHBG						
SHBG (nmol/L)	E4/DRSP	64.8 (25.3, 117.9)	97.0 (45.5, 185.7)	51.5 (−23.0, 132.0)*	87.2 (52.7, 196.0)	55.0 (−22.0, 171.0)*
	EE/LNG	67.3 (27.1, 144.4)	118.6 (58.5, 187.6)	67.0 (−10.0, 313.0)*	119.8 (65.2, 191.4)	74.0 (−17.0, 261.0)*
	EE/DRSP	70.6 (36.2, 125.6)	245.9 (164.0, 382.0)	239.5 (128.0, 608.0)*,#	264.3 (162.3, 447.4)	251.0 (122.0, 637.0)*,#

APC = activated protein C; ETP = endogenous thrombin potential; SHBG = sex hormone-binding globulin; CFB = Change from baseline; TF, tissue factor; TFPI, tissue factor pathway inhibitor; t-PA, tissue plasminogen activator inhibitor; vWF, von Willebrand Factor.
[1] Data at Cycle 6 or end of treatment.
* Different versus baseline, $p < 0.05$ using a signed rank test.
# Different from treatment with 15 mg E4/3 mg DRSP, $p < 0.05$ using the Dwass–Steel–Critchlow–Fligner test.

with EE/DRSP this change could almost be qualified as a protein S deficiency (*i.e.* levels around 50%). However, protein S activity assays have shown decreased protein S levels in 10–15 percent of healthy subjects, and that values may return to normal when the test is repeated on a new sample [20]. The statistically significant decrease of TFPI levels observed for EE/DRSP, *i.e.* −20% versus −8.5%, and −6% for E4/DRSP and EE/LNG (Table 3), was in line with TFPI levels observed in the literature [3]. Low plasma TFPI is probably a weak risk factor for VTE [22], but it remains an open question whether a reduction of TFPI during COC use is associated with an increased risk of VTE.

Concerning the fibrinolytic system, the change in tPA levels with E4/DRSP was smaller compared with EE/LNG or EE/DRSP (−7%, −33%, and −39.5%, respectively). The changes in PAI-1 levels were not different between the 3 treatments (+20%, +0%, and +0%, for E4/DRSP, EE/LNG and EE/DRSP, respectively) while all treatments induced a significant increase in plasminogen levels (+12%, +40%, and +35.5% for E4/DRSP, EE/LNG and EE/DRSP, respectively) (Table 3). These results do not allow any conclusions to be drawn regarding a potential hypo- or hyper-fibrinolytic profile of E4/DRSP but do underline the weak impact of E4/DRSP on fibrinolysis markers. In addition, there is little evidence that changes to fibrinolysis parameters are important risk factors for VTE.

The change in nAPCsr, as determined by the ETP-based APCr, was statistically significant, being lower for E4/DRSP (+30%) in comparison with EE/LNG (+165%), and EE/DRSP (+219%) (Table 4). Activated protein C resistance, determined with an ETP-based assay, has been considered to be a suitable marker to assess the thrombogenicity of COCs for two decades [12,20] and the extent of ETP-based APCr is related to the thrombotic risk of newer COC preparations, the contraceptive patch, hormone replacement therapy, pregnancy, and other VTE risk states [23,24]. In addition, a relationship between an increase in the nAPCsr and the risk of VTE was reported by Morimont et al. [25]. In their study, mean nAPCsr values for different COC combinations ranged from 3.75 to 5.40 while in non-users the mean nAPCsr (±SD) was 1.68 (±0.88) [25]. Modeling showed that these nAPCsr values were exponentially related to the relative VTE risk of COCs seen in epidemiological studies (r-square = 0.95), but clearly prospective data are needed to confirm the predictive value of the nAPCsr for the assessment of VTE risk.

The increase of D-dimer was relatively small (+4%, +7% and +0% for E4/DRSP, EE/LNG and EE/DRSP, respectively). The tight dispersion around the median value at baseline in the E4/DRSP group compared with the high dispersion in the EE/LNG and EE/DRSP groups may explain the significance observed for E4/DRSP, which was not observed for EE/LNG, despite the numerically higher values for the latter. D-dimer is mainly used for diagnosing patients who present with a low-medium clinical probability of having VTE, and many variables may increase the D-dimers transiently. Therefore, changes in D-dimer should not be regarded as predictive of any thrombotic risk [21]. Prothrombin fragment 1 + 2, which represents a direct marker of ongoing coagulation since it results in the catalytic conversion of inactive prothrombin into active thrombin, was increased for E4/DRSP (+23%) but this increase was significantly lower in comparison with EE/LNG (+71%) and EE/DRSP (+64%). However, the absolute changes were low and show high dispersion, especially in the EE/LNG arm (Table 4).

4.2. Differential effect of estetrol and ethinylestradiol on hemostasis and liver biomarkers

EE/DRSP has been associated with a higher risk of VTE than EE/LNG [5]. The use of E4 instead of EE in combination with DRSP appears to reduce the effect on hemostatic parameters, including the nAPCsr [15]. E4/DRSP was reported to have a neutral profile on the liver compared to EE/DRSP [22]. This also translated into a minimal effect on triglycerides, angiotensinogen and SHBG, although tested at lower E4 doses than in this study [23]. The mild increase of SHBG observed with E4/DRSP, which was significantly less than the change of SHBG with EE/DRSP, suggests a lower estrogenic effect of E4 on the liver. The lower effect observed with EE/LNG compared with EE/DRSP is explained by the androgenic properties of LNG, which are absent with DRSP [10]. This enables LNG to

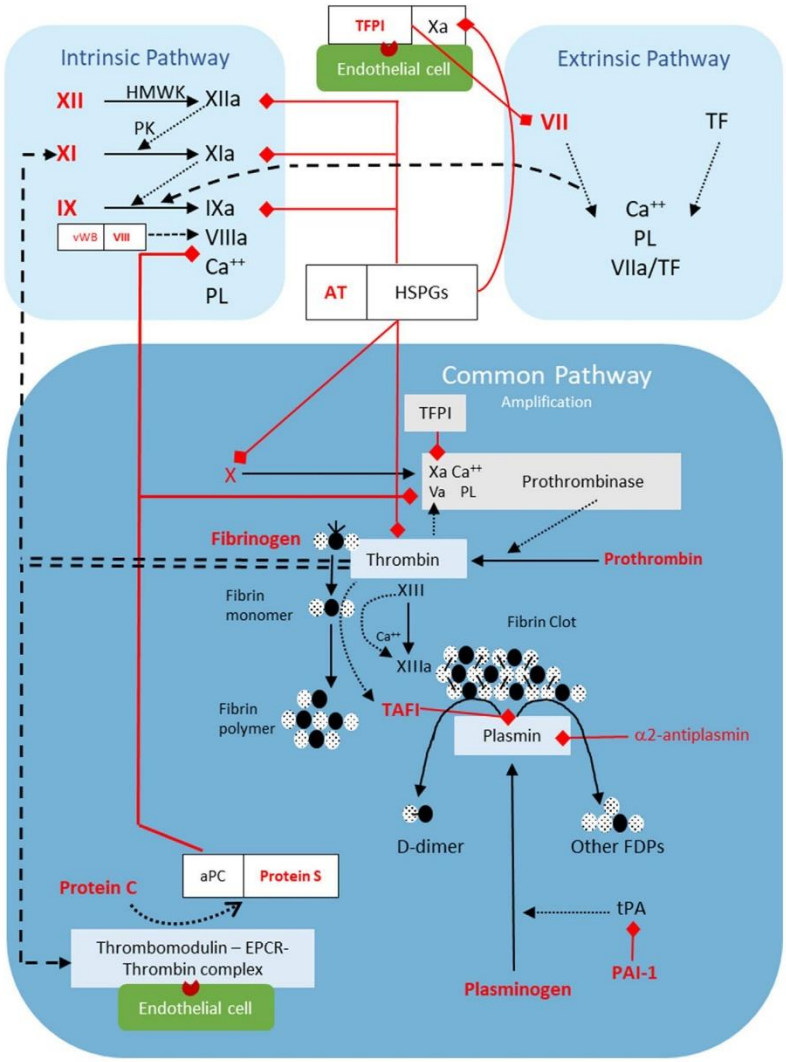

Fig. 2. A simplified scheme of the coagulation and fibrinolysis pathways. *Factor or proteins in orange are those suspected to be altered by COC treatments. Dashed black lines represent activation while black lines represent factor or protein activation. Red lines represent inhibitory actions. Abbreviations: aPC, activated protein C; AT, antithrombin; Ca^{++}, calcium; EPCR, endothelial protein C receptor; FDP, fibrin degradation product; HMWK, high molecular weight kininogen; HSPG, heparan sulfate proteoglycan; PAI-1, plasminogen activator inhibitor-1; PK, pre-kallikrein; PL, phospholipids; TAFI, thrombin activatable fibrinolysis inhibitor.*

counteract the effects of EE on the liver, thereby reducing total estrogenicity and thrombogenicity [4,7].

4.3. Strengths and limitations

This study was conducted with a limited number of subjects with no former sample size calculation. A sample size of 30–40 patients per treatment arm was chosen since several studies have shown that this study size was sufficient to estimate clinically meaningful changes of hemostasis parameters during COC use. Due to the exploratory nature of the study, no correction for multiplicity was made. A classical Bonferroni adjustment would have resulted in a significance level of 0.003 (0.05/17), if such adjustment had been performed with the 17 different parameters (coagulation factors and SHBG) assessed in this study. It is worth emphasizing that all values of the pairwise comparisons indicated as $p < 0.05$ were $p < 0.003$ (except for tPA, $p = 0.0046$ for the difference between E4/DRSP and EE/LNG) confirming the robustness of the findings.

5. Conclusion

In this study, changes in hemostasis parameters after treatment with 6 cycles of E4 DRSP were smaller or similar to those observed for EE/LNG. More pronounced changes were observed versus EE/DRSP, which supports the hypothesis that the effect of COCs on hemostasis parameters is mainly mediated by the estrogenic component. The neutral profile of E4/DRSP on hemostasis parameters suggests that it is less likely to be associated with VTE risk, but this has to be confirmed by data obtained from post-marketing surveillance program assessing the clinical occurrence of VTE events.

Table 2
Procoagulant factors: Median (min, max) values at baseline, at cycle 3 and at cycle 6 and changes from baseline (%) at cycle 3 and cycle 6.

Parameter	Treatment	Baseline	Cycle 3		Cycle 6[1]	
		Value	Value	CFB (%)	Value	CFB (%)
Fibrinogen (mg/dL)	E4/DRSP	240 (173, 355)	258 (153, 372)	3.5 (−23.0, 77.0)	262 (197, 370)	10.0 (−24.0, 54.0)*
	EE/LNG	239 (144, 472)	274 (182, 424)	8.0 (−38.0, 110.0)*	280 (168, 430)	5.0 (−35.0, 95.0)
	EE/DRSP	229 (176, 409)	291 (190, 388)	22.0 (−36.0, 69.0)*,#	280 (225, 436)	16.0 (−44.0, 58.0)*
Prothrombin (%)	E4/DRSP	86 (67, 106)	92 (69, 109)	9.0 (−11.0, 24.0)*	91 (74, 107)	7.0 (−12.0, 26.0)*
	EE/LNG	87 (72, 112)	101 (83, 119)	13.0 (−1.0, 35.0)*,#	96 (84, 129)	13.0 (−7.0, 32.0)*
	EE/DRSP	89 (77, 112)	101 (88, 125)	14.5 (1.0, 33.0)*,#	101 (81, 117)	7.0 (−4.0, 49.0)*
Factor VII (%)	E4/DRSP	94 (61, 166)	92 (54, 133)	−4.0 (−30.0, 26.0)*	95 (68, 141)	−3.0 (−31.0, 44.0)
	EE/LNG	93 (53, 127)	83 (56, 118)	−7.0 (−41.0, 43.0)*	80 (53, 128)	−5.0 (−38.0, 47.0)*
	EE/DRSP	94 (64, 130)	116 (56, 187)	18.5 (−16.0, 65.0)*,#	113 (64, 178)	20.0 (−14.0, 49.0)*,#
Factor VIII (%)	E4/DRSP	114 (85, 255)	135 (90, 268)	11.0 (−28.0, 58.0)*	125 (80, 226)	5.0 (−33.0, 74.0)
	EE/LNG	115 (74, 217)	126 (90, 231)	9.0 (−35.0, 62.0)	112 (70, 209)	3.0 (−38.0, 79.0)
	EE/DRSP	110 (72, 203)	136 (80, 226)	20.5 (−15.0, 56.0)*	118 (68, 217)	9.0 (−33.0, 66.0)
Von Willebrand factor (%)	E4/DRSP	103 (60, 240)	106 (70, 212)	5.0 (−21.0, 39.0)*	108 (63, 202)	5.0 (−26.0, 52.0)
	EE/LNG	96 (56, 295)	94 (52, 266)	−2.0 (−41.0, 33.0)	97 (44, 263)	−2.0 (−41.0, 38.0)
	EE/DRSP	88 (55, 154)	94 (60, 167)	7.5 (−15.0, 42.0)*	96 (62, 192)	13.0 (−19, 51.0)*

CFB = Change from baseline.
[1] Data at Cycle 6 or end of treatment.
* Different versus baseline, $p < 0.05$ using a signed rank test.
# Different from treatment with 15 mg E4/3 mg DRSP, $p < 0.05$ using the Dwass–Steel–Critchlow–Fligner test.

Table 3
Anticoagulant proteins and fibrinolytic proteins: Median (min, max) values at baseline, cycle 3 and cycle 6 and changes from baseline (%) at cycle 3 and cycle 6.

Parameter	Treatment	Baseline	Cycle 3		Cycle 6[1]	
		Value	Value	CFB (%)	Value	CFB (%)
Anticoagulant proteins						
Antithrombin (%)	E4/DRSP	96 (87, 110)	97 (87, 113)	0.0 (−11.0, 13.0)	98 (85, 113)	−1.0 (−9.0, 13.0)
	EE/LNG	99 (64, 124)	96 (64, 112)	−2.0 (−17.0, 14.0)*	94 (66, 117)	−5.0 (−14.0, 13.0)*
	EE/DRSP	97 (88, 114)	96 (83, 107)	−2.5 (−13.0, 14.0)	97 (83, 116)	−3.5 (−17.0, 16.0)
Protein S activity (%)	E4/DRSP	93 (74, 134)	92 (72, 145)	1.0 (−22.0, 33.0)	89 (69, 126)	−4.0 (−23.0, 30.0)
	EE/LNG	105 (82, 142)	101 (80, 145)	−2.0 (−32.0, 59.0)	96 (79, 133)	−5.0 (−29.0, 35.0)*
	EE/DRSP	104 (65, 149)	80 (55, 109)	−26.0 (−41.0, −6.0)*,#	74 (51, 106)	−30.5 (−45.0, −8.0)*,#
Protein S free (%)	E4/DRSP	84 (59, 111)	90 (70, 114)	8.0 (−10.0, 34.0)*	87 (61, 117)	5.0 (−19.0, 32.0)
	EE/LNG	94 (76, 121)	99 (70, 136)	2.0 (−27.0, 66.0)	92 (71, 125)	−3.0 (−24.0, 29.0)*
	EE/DRSP	88 (69, 134)	71 (53, 103)	−21.0 (−36.0, 0.0)*,#	69 (49, 88)	−22.5 (−46.0, 1.0)*,#
Protein C (%)	E4/DRSP	95 (73, 141)	94 (73, 137)	1.0 (−14.0, 32.0)	91 (72, 140)	2.0 (−19.0, 26.0)
	EE/LNG	98 (71, 135)	106 (83, 142)	12.0 (−11.0, 34.0)*,#	101 (75, 137)	7.0 (−14.0, 39.0)*
	EE/DRSP	98 (69, 122)	111 (88, 171)	19.5 (−9.0, 46.0)*,#	114 (87, 183)	17.5 (0.0, 56.0)*,#
TFPI free (U/mL)	E4/DRSP	1.1 (0.5, 1.6)	1.0 (0.6, 1.6)	9.0 (−56.0, 74.0)	1.0 (0.6, 1.6)	−8.5 (−53.0, 52.0)
	EE/LNG	1.0 (0.7, 1.6)	1.1 (0.6, 1.6)	−3.0 (−46.0, 100.0)	1.0 (0.6, 1.6)	−6.0 (−58.0, 63.0)
	EE/DRSP	1.0 (0.7, 1.6)	1.0 (0.6, 1.6)	−19.0 (−58.0, 98.0)	1.0 (0.5, 1.3)	−20.0 (−49.0, 30.0)*
Fibrinolytic proteins						
Plasminogen (%)	E4/DRSP	94 (68, 123)	107 (72, 126)	12.0 (−12.0, 31.0)*	108 (82, 131)	12.0 (−5.0, 35.0)*
	EE/LNG	96 (78, 127)	135 (111, 162)	45.0 (13.0, 65.0)*,#	136 (110, 173)	40.0 (15.0, 80.0)*,#
	EE/DRSP	99 (78, 123)	131 (111, 162)	32.0 (9.0, 78.0)*,#	132 (114, 172)	35.5 (6.0, 68.0)*,#
PAI-1 (U/mL)	E4/DRSP	0.9 (0.5, 5.5)	1.1 (0.5, 3.3)	0.0 (−68.0, 560.0)	1.3 (0.5, 3.7)	20.0 (−68.0, 400.0)
	EE/LNG	0.5 (0.5, 6.2)	0.5 (0.5, 2.5)	0.0 (−92.0, 400.0)	0.5 (0.5, 1.5)	0.0 (−92.0, 200.0)
	EE/DRSP	0.7 (0.5, 6.2)	0.5 (0.5, 6.2)	0.0 (−89.0, 120.0)*	0.6 (0.5, 5.2)	0.0 (−87.0, 240.0)
t-PA (ng/mL)	E4/DRSP	4.5 (2.0, 10.6)	3.8 (2.2, 6.3)	−16.0 (−51.0, 104.0)*	4.1 (2.1, 8.3)	−7.0 (−52.0, 79.0)
	EE/LNG	4.3 (1.9, 12.7)	2.7 (1.6, 4.7)	−35.0 (−74.0, 34.0)*	2.8 (1.2, 4.4)	−33.0 (−84.0, 29.0)*,#
	EE/DRSP	4.4 (1.5, 13.1)	2.9 (1.8, 8.7)	−35.5 (−70.0, 93.0)*	2.8 (1.0, 7.0)	−39.5 (−72.0, 80.0)*,#

TFPI = tissue factor pathway inhibitor; PAI-1 = plasminogen activator inhibitor-1; t-PA = tissue plasminogen activator; CFB = Change from baseline.
[1] Data at Cycle 6 or end of treatment.
* Different versus baseline, $p < 0.05$ using a signed rank test.
# Different from treatment with 15 mg E4/3 mg DRSP, $p < 0.05$ using the Dwass–Steel–Critchlow–Fligner test.

4.1. Impact of E4/DRSP on hemostasis biomarkers

Changes in levels of procoagulant proteins were small for E4/DRSP and EE/LNG. For some subjects in the EE/DRSP group, levels of FVII slightly exceeded the threshold that is used to classify subjects with a higher thrombotic risk (*i.e.* >170% of FVII levels observed in the normal population), although there is little evidence suggesting that a greater FVII level is a risk factor for VTE [19]. None of these subjects had FVII levels above the threshold at baseline (Table 2).

No relevant changes were observed for the anticoagulant factors antithrombin, protein S (free and activity), protein C, and TFPI levels with E4/DRSP (Table 3). Levels and activity of protein S were decreased for EE/LNG and EE/DRSP, and in some subjects treated

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.contraception.2020.08.015.

References

[1] Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A 1993;90:1004–8.
[2] Wu O, Robertson L, Langhorne P, et al. Oral contraceptives, hormone replacement therapy, thrombophilias and risk of venous thromboembolism: a systematic review: The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) Study. Thromb Haemost 2005;94:17–25.
[3] Rosing J, Tans G, Nicolaes GA, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol 1997;97:233–8.
[4] Tchaikovski SN, Rosing J. Mechanisms of estrogen-induced venous thromboembolism. Thromb Res 2010;126:5–11.
[5] Lidegaard O, Nielsen LH, Skovlund CW, Skjeldestad FE, Lokkegaard E. Risk of venous thromboembolism from use of oral contraceptives containing different progestogens and oestrogen doses: Danish cohort study, 2001–9. BMJ 2011;343:d6423.
[6] Westhoff CL, Pike MC, Cremers S, Eisenberger A, Thomassen S, Rosing J. Endogenous thrombin potential changes during the first cycle of oral contraceptive use. Contraception 2017;95:456–63.
[7] Farris M, Bastianelli C, Rosato E, Brosens I, Benagiano G. Pharmacodynamics of combined estrogen-progestin oral contraceptives: 2. effects on hemostasis. Expert Rev Clin Pharmacol 2017;10:1129–44.
[8] Agren UM, Anttila M, Maenpaa-Liukko K, et al. Effects of a monophasic combined oral contraceptive containing nomegestrol acetate and 17beta-oestradiol compared with one containing levonorgestrel and ethinylestradiol on haemostasis, lipids and carbohydrate metabolism. Eur J Contracept Reprod Health Care 2011;16:444–57.
[9] Dragoman MV, Tepper NK, Fu R, Curtis KM, Chou R, Gaffield ME. A systematic review and meta-analysis of venous thrombosis risk among users of combined oral contraception. Int J Gynaecol Obstet 2018;141:287–94.
[10] Gaussem P, Alhenc-Gelas M, Thomas JL, et al. Haemostatic effects of a new combined oral contraceptive, nomegestrol acetate/17beta-estradiol, compared with those of levonorgestrel/ethinyl estradiol. A double-blind, randomised study. Thromb Haemost 2011;105:560–7.
[11] Fruzzetti F, Cagnacci A. Venous thrombosis and hormonal contraception: what's new with estradiol-based hormonal contraceptives?. Open Access J Contracept 2018;9:75–9.
[12] Heinemann K, Franke C, Moehner S, Do Minh T, Dinger J. Cardiovascular safety in users of different combined oral contraceptives – Final results from the INAS-SCORE study. Abstract FC-03. Eur J Contracept Reprod Health Care 2018;23:40.
[13] Holinka CF, Diczfalusy E, Coelingh Bennink HJ. Estetrol: a unique steroid in human pregnancy. The J Steroid Biochem Mol Biol 2008;110:138–43.
[14] Coelingh Bennink HJT, Holinka CF, Diczfalusy E. Estetrol review: profile and potential clinical applications. Climacteric 2008;11(Suppl 1):47–58.
[15] Kluft C, Zimmerman Y, Mawet M, et al. Reduced hemostatic effects with drospirenone-based oral contraceptives containing estetrol vs. ethinyl estradiol. Contraception 2017;95:140–7.
[16] Apter D, Zimmerman Y, Beekman L, et al. Bleeding pattern and cycle control with estetrol-containing combined oral contraceptives: results from a phase II, randomised, dose-finding study (FIESTA). Contraception 2016;94:366–73.
[17] Apter D, Zimmerman Y, Beekman L, Mawet M, Maillard C, Foidart J-M, Coelingh Bennink HJT. Estetrol combined with drospirenone: an oral contraceptive with high acceptability, user satisfaction, well-being and favourable body weight control. Eur J Contracept Reprod Health Care 2017;22:260–7.
[18] Curvers J, Thomassen MC, Rimmer J, et al. Effects of hereditary and acquired risk factors of venous thrombosis on a thrombin generation-based APC resistance test. Thromb Haemost 2002;88:5–11.
[19] Kluft C, Meijer P, LaGuardia KD, Fisher AC. Comparison of a transdermal contraceptive patch vs. oral contraceptives on hemostasis variables. Contraception 2008;77:77–83.
[20] Klipping C, Duijkers I, Parke S, Mellinger U, Serrani M, Junge W. Hemostatic effects of a novel estradiol-based oral contraceptive: an open-label, randomized, crossover study of estradiol valerate/dienogest versus ethinylestradiol/levonorgestrel. Drugs R D 2011;11:159–70.
[21] Favresse J, Lippi G, Roy PM, et al. D-dimer: Preanalytical, analytical, postanalytical variables, and clinical applications. Crit Rev Clin Lab Sci 2018;55:548–77.
[22] Abot A, Fontaine C, Buscato M, et al. The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation. EMBO Mol Med 2014;6:1328–46.
[23] Mawet M, Maillard C, Klipping C, Zimmerman Y, Foidart JM, Coelingh Bennink HJ. Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives. Eur J Contracept Reprod Health Care 2015;20:463–75.
[24] Duijkers IJ, Klipping C, Zimmerman Y, et al. Inhibition of ovulation by administration of estetrol in combination with drospirenone or levonorgestrel: results of a phase II dose-finding pilot study. Eur J Contracept Reprod Health Care 2015;20:476–89.
[25] Morimont L, Dogńe JM, Douxfils J. Letter to the Editors-in-Chief in response to the article of Abou-Ismail, et al. entitled "Estrogen and thrombosis: A bench to bedside review" (Thrombosis Research 192 (2020) 40-51). Thromb Res. 2020;193:221–3.
[26] Douxfils J, Morimont L, Delvigne AS, et al. Validation and standardization of the ETP-based activated protein C resistance test for the clinical investigation of steroid contraceptives in women: an unmet clinical and regulatory need. Clin Chem Lab Med 2020;58:294–305.

6.3. Discussion

In addition to the data delivered in the article itself, this study has delivered important data:

1. To our knowledge, this was the first time that the 4th generation EE/DRSP was directly compared to the 2nd generation EE/LNG in terms of change in hemostasis parameters. Indeed, the European authorities request an assessment of the hemostasis parameters with a new COC before seeking marketing authorization and this study must be comparative. However, in its guideline, EMA leaves the choice between two comparators, namely a 3rd generation COC (e.g. EE/DSG) or a 2nd generation (EMA 2006). The hemostasis study conducted with EE/DRSP used EE/DSG as comparator, and therefore no scientific article until today was available that directly compared the hemostasis impact of 4th and 2nd generation COCs (Kluft, et al. 2006).
2. Our study will also probably allow to increase the predictability of hemostasis parameters. Indeed, as outlined in Chapter 1, Section 4.7, a big step forward in the estro-progestin field would be to delineate one (or a combination of) hemostasis parameter(s) able to predict the real risk of VTE with a specific combination without having to conduct a large and long post-marketing study. Our study included three COCs: for the two marketed ones (EE/LNG and EE/DRSP), the risk of VTE is well established in the literature. In our study we used state-of-the art bioassays to measure the hemostasis parameters which was not always the case in former studies. Our data are therefore very useful to seek such correlation and represent an important next step for the scientific community.

The EMA guideline recommends evaluating hemostasis changes on a 6 cycle basis. However, it appears that the changes are already visible after 3 cycles of treatment without further changes. This is also seen in similar studies conducted for other COCs (Agren, et al. 2011; Junge, et al. 2011). We think that this should be taken into account by the health authorities in order to reduce the length of these studies and avoid unnecessary exposition of the subjects.

CHAPTER 3: DISCUSSION

Combined oral contraceptive is a birth control method combining two steroids, namely a progestin and an estrogen. Most COCs contain EE, a potent synthetic estrogen, as estrogenic compound. Use of COC is very popular for two reasons: first, it has a high contraceptive efficacy and secondly, it displays several non-contraceptive benefits such as improvement of menstrual bleeding issues, dysmenorrhea and acne. However COC use is associated with rare but potentially fatal VTEs. This VTE risk is proportional to the EE dose in the combination and the risk has decreased with the use of lower doses (from 50 mcg to 20 mcg). A further lowering in the content of EE is associated with an inadequate bleeding pattern. Therefore, research in women's health has turned to select estrogen compounds which would display a safer profile: two E2-containing COCs have therefore been launched during this last decade. The VTE risk associated with these COCs is probably close to that of second generation COCs but they fail to become popular among women and clinicians probably because of a less comfortable bleeding pattern in comparison to the EE-containing COCs. The type of progestin, by modulating the overall estrogenicity of the combination, also influences the VTE risk: the incidence of VTE is lower with more androgenic progestins than with less or anti-androgenic progestins.

Among all the available progestins, DRSP is a special one: it is a spironolactone derivative and it displays both anti-mineralocorticoid and anti-androgenic properties. A first COC combining 3 mg DRSP with 30 mcg EE was launched in 2001 and became rapidly very popular because of its numerous non-contraceptive benefits: significant improvement of seborrhea and acne, significant improvement of PMDD, no alteration (and even improvement) of the cholesterol profile, no weight gain (and often even a small body weight reduction in the first cycles of use), and no blood pressure increase. Since the launch of this first EE/DRSP combination, two additional EE/DRSP combinations were developed containing 20 mcg of EE (one administered in a 21/7-day regimen and the other administered in a 24/4-day regimen [YAZ®]). They represent the so-called *fourth generation* COCs. However, cohort studies have suggested that EE/DRSP use is associated with a two-fold higher incidence of VTE than EE/LNG preparations. Therefore, use of COCs containing the androgenic second generation progestins are recommended in first intention (EE/LNG) but, due to their androgenic activity leading to acne, hirsutism, oily hair and/or weight gain, these COCs are often less appreciated by women and physicians.

Estetrol is a naturally occurring estrogen only produced by the fetal liver during human pregnancy. Its PK properties make E4 suitable to be used in clinical settings: high oral bioavailability, insignificant binding to circulating proteins, no active metabolites and long terminal half-life. Estetrol displays an agonistic activity on the nuclear ERα but, unlike E2 and

EE, E4 is an antagonist of the membrane ERα. This different profile of agonistic/antagonistic activities is responsible for the distinct profile of E4 in comparison to the other available estrogens as shown in animal and *in vitro* studies. The main results are summarized in Table 19 below.

Table 19. Common and distinct estrogenic profile of estetrol in comparison with estradiol and ethinyelestradiol.

Properties common to E4, E2 and EE	Properties specific to E4
Endometrial proliferation	Antagonization of the proliferative activity of E2 on normal and cancerous breast cancer tissue
Vaginal epithelium restauration	Weak modification of the liver synthesis activity
Ovulation inhibition	Antiovulatory effect
Suppression of hot flushes	Persistence of endothelial protection in aged animals
Atheroprotection	
Prevention of neo-intimal hyperplasia after angioplasty	
Vasorelaxing effect	

E4, estetrol; E2, estradiol; EE, ethinylestradiol

A new COC with E4 as estrogen compound is currently under development. This project is called the Estelle project and is the subject of this thesis. Estetrol is a new chemical entity, meaning that no drug containing E4 has currently received commercialization approval by any health authorities. Therefore, a complete characterization of the molecule is necessary before seeking any market authorization application. This includes *in vitro* studies, testing of the drug in different animal species (rat, rabbit, monkey, fish) for different period of time (up to two years), and clinical studies in human going from phase 1 trials (first-in-human studies intended to evaluate the tolerance to the drug) to phase 4 trials (post-marketing surveillance studies). Developing a new chemical entity also necessitates regular contact with the health authorities, particularly with the EMA and the FDA, in order to obtain their feedback on the already generated data and to seek their approval to move forward to the next steps of the development. In the context of developing a new hormonal contraceptive, specific guidelines edited by the EMA and the FDA have also to be followed describing the minimum data to generate before submitting the dossier.

The phase 2 dose-finding program of the Estelle project aimed at selecting the right dose of E4 and the progestin to be associated to E4 to form the COC intended to be further evaluated in the phase 3 program. The results of this dose-finding process were published in 5 articles, discussed in this work. The 15 mg E4/3 mg DRSP was selected as final combination to be

further evaluated, taking into account two aspects: the contraceptive efficacy and the safety aspects.

1. Contraceptive efficacy

There was no ovulation in any combination tested, demonstrating that a dose of 5 mg E4 combined with a progestin is sufficient to achieve a complete ovulation inhibition. Consequently, among the tested doses of E4, 5 mg E4 is considered as the minimal effective dose. However, we choose a higher dose of E4 in the final composition as, above 10 mg E4, a lower degree of residual ovarian activity was achieved and consequently a better bleeding pattern.
A reliable contraceptive efficacy is expected from this E4-COC for three reasons. First, by having chosen 15 mg E4, we will use 3 times the dose needed for ovulation inhibition. The safety margin is therefore very high. Secondly, the half-life of both E4 and DRSP is around 30 hours. This means that, even if a pill is missed, the plasma concentrations of both compounds are above the minimal effective dose. Finally, animal studies have demonstrated that the ovulation process necessitates the activation of the membrane ERα. Estetrol is an antagonist of this receptor. This most probably contributes to the high contraceptive potential of an E4-containing COC (Abot, et al. 2014). These three aspects explain the total absence of ovulation detected during our study, a scenario rarely observed based on the literature reports of studies evaluating ovulation inhibition with other COCs.

2. Safety aspects

Even if the global tolerance to E4 was good with all the doses tested across the dose-finding program, some specific safety aspects helped in the final dose selection: the bleeding pattern, the tolerance to the combination, and the potential VTE risk.

2.1. Bleeding pattern

The bleeding pattern was optimal with two combinations, namely 15 mg E4/DRSP and 20 mg E4/LNG: both combinations were associated with less than 20% of the treated subjects reporting intermenstrual bleeding while withdrawal bleeding occurred in more than 80% of the subjects treated. A total absence of withdrawal bleeding under hormonal contraception is often seen as an advantage. This is comfortable when amenorrhea is constant and predictable (*e.g.* the amenorrhea often reported by LNG intrauterine system users). However, when occurrence of amenorrhea is irregular and unpredictable, a pregnancy has to be ruled out which is a reason of unnecessary anxiety for the user and her partner. Therefore, the very low incidence of amenorrhea with the 15 mg E4/DRSP combination (3.5% versus 14.0% with 20 mg E4/LNG and 27.1% with the commercialized comparator - Qlaira®) was an additional reason to prefer this combination over the 20 mg E4/LNG.

2.2. General tolerance, user satisfaction and body weight

The second phase 2 trial was conducted on a sufficiently long period (6 cycles) and a sufficiently large population (between 75 and 80 subjects per group) to use the safety data for the dose selection. Incidence of adverse events was the lowest among the users of the 15 mg E4/DRSP combination in comparison with the other groups, including the comparator Qlaira®. In addition, the incidence of adverse events leading to subject discontinuation of the trial was much lower in the 15 mg E4/DRSP group than in the other E4-containing combinations groups: 6.3% versus 10.7 to 15.6%. This demonstrated a better tolerance to the 15 mg E4/DRSP combination. User satisfaction and willingness to continue on the current COC was higher with E4/DRSP combinations versus E4/LNG ones, demonstrating the superiority of DRSP on LNG for these aspects. Finally, in accordance with the well-documented DRSP properties, the E4/DRSP combinations were also associated with more positive impact on body weight than the E4/LNG combinations.

2.3. VTE risk

Based on the bleeding pattern and the safety aspects, it was evident that the 15 mg E4/DRSP combination was the best combination to be selected. However, having chosen DRSP as progestin automatically raised questions regarding the incidence of VTE as DRSP-containing COCs currently on the market are associated with the highest VTE risk. Our data confirmed that it is well the estrogen that dramatically drives the hemostasis changes seen with estro-progestin combinations, and consequently, the choice of estrogen is responsible for the VTE risk. The head-to-head comparison between 15 mg E4 and 20 mcg EE (both combined with the same progestin, 3 mg DRSP) shows a major difference between the two molecules in terms of synthesis of hemostasis parameters and estrogenicity marker (SHBG). Estetrol had globally a neutral effect on these parameters. Regarding the comparison with the currently safest preparation, namely EE/LNG, the changes recorded with E4/DRSP were generally similar to the second generation. However, if we admit the high predictable value of APCr, E4/DRSP could even be associated with a lower VTE risk than a second generation COC.

CHAPTER 4: CONCLUSION

The use of DRSP in contraception has significantly modified the contraceptive field. Indeed, the well-established non-contraceptive benefits of DRSP allowed for new therapeutics perspective such as treatment of acne, improvement of PMDD, stabilization of body weight and blood pressure, and an overall improvement for women suffering from lasting side effects associated with more androgenic progestins.

Epidemiological evidence shows an increase VTE risk with EE/DRSP combinations. These data have been largely discussed in the scientific community but also in the lay press. Therefore, clinicians became reluctant to prescribe these COCs and, although well appreciated, users were no longer reassured by using these products. However, contraceptive efficacy largely relies on user compliance and it is crucial to develop products associated with a high tolerance to insure compliance.

The results obtained so far in the Estelle project authorize to think that E4 could be the solution to the VTE issue associated with the use of EE/DRSP COCs. The 15 mg E4/DRSP combination could be seen as the combination of the right estrogen with the best progestin.

CHAPTER 5: PERSPECTIVES

1. Perspectives of the Estelle Project

The first major perspective of the Estelle project is to obtain the marketing authorization from the national medicines agencies, starting with both the EMA and the FDA, and then extending the authorization to the rest of the world.

Given the success of the phase 2 program described in this work, the project was continued with a large phase 3 program intended to assess the contraceptive efficacy of the 15 mg E4/3 mg DRSP combination and the safety, tolerance and side-effects.

Two phase 3 studies with similar design were conducted in parallel: one in Europe and Russia, and the other in the USA and Canada. The results of the European study were exposed at the ESG congress in October 2019 in Vienna and the abstract is reported below.

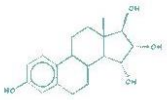
Estetrol 15 mg combined with drospirenone 3 mg is an effective oral contraceptive: results from the E4Freedom EU/RU phase 3 trial

Authors: Mitchell D. Creinin[1], Marie Mawet[2], Sophie Ledant[2], Maud Jost[2], Jean-Michel Foidart[2,3]

[1] *Department of Obstetrics and Gynecology, University of California, Davis, Sacramento, CA, USA*

[2] *Mithra Pharmaceuticals, Liège, Belgium*

[3] *University of Liège, Liège, Belgium*



Objective: To assess the contraceptive efficacy and safety of estetrol (E4) 15 mg / drosperinone (DRSP) 3 mg in a 24/4-day regimen, during 1 year of usage.

Methods: This multicentre, open-label, phase 3 trial was conducted in 69 centres across Europe and Russia. Healthy women, 18-50 years with regular cycles and a BMI between 18 and 35 kg/m2 were enrolled for up to 13 consecutive cycles. We evaluated on-treatment pregnancies in women 18-35 and 18-50 years (measured by the Pearl Index (PI) and the method failure PI in at-risk cycles (cycles with no other contraceptive use), and the cumulative pregnancy rates at cycle 13 for both age groups.

Results: In total 1.577 subjects were enrolled of whom 1,553 (98.5%) started study treatment and 1,218 (77%) completed 13 cycles. The primary efficacy population included 1,353 women age 18-35 years of whom 1,052 (78%) completed 13 cycles. Five on-treatment pregnancies occurred of which 3 were considered method failure. The PI for the primary efficacy population with 14,759 at risk cycles was 0.44 (95% CI: 0.14; 1.03). The PI for all subjects (18-50 years) with 17,037 at risk cycles was 0.38 (95% CI: 0.12; 0.89). Method failure PIs were 0.26 (95% CI: 0.05; 0.77) and 0.23 (95% CI: 0.05; 0.67) for the age groups 18-35 and 18-50 years, respectively. The cumulative 13-cycle pregnancy rate for subjects aged 18-35 years was 0.45% (95%: CI 0.19; 1.09) and for subjects aged 18-50 years was 0.28% (95% CI: 0.09; 0.86). Among the 89 women with a BMI ≥ 30 mg/kg^2, no on-treatment pregnancies occurred.

Conclusion: Estetrol 15 mg combined with drospirenone 3 mg is a highly effective oral contraceptive. The probability of contraceptive protection with 1 year of treatment is 99.5%.

The results of the North American study are in publishing process.

Altogether, the data obtained during the phase 3 program confirm the high contraceptive efficacy and the excellent safety of 15 mg E4/3 mg DRSP. This paves the route to a new area in the field of COC and there is no doubt that the coming years will deliver new interesting data on this innovative combination.

2. Perspectives for the 15 mg E4/3 mg DRSP combination

The particular properties of both E4 and DRSP allow to plan a series of other additional developments for 15 mg E4/3 mg DRSP:

2.1. Administration in populations at higher cardiovascular risk

The potential safer cardiovascular profile of E4/DRSP makes this combination a good option for women in whom the prescription of an estro-progestin preparation is currently not recommended. This is notably the case of some women suffering from PCOS and of perimenopausal women.

Polycystic ovary syndrome (PCOS) is an heterogenous syndrome that may encompass ovulation dysfunction, hyperandrogenism, and polycystic ovaries. Hyperandrogenism is clinically translated by the presence of acne, hirsutism and alopecia. Women with PCOS also often present insulin resistance (which may evolve to type 2 diabetes), dyslipidemia, with or without overweight/obesity (Rosenfield). These abnormalities concur to a higher cardiovascular risk and, therefore, the safety of prescribing oestro-progestin preparations to these women is debated (Carmina 2013).

In line with the antiandrogenic activity of DRSP, previous data generated with DRSP combined with EE have shown that such a combination significantly improves hyperandrogenism in women with PCOS. It also decreases the LDL/HDL ratio and improves insulin resistance (Li, Ren, and Sun 2017). However, the fear of cardiovascular events often precludes the use of EE/DRSP in women with PCOS. Replacing EE by E4 could solve the issue as the advantages of DRSP on androgenic symptoms would be maintained while the global cardiovascular risk of the COC would decrease.

Contraception in *perimenopausal women* remains also a challenge in clinical practice. Perimenopause is the period in a woman's life that precedes menopause. It is clinically characterized by the onset of irregular ovulations which is responsible for estrogen deficiency symptoms (*i.e.* hot flushes, night sweats, sleep disturbances, loss of libido, mood alteration, etc) and abnormal menstrual bleeding (mainly heavy menstrual bleeding and irregular bleeding episodes).

As long as ovulations still occur, perimenopausal women remain at risk of getting pregnant. Unplanned pregnancy rate is high in women above 40 years old and resulted, for example, in almost 30% of elective abortion in England in 2015. It is also important to bear in mind that pregnancy in women older than 40 years represents a significant health concern for both mother and child: overall maternal mortality is 3 times higher than in women below 25 years of age, and risk of stillbirth/perinatal mortality is also significantly increased. The risk of congenital abnormalities is also much higher than in young women (*e.g.* the risk of Down syndrome is 1 in 146 in women 40 to 44 years old versus 1 in 1,544 in a 20 years old woman) (FSRH 2019a).

The use of a COC during perimenopause is thus an attractive therapeutic option: beside its high contraceptive efficacy, COC will improve the bleeding pattern and the estrogen compound will

solve the estrogen deficiency symptoms. Although very effective in terms of contraception, the other currently available contraceptive methods are not able to achieve all these goals: progestin only contraceptives (*e.g.* progestin only pills, LNG-IUS) do not solve the estrogen deficiency syndrome; copper IUD is often associated with a deterioration of the bleeding pattern; and male or female sterilization do not improve bleeding pattern nor estrogen deficiency syndrome.

However, many health practitioners do not recommend the use of COC in women above 40 years old. First, as discussed in the introduction of this thesis, the overall risk of cardiovascular events under COC increases with the age of the women. For example, all types of COCs combined, the incidence of VTE triples above 40 years of age (incidence of VTE is < 5/10 000 women-years in women below 25 years old versus >15/10 000 women-years in women above 40 years old) (Lidegaard, et al. 2011). A second reason why prescribers are so reluctant to use COC in perimenopausal women is the fear of inducing breast cancer in this population, even though this does not seem to be supported by current epidemiological data.

Here also, E4/DRSP is an optimal alternative as both the cardiovascular profile and the breast safety seem to be improved by replacing EE by E4. Research in this population is therefore promising.

2.2. Modification of the administration regimen

The E4/DRSP combination is currently administrated in a 24/4-day regimen, an administration scheme associated with a cyclical regular menstrual bleeding. Biologically speaking, there is no reason to maintain monthly withdrawal bleeds under COC. The choice is more cultural and social, regular menstruation being considered as a sign of fertility and of general good health. Longer administration regimens have thus been developed: extended-cycle regimens are defined as administration of active tablets for more than 28 days followed by a scheduled free-interval (*e.g.* 84/7-day regimen), and continuous regimen consists in the administration of active tablets without hormone free interval. There are many clinical benefits of using these extended/continuous regimens: first, they offer a greater comfort for the users with less bleeding (and even amenorrhea in certain women) and a lower risk of compliance issue. They are also associated with a significative improvement in menstrual-associated symptoms (*e.g.* dysmenorrhea and catamenial migraine) and endometriosis (Edelman, et al. 2014). Importantly, no increased risk of VTE nor metabolic impairment have been recorded with these extended administration regimens (Rad, et al. 2011). Future development of an E4-based continuous COC is perfectly conceivable with a specific focus on the impact of such regimen on bleeding pattern, dysmenorrhea and compliance.

2.3. Long acting reversible contraception

Although effective contraceptive methods are available, women in developed countries are still affected by a high rate of unplanned pregnancies. In Western Europe, 34% of pregnancies are unintended and this value increases to 45% in the USA and 54% in Eastern Europe (Group 2018; Finer and Zolna 2016).

In the face of this large and constant number of unwanted pregnancies despite effective contraceptive means, the development of methods with a better compliance has become crucial and mandatory. Long acting reversible contraceptives (LARCs) are a category of safe and very effective contraceptive methods that do not require user involvement once inserted. This includes subdermal implants and intrauterine contraceptive methods (Copper device or Levonorgestrel-releasing hormones system). Most LARCs are associated with a one year failure rate < 0.1%. Currently available LARCs do not contain estrogen which may explain some of their side effects, notably on BMD and mood alterations (Wu, Moniz, and Ursu 2018). Introduction of estrogen in this type of long lasting contraception is a challenge for manufacturer but new technologies in this area are in constant development. The safe estrogen E4 could possibly find its place in this field.

3. Estetrol in Menopause

Besides contraception, E4 has also been studied in post-menopausal women presenting VMS. A phase 2 dose-finding study has been conducted among 257 post-menopausal women moderately to severely affected by VMS. They were randomly assigned to either 2.5 mg E4, 5 mg E4, 10 mg E4, 15 mg E4 or placebo. Treatment was administrated for 12 weeks. Results showed that both frequency and severity of VMS decreased proportionally with the E4 dose. The minimum effective dose in this study was 15 mg E4 which was associated with a 66% reduction in VMS frequency at treatment week 4 (versus 49% in the placebo group) and with a 82% reduction in VMS frequency at week 12 (versus 65% in the placebo group). The treatment was safe and well tolerated (Gaspard, et al. 2020).

A phase 3 program is currently ongoing that evaluates the effects of 15 mg E4 and 20 mg E4 on moderate to severe VMS in post-menopausal women. The results of this program are expected in August 2021 (ClinicalTrial 2019).

In the menopause field, the possible perspectives for E4 may also include a fixed combined product with a progestin and a specific treatment for vulvo-vaginal atrophy.

4. Perspectives in non-gynecological fields

4.1. Hypoxic ischemic encephalopathy

As described in Chapter 1, Section 7.9, interesting pre-clinical data were generated in animal models of neonatal hypoxic ischemic encephalopathy (HIE), a condition characterized by decreasing O2 concentration and blood flow to the brain, leading to cerebral cell death (Toro-Urrego, et al. 2019). The data obtained in animals with E4 strongly suggests a protective action

of E4 in this pathology. At the end of the pregnancy, the fetal's blood concentration in E4 is high and one may suppose that administrating the same compound in the days following the birth will not compromise the safety of the new born, while recreating the favourable environment for optimal brain protection and development. Therefore, E4 is seen as a relatively safe drug in this vulnerable population.
Recently, the EMA and the FDA have granted Orphan Drug designation for E4 in the treatment of HIE based on these promising preclinical results (Mithra 2019).

4.2. E4 Impact on the environment

Today, there is a growing concern related to the impact of endocrine disruptors in the environment. Ethinylestradiol is present in 97% of marketed COCs. Once released in the environment (through the urine and feces of EE consumers), EE remains resistant to degradation and inactivation. Because of these properties, EE persists and accumulates in the environment and is therefore considered to be a strong *environmental endocrine disruptor*. Over 700 kg of EE are discharged every year in the waters solely from COC intakes. This unwanted exposure to EE can lead to epigenetic and transgenerational effects (Mithra 2020).
Extensive testing in various fish species revealed adverse effects of the natural estrogens (E1, E2) and of the synthetic EE occurring at levels as low as 1 ng/L. These effects include reduced egg production, reduced testicular growth, delayed maturation, development of ova-testes in males and development of the populations with skewed female to male ratios (*i.e.* feminization). Estetrol on the other hand had no such adverse effects in a study covering the life span of a representative test species up to 32,000 ng/L (Mithra 2020). Estetrol may thus be considered as the first "environmental friendly estrogen" and thus becoming the leader in a world caring more than ever in its impact on the environment.

5. Perspectives Conclusion: the End of the Beginning

Today, only a few experts still remember that before the advent of EE, mestranol was the estrogen compound used in COC. Although associated with several unwanted (and sometimes life threatening) side effects, EE has remained the only estrogen used in combined hormonal contraception for decades. Estetrol, with its high efficacy and expected safer profile, has the potential to replace this synthetic estrogen in all the gynaecological applications where exogenous estrogens are used.
Estrogens moved from the field of physiology to the field of pharmacology more than 80 years ago. Progress has since been impressive both considering the chemistry of derivatives, their pharmacology and their indications. However no new estrogen has been introduced after 1943. No doubt that E4 is one most important step of this journey. We believe that the story is far from being finished not only in the gynaecologic domain but also in other domains of medicine as knowledge increases considering for instance the physiology of estrogens membrane and nuclear receptors. Estetrol will undoubtedly be a leader in these new exciting fields.

REFERENCES

Abot, A., et al. 2014. "The Uterine and Vascular Actions of Estetrol Delineate a Distinctive Profile of Estrogen Receptor Alpha Modulation, Uncoupling Nuclear and Membrane Activation." *EMBO Mol Med* 6, no. 10 (Oct): 1328-46. http://dx.doi.org/10.15252/emmm.201404112.

Acevedo-Rodriguez, A., et al. 2018. "Emerging Insights into Hypothalamic-Pituitary-Gonadal Axis Regulation and Interaction with Stress Signalling." *J Neuroendocrinol* 30, no. 10 (Oct): e12590. http://dx.doi.org/10.1111/jne.12590.

Adlanmerini, M., et al. 2014. "Mutation of the Palmitoylation Site of Estrogen Receptor Alpha in Vivo Reveals Tissue-Specific Roles for Membrane Versus Nuclear Actions." *Proc Natl Acad Sci U S A* 111, no. 2 (Jan 14): E283-90. http://dx.doi.org/10.1073/pnas.1322057111.

Agren, U. M., et al. 2011. "Effects of a Monophasic Combined Oral Contraceptive Containing Nomegestrol Acetate and 17beta-Oestradiol Compared with One Containing Levonorgestrel and Ethinylestradiol on Haemostasis, Lipids and Carbohydrate Metabolism." *Eur J Contracept Reprod Health Care* 16, no. 6 (Dec): 444-57. http://dx.doi.org/10.3109/13625187.2011.604450.

Ahrendt, H. J., et al. 2009. "Bleeding Pattern and Cycle Control with an Estradiol-Based Oral Contraceptive: A Seven-Cycle, Randomized Comparative Trial of Estradiol Valerate/Dienogest and Ethinyl Estradiol/Levonorgestrel." *Contraception* 80, no. 5 (Nov): 436-44. http://dx.doi.org/10.1016/j.contraception.2009.03.018.

Alhenc-Gelas, M., et al. 2004. "Impact of Progestagens on Activated Protein C (Apc) Resistance among Users of Oral Contraceptives." *J Thromb Haemost* 2, no. 9 (Sep): 1594-600. http://dx.doi.org/10.1111/j.1538-7836.2004.00894.x.

Amiri, M., et al. 2020. "A Comparison of the Effects of Oral Contraceptives on the Clinical and Biochemical Manifestations of Polycystic Ovary Syndrome: A Crossover Randomized Controlled Trial." *Hum Reprod* 35, no. 1 (01): 175-186. http://dx.doi.org/10.1093/humrep/dez255.

Anderson, G. L., et al. 2004. "Effects of Conjugated Equine Estrogen in Postmenopausal Women with Hysterectomy: The Women's Health Initiative Randomized Controlled Trial." *JAMA* 291, no. 14 (Apr 14): 1701-12. http://dx.doi.org/10.1001/jama.291.14.1701.

Apter, D., et al. 2016. "Bleeding Pattern and Cycle Control with Estetrol-Containing Combined Oral Contraceptives: Results from a Phase Ii, Randomised, Dose-Finding Study (Fiesta)." *Contraception* 94, no. 4 (Oct): 366-73. http://dx.doi.org/10.1016/j.contraception.2016.04.015.

Archer, D. F., et al. 2019. "Efficacy of the 1-Year (13-Cycle) Segesterone Acetate and Ethinylestradiol Contraceptive Vaginal System: Results of Two Multicentre, Open-Label, Single-Arm, Phase 3

Trials." *Lancet Glob Health* 7, no. 8 (Aug): e1054-e1064. http://dx.doi.org/10.1016/S2214-109X(19)30265-7.

Arnal, J. F., et al. 2017. "Membrane and Nuclear Estrogen Receptor Alpha Actions: From Tissue Specificity to Medical Implications." *Physiol Rev* 97, no. 3 (Jul 1): 1045-1087. http://dx.doi.org/10.1152/physrev.00024.2016.

Aronson, Jeffrey K. 2009. *Meyler's Side Effects of Endocrine and Metabolic Drugs*: Elsevier.

Azzopardi, D. V., et al. 2009. "Moderate Hypothermia to Treat Perinatal Asphyxial Encephalopathy." *N Engl J Med* 361, no. 14 (Oct 1): 1349-58. http://dx.doi.org/10.1056/NEJMoa0900854.

Bagot, C. N., et al. 2010. "The Effect of Estrone on Thrombin Generation May Explain the Different Thrombotic Risk between Oral and Transdermal Hormone Replacement Therapy." *J Thromb Haemost* 8, no. 8 (Aug): 1736-44. http://dx.doi.org/10.1111/j.1538-7836.2010.03953.x.

Bahamondes, L., M. Valeria Bahamondes, and L. P. Shulman. 2015. "Non-Contraceptive Benefits of Hormonal and Intrauterine Reversible Contraceptive Methods." *Hum Reprod Update* 21, no. 5 (Sep-Oct): 640-51. http://dx.doi.org/10.1093/humupd/dmv023.

Barton, R.A. 2006. "Primate Brain Evolution: Integrating Comparative, Neurophysiological, and Ethological Data." *Evolutionary anthropology* 15, no. 6: 224-236.

Bauer, Kenneth. 2020. "Overview of the Causes of Venous Thrombosis." In *Uptodate*.

Bayer HealthCare Pharmaceuticals, Inc. 2001. "Product Information Yaz (Ethinylestradiol/Drospirenone)."

---. 2010. "Product Information Natazia (Estradiol Valerate/Dienogest)."

Beasley, A., et al. 2012. "The Effect of Obesity and Low-Dose Oral Contraceptives on Carbohydrate and Lipid Metabolism." *Contraception* 85, no. 5 (May): 446-52. http://dx.doi.org/10.1016/j.contraception.2011.09.014.

Belisle, S., J. G. Lehoux, and J. Brault. 1980. "The Metabolism of Androstenedione in Human Pregnancy: The Use of Constant Infusion of Unlabeled Steroid to Assess Its Metabolic Clearance Rate, Its Production Rate, and Its Conversion into Androgens and Estrogens." *Am J Obstet Gynecol* 136, no. 8 (Apr 15): 1030-5. http://dx.doi.org/10.1016/0002-9378(80)90632-8.

Belisle, S., I. Schiff, and D. Tulchinsky. 1980. "The Use of Constant Infusion of Unlabeled Dehydroepiandrosterone for the Assessment of Its Metabolic Clearance Rate, Its Half-Life, and Its Conversion into Estrogens." *J Clin Endocrinol Metab* 50, no. 1 (Jan): 117-21. http://dx.doi.org/10.1210/jcem-50-1-117.

Benoit, T., et al. 2017. "Estetrol, a Fetal Selective Estrogen Receptor Modulator, Acts on the Vagina of Mice through Nuclear Estrogen Receptor A Activation." *Am J Pathol* 187, no. 11 (Nov): 2499-2507. http://dx.doi.org/10.1016/j.ajpath.2017.07.013.

Bergendal, A., et al. 2014. "Association of Venous Thromboembolism with Hormonal Contraception and Thrombophilic Genotypes." *Obstet Gynecol* 124, no. 3 (Sep): 600-9. http://dx.doi.org/10.1097/AOG.0000000000000411.

Billon-Gales, A., et al. 2009. "Endothelial Estrogen Receptor-Alpha Plays a Crucial Role in the Atheroprotective Action of 17beta-Estradiol in Low-Density Lipoprotein Receptor-Deficient Mice." *Circulation* 120, no. 25 (Dec 22): 2567-76. http://dx.doi.org/10.1161/CIRCULATIONAHA.109.898445.

Blair, R. M., et al. 2000. "The Estrogen Receptor Relative Binding Affinities of 188 Natural and Xenochemicals: Structural Diversity of Ligands." *Toxicol Sci* 54, no. 1 (Mar): 138-53. http://dx.doi.org/10.1093/toxsci/54.1.138.

Bosetti, C., et al. 2009. "Oral Contraceptives and Colorectal Cancer Risk: A Systematic Review and Meta-Analysis." *Hum Reprod Update* 15, no. 5 (Sep-Oct): 489-98. http://dx.doi.org/10.1093/humupd/dmp017.

Bottiger, L. E., et al. 1980. "Oral Contraceptives and Thromboembolic Disease: Effects of Lowering Oestrogen Content." *Lancet* 1, no. 8178 (May 24): 1097-101. http://dx.doi.org/10.1016/s0140-6736(80)91550-0.

Burkman, R., C. Bell, and D. Serfaty. 2011. "The Evolution of Combined Oral Contraception: Improving the Risk-to-Benefit Ratio." *Contraception* 84, no. 1 (Jul): 19-34. http://dx.doi.org/10.1016/j.contraception.2010.11.004.

Burkman, R., J. J. Schlesselman, and M. Zieman. 2004. "Safety Concerns and Health Benefits Associated with Oral Contraception." *Am J Obstet Gynecol* 190, no. 4 Suppl (Apr): S5-22. http://dx.doi.org/10.1016/j.ajog.2004.01.061.

Caine, Y. G., et al. 1992. "Coagulation Activation Following Estrogen Administration to Postmenopausal Women." *Thromb Haemost* 68, no. 4 (Oct): 392-5.

Cantineau, R., et al. 1985. "15- and 16-Hydroxylations of Androgens and Estrogens in the Human Fetal Liver: A Critical Step in Estetrol Biosynthesis." *J Steroid Biochem* 22, no. 2 (Feb): 195-201. http://dx.doi.org/10.1016/0022-4731(85)90112-8.

Carmina, E. 2013. "Oral Contraceptives and Cardiovascular Risk in Women with Polycystic Ovary Syndrome." *J Endocrinol Invest* 36, no. 5 (May): 358-63. http://dx.doi.org/10.3275/8882.

Castellanos, Mar. 2011. "Antiplatelet Therapy for Secondary Prevention of Stroke." In *Stroke (5th Edition)*, 1147-1172.

CDC. 2016. "Us Medical Eligibility Criteria (Us Mec) for Contraceptive Use." Accessed 31 JUL 2020, 2020. https://www.cdc.gov/reproductivehealth/contraception/mmwr/mec/summary.html.

ClinicalTrial. 2019. "Estetrol for the Treatment of Moderate to Severe Vasomotor Symptoms in Postmenopausal Women (E4comfort Study I)." Accessed 13 AUG 2020, https://clinicaltrials.gov/ct2/show/record/NCT04209543?term=menopause&cond=estetrol&draw=2&rank=3.

Coelingh Bennink, F., et al. 2008. "Maternal and Fetal Estetrol Levels During Pregnancy." *Climacteric* 11 Suppl 1: 69-72. http://dx.doi.org/10.1080/13697130802056321.

Coelingh Bennink, H. J., et al. 2008a. "Oral Bioavailability and Bone-Sparing Effects of Estetrol in an Osteoporosis Model." *Climacteric* 11 Suppl 1: 2-14. http://dx.doi.org/10.1080/13697130701798692.

Coelingh Bennink, H. J., C. F. Holinka, and E. Diczfalusy. 2008. "Estetrol Review: Profile and Potential Clinical Applications." *Climacteric* 11 Suppl 1: 47-58. http://dx.doi.org/10.1080/13697130802073425.

Coelingh Bennink, H. J., et al. 2008b. "Ovulation Inhibition by Estetrol in an in Vivo Model." *Contraception* 77, no. 3 (Mar): 186-90. http://dx.doi.org/10.1016/j.contraception.2007.11.014.

Coelingh Bennink, H. J. T., et al. 2017a. "Pharmacodynamic Effects of the Fetal Estrogen Estetrol in Postmenopausal Women: Results from a Multiple-Rising-Dose Study." *Menopause* 24, no. 6 (Jun): 677-685. http://dx.doi.org/10.1097/GME.0000000000000823.

---. 2017b. "Pharmacokinetics of the Fetal Estrogen Estetrol in a Multiple-Rising-Dose Study in Postmenopausal Women." *Climacteric* 20, no. 3 (Jun): 285-289. http://dx.doi.org/10.1080/13697137.2017.1291608.

Coelingh Bennink, H. J., et al. 2016. "Clinical Effects of the Fetal Estrogen Estetrol in a Multiple-Rising-Dose Study in Postmenopausal Women." *Maturitas* 91 (Sep): 93-100. http://dx.doi.org/10.1016/j.maturitas.2016.06.017.

Creinin, M. D., and J. T. Jensen. 2020. "Oral Contraceptive Generations - Time to Stop Using a Marketing Myth to Define Nomenclature." *Contraception* (Jun). http://dx.doi.org/10.1016/j.contraception.2020.05.017.

de Bastos, M., et al. 2014. "Combined Oral Contraceptives: Venous Thrombosis." *Cochrane Database Syst Rev*, no. 3 (Mar 3): CD010813. http://dx.doi.org/10.1002/14651858.CD010813.pub2.

De Caterina, R., et al. 2013. "General Mechanisms of Coagulation and Targets of Anticoagulants (Section I). Position Paper of the Esc Working Group on Thrombosis--Task Force on

Anticoagulants in Heart Disease." *Thromb Haemost* 109, no. 4 (Apr): 569-79. http://dx.doi.org/10.1160/TH12-10-0772.

De Leo, V., et al. 2016. "Hormonal Contraceptives: Pharmacology Tailored to Women's Health." *Hum Reprod Update* 22, no. 5 (Sep): 634-46. http://dx.doi.org/10.1093/humupd/dmw016.

de Visser, M. C., et al. 2005. "Determinants of the Aptt- and Etp-Based Apc Sensitivity Tests." *J Thromb Haemost* 3, no. 7 (Jul): 1488-94. http://dx.doi.org/10.1111/j.1538-7836.2005.01430.x.

Dhamad, A. E., et al. 2016. "Systematic Proteomic Identification of the Heat Shock Proteins (Hsp) That Interact with Estrogen Receptor Alpha (Eralpha) and Biochemical Characterization of the Eralpha-Hsp70 Interaction." *PLoS One* 11, no. 8: e0160312. http://dx.doi.org/10.1371/journal.pone.0160312.

Dhont, M. 2010. "History of Oral Contraception." *Eur J Contracept Reprod Health Care* 15 Suppl 2 (Dec): S12-8. http://dx.doi.org/10.3109/13625187.2010.513071.

Dinger, J., et al. 2010. "Risk of Venous Thromboembolism and the Use of Dienogest- and Drospirenone-Containing Oral Contraceptives: Results from a German Case-Control Study." *J Fam Plann Reprod Health Care* 36, no. 3 (Jul): 123-9. http://dx.doi.org/10.1783/147118910791749416.

Dinger, J., K. Bardenheuer, and K. Heinemann. 2014. "Cardiovascular and General Safety of a 24-Day Regimen of Drospirenone-Containing Combined Oral Contraceptives: Final Results from the International Active Surveillance Study of Women Taking Oral Contraceptives." *Contraception* 89, no. 4 (Apr): 253-63. http://dx.doi.org/10.1016/j.contraception.2014.01.023.

Dinger, J. C., L. A. Heinemann, and D. Kuhl-Habich. 2007. "The Safety of a Drospirenone-Containing Oral Contraceptive: Final Results from the European Active Surveillance Study on Oral Contraceptives Based on 142,475 Women-Years of Observation." *Contraception* 75, no. 5 (May): 344-54. http://dx.doi.org/10.1016/j.contraception.2006.12.019.

Dossus, L., et al. 2010. "Reproductive Risk Factors and Endometrial Cancer: The European Prospective Investigation into Cancer and Nutrition." *Int J Cancer* 127, no. 2 (Jul 15): 442-51. http://dx.doi.org/10.1002/ijc.25050.

Douxfils, J., et al. 2020. "Validation and Standardization of the Etp-Based Activated Protein C Resistance Test for the Clinical Investigation of Steroid Contraceptives in Women: An Unmet Clinical and Regulatory Need." *Clin Chem Lab Med* 58, no. 2 (Jan): 294-305. http://dx.doi.org/10.1515/cclm-2019-0471.

Dutertre, M., and C. L. Smith. 2000. "Molecular Mechanisms of Selective Estrogen Receptor Modulator (Serm) Action." *J Pharmacol Exp Ther* 295, no. 2 (Nov): 431-7.

Edelman, A., et al. 2014. "Continuous or Extended Cycle Vs. Cyclic Use of Combined Hormonal Contraceptives for Contraception." *Cochrane Database Syst Rev*, no. 7 (Jul): CD004695. http://dx.doi.org/10.1002/14651858.CD004695.pub3.

Elkazaz, A. Y., and K. Salama. 2015. "The Effect of Oral Contraceptive Different Patterns of Use on Circulating Igf-1 and Bone Mineral Density in Healthy Premenopausal Women." *Endocrine* 48, no. 1 (Feb): 272-8. http://dx.doi.org/10.1007/s12020-014-0290-2.

2006. *Guideline on Clinical Investigation of Steroid Contraceptives in Women*, by EMA.

---. 2012. "European Comission Final Decision." Accessed 20/04/2020, 2020. https://www.ema.europa.eu/en/medicines/human/referrals/yaz-244.

Endrikat, J., et al. 2003. "A Meta-Analysis on the Correlation between Ovarian Activity and the Incidence of Intermenstrual Bleeding During Low-Dose Oral Contraceptive Use." *Gynecol Endocrinol* 17, no. 2 (Apr): 107-14.

FDA. 2011. "Fda Drug Safety Communication: Updated Information About the Fda-Funded Study on Risk of Blood Clots in Women Taking Birth Control Pills Containing Drospirenone." Accessed 18/05/2020, https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-updated-information-about-fda-funded-study-risk-blood-clots-women.

---. 2019. "Guidance for Industry - Establishing Effectiveness and Safety for Hormonal Drug Products Intended to Prevent Pregnancy."

Feigelson, H. S., and B. E. Henderson. 1996. "Estrogens and Breast Cancer." *Carcinogenesis* 17, no. 11 (Nov): 2279-84. http://dx.doi.org/10.1093/carcin/17.11.2279.

Finer, L. B., and M. R. Zolna. 2016. "Declines in Unintended Pregnancy in the United States, 2008-2011." *N Engl J Med* 374, no. 9 (Mar): 843-52. http://dx.doi.org/10.1056/NEJMsa1506575.

Foidart, J. M., et al. 2000. "A Comparative Investigation of Contraceptive Reliability, Cycle Control and Tolerance of Two Monophasic Oral Contraceptives Containing Either Drospirenone or Desogestrel." *Eur J Contracept Reprod Health Care* 5, no. 2 (Jun): 124-34. http://dx.doi.org/10.1080/13625180008500387.

Fournier, A., F. Berrino, and F. Clavel-Chapelon. 2008. "Unequal Risks for Breast Cancer Associated with Different Hormone Replacement Therapies: Results from the E3n Cohort Study." *Breast Cancer Res Treat* 107, no. 1 (Jan): 103-11. http://dx.doi.org/10.1007/s10549-007-9523-x.

FSRH. 2019a. *Contraception for Women Aged over 40 Years*.

---. 2019b. "Fsrh Clinical Guideline: Combined Hormonal Contraception." Accessed 08/05/2020, https://www.fsrh.org/standards-and-guidance/documents/combined-hormonal-contraception/.

Gallo, M. F., et al. 2013. "20 Microg Versus >20 Microg Estrogen Combined Oral Contraceptives for Contraception." *Cochrane Database Syst Rev*, no. 8 (Aug 1): CD003989. http://dx.doi.org/10.1002/14651858.CD003989.pub5.

Garcia, C. R., G. Pincus, and J. Rock. 1956. "Effects of Certain 19-nor Steroids on the Normal Human Menstrual Cycle." *Science* 124, no. 3227 (Nov 2): 891-3. http://dx.doi.org/10.1126/science.124.3227.891.

Gaspard, U., et al. 2004. "A Randomized Study on the Influence of Oral Contraceptives Containing Ethinylestradiol Combined with Drospirenone or Desogestrel on Lipid and Lipoprotein Metabolism over a Period of 13 Cycles." *Contraception* 69, no. 4 (Apr): 271-8. http://dx.doi.org/10.1016/j.contraception.2003.11.003.

---. 2003. "A Randomized Study over 13 Cycles to Assess the Influence of Oral Contraceptives Containing Ethinylestradiol Combined with Drospirenone or Desogestrel on Carbohydrate Metabolism." *Contraception* 67, no. 6 (Jun): 423-9. http://dx.doi.org/10.1016/s0010-7824(02)00537-1.

---. 2020. "A Multicenter, Randomized Study to Select the Minimum Effective Dose of Estetrol (E4) in Postmenopausal Women (E4relief): Part 1. Vasomotor Symptoms and Overall Safety." *Menopause* (May). http://dx.doi.org/10.1097/GME.0000000000001561.

Gerard, C., et al. 2015a. "Estetrol Is a Weak Estrogen Antagonizing Estradiol-Dependent Mammary Gland Proliferation." *J Endocrinol* 224, no. 1 (Jan): 85-95. http://dx.doi.org/10.1530/JOE-14-0549.

---. 2015b. "Combined Estrogenic and Anti-Estrogenic Properties of Estetrol on Breast Cancer May Provide a Safe Therapeutic Window for the Treatment of Menopausal Symptoms." *Oncotarget* 6, no. 19 (Jul 10): 17621-36. http://dx.doi.org/10.18632/oncotarget.4184.

Gerstman, B. B., et al. 1991. "Oral Contraceptive Estrogen Dose and the Risk of Deep Venous Thromboembolic Disease." *Am J Epidemiol* 133, no. 1 (Jan): 32-7. http://dx.doi.org/10.1093/oxfordjournals.aje.a115799.

Gharib, S. D., et al. 1990. "Molecular Biology of the Pituitary Gonadotropins." *Endocr Rev* 11, no. 1 (Feb): 177-99. http://dx.doi.org/10.1210/edrv-11-1-177.

Giretti, M. S., et al. 2014. "Effects of Estetrol on Migration and Invasion in T47-D Breast Cancer Cells through the Actin Cytoskeleton." *Front Endocrinol (Lausanne)* 5: 80. http://dx.doi.org/10.3389/fendo.2014.00080.

Giribela, C. R., et al. 2015. "Effects of a Combined Oral Contraceptive Containing 20 Mcg of Ethinylestradiol and 3 Mg of Drospirenone on the Blood Pressure, Renin-Angiotensin-Aldosterone System, Insulin Resistance, and Androgenic Profile of Healthy Young Women." *Gynecol Endocrinol* 31, no. 11: 912-5. http://dx.doi.org/10.3109/09513590.2015.1062860.

Gluckman, P. D., et al. 2005. "Selective Head Cooling with Mild Systemic Hypothermia after Neonatal Encephalopathy: Multicentre Randomised Trial." *Lancet* 365, no. 9460 (Feb 19-25): 663-70. http://dx.doi.org/10.1016/S0140-6736(05)17946-X.

Gronich, N., I. Lavi, and G. Rennert. 2011. "Higher Risk of Venous Thrombosis Associated with Drospirenone-Containing Oral Contraceptives: A Population-Based Cohort Study." *CMAJ* 183, no. 18 (Dec 13): E1319-25. http://dx.doi.org/10.1503/cmaj.110463.

Group, ESHRE Capri Workshop. 2018. "Why after 50 Years of Effective Contraception Do We Still Have Unintended Pregnancy? A European Perspective." *Hum Reprod* 33, no. 5 (05): 777-783. http://dx.doi.org/10.1093/humrep/dey089.

GuttmacherInstitute. 2020. "Contraceptive Use in the United States." *https://www.guttmacher.org/fact-sheet/contraceptive-use-united-states*.

Hammond, G. L., et al. 2008. "Estetrol Does Not Bind Sex Hormone Binding Globulin or Increase Its Production by Human Hepg2 Cells." *Climacteric* 11 Suppl 1: 41-6. http://dx.doi.org/10.1080/13697130701851814.

Hannaford, P. C., et al. 2010. "Mortality among Contraceptive Pill Users: Cohort Evidence from Royal College of General Practitioners' Oral Contraception Study." *BMJ* 340 (Mar 11): c927. http://dx.doi.org/10.1136/bmj.c927.

Harvey, John. 2012. *Veterinary Hematology*.

Havrilesky, L. J., et al. 2013. "Oral Contraceptive Pills as Primary Prevention for Ovarian Cancer: A Systematic Review and Meta-Analysis." *Obstet Gynecol* 122, no. 1 (Jul): 139-47. http://dx.doi.org/10.1097/AOG.0b013e318291c235.

Heegaard, A. M., et al. 2008. "Estrogenic Uterovaginal Effects of Oral Estetrol in the Modified Allen-Doisy Test." *Climacteric* 11 Suppl 1: 22-8. http://dx.doi.org/10.1080/13697130701842490.

Heikkila, J., and T. Luukkainen. 1971. "Urinary Excretion of Estriol and 15 Alpha-Hydroxyestriol in Complicated Pregnancies." *Am J Obstet Gynecol* 110, no. 4 (Jun 15): 509-21. http://dx.doi.org/10.1016/0002-9378(71)90692-2.

Heldring, N., et al. 2007. "Estrogen Receptors: How Do They Signal and What Are Their Targets." *Physiol Rev* 87, no. 3 (Jul): 905-31. http://dx.doi.org/10.1152/physrev.00026.2006.

Herrmann, M., and M. J. Seibel. 2010. "The Effects of Hormonal Contraceptives on Bone Turnover Markers and Bone Health." *Clin Endocrinol (Oxf)* 72, no. 5 (May): 571-83. http://dx.doi.org/10.1111/j.1365-2265.2009.03688.x.

Hickey, M., R. Hart, and J. A. Keelan. 2014. "The Relationship between Umbilical Cord Estrogens and Perinatal Characteristics." *Cancer Epidemiol Biomarkers Prev* 23, no. 6 (Jun): 946-52. http://dx.doi.org/10.1158/1055-9965.EPI-13-1321.

Hilgers, R. H., et al. 2012. "Vasorelaxing Effects of Estetrol in Rat Arteries." *J Endocrinol* 215, no. 1 (Oct): 97-106. http://dx.doi.org/10.1530/JOE-12-0009.

Holinka, C. F., M. Brincat, and H. J. Coelingh Bennink. 2008. "Preventive Effect of Oral Estetrol in a Menopausal Hot Flush Model." *Climacteric* 11 Suppl 1: 15-21. http://dx.doi.org/10.1080/13697130701822807.

Hoogland, H. J., and S. O. Skouby. 1993. "Ultrasound Evaluation of Ovarian Activity under Oral Contraceptives." *Contraception* 47, no. 6 (Jun): 583-90. http://dx.doi.org/10.1016/0010-7824(93)90025-3.

Hugon-Rodin, J., et al. 2017. "Sex Hormone-Binding Globulin and Thrombin Generation in Women Using Hormonal Contraception." *Biomarkers* 22, no. 1 (Feb): 81-85. http://dx.doi.org/10.1080/1354750X.2016.1204010.

Inman, W. H., et al. 1970. "Thromboembolic Disease and the Steroidal Content of Oral Contraceptives. A Report to the Committee on Safety of Drugs." *Br Med J* 2, no. 5703 (Apr 25): 203-9. http://dx.doi.org/10.1136/bmj.2.5703.203.

International Planned Parenthood Federation, M. 2013. "Imap Short Statement on the Safety of Third and Fourth Generation Combined Oral Contraceptives." *IPPF Medical Bulletin*.

James, A. H. 2017. "Pregnancy, Contraception and Venous Thromboembolism (Deep Vein Thrombosis and Pulmonary Embolism)." *Vasc Med* 22, no. 2 (Apr): 166-169. http://dx.doi.org/10.1177/1358863X17690601.

Jensen, J. T. 2010. "Evaluation of a New Estradiol Oral Contraceptive: Estradiol Valerate and Dienogest." *Expert Opin Pharmacother* 11, no. 7 (May): 1147-57. http://dx.doi.org/10.1517/14656561003724713.

Jick, S. S., and R. K. Hernandez. 2011. "Risk of Non-Fatal Venous Thromboembolism in Women Using Oral Contraceptives Containing Drospirenone Compared with Women Using Oral Contraceptives Containing Levonorgestrel: Case-Control Study Using United States Claims Data." *BMJ* 342 (Apr 21): d2151. http://dx.doi.org/10.1136/bmj.d2151.

Jordan, WM, and JK Anand. 1961. "Pulmonary Embolism." *The Lancet* 278: 1146-1147.

Junge, W., et al. 2011. "Metabolic and Haemostatic Effects of Estradiol Valerate/Dienogest, a Novel Oral Contraceptive: A Randomized, Open-Label, Single-Centre Study." *Clin Drug Investig* 31, no. 8: 573-584. http://dx.doi.org/10.2165/11590220-000000000-00000.

Kanasaki, H., et al. 2017. "How Is Gnrh Regulated in Gnrh-Producing Neurons? Studies Using Gt1-7 Cells as a Gnrh-Producing Cell Model." *Gen Comp Endocrinol* 247 (Jun 1): 138-142. http://dx.doi.org/10.1016/j.ygcen.2017.01.025.

Kelly, S., et al. 2010. "Effects of Oral Contraceptives Containing Ethinylestradiol with Either Drospirenone or Levonorgestrel on Various Parameters Associated with Well-Being in Healthy Women: A Randomized, Single-Blind, Parallel-Group, Multicentre Study." *Clin Drug Investig* 30, no. 5: 325-36. http://dx.doi.org/10.2165/11535450-000000000-00000.

Klipping, C., et al. 2008. "Suppression of Ovarian Activity with a Drospirenone-Containing Oral Contraceptive in a 24/4 Regimen." *Contraception* 78, no. 1 (Jul): 16-25. http://dx.doi.org/10.1016/j.contraception.2008.02.019.

Kluft, C., et al. 2006. "A Prospective Study on the Effects on Hemostasis of Two Oral Contraceptives Containing Drospirenone in Combination with Either 30 or 20 Microg Ethinyl Estradiol and a Reference Containing Desogestrel and 30 Microg Ethinyl Estradiol." *Contraception* 73, no. 4 (Apr): 336-43. http://dx.doi.org/10.1016/j.contraception.2005.09.015.

Koltun, W., et al. 2008. "Efficacy and Safety of 3 Mg Drospirenone/20 Mcg Ethinylestradiol Oral Contraceptive Administered in 24/4 Regimen in the Treatment of Acne Vulgaris: A Randomized, Double-Blind, Placebo-Controlled Trial." *Contraception* 77, no. 4 (Apr): 249-56. http://dx.doi.org/10.1016/j.contraception.2007.11.003.

Kujovich, J. L. 2011. "Factor V Leiden Thrombophilia." *Genet Med* 13, no. 1 (Jan): 1-16. http://dx.doi.org/10.1097/GIM.0b013e3181faa0f2.

Kundu, N., and M. Grant. 1976. "Radioimmunoassay of 15alpha-Hydroxyestriol (Estetrol) in Pregnancy Serum." *Steroids* 27, no. 6 (Jun): 785-96. http://dx.doi.org/10.1016/0039-128x(76)90138-0.

Kundu, N., et al. 1981. "Comparison of Serum Unconjugated Estriol and Estetrol in Normal and Complicated Pregnancies." *Obstet Gynecol* 58, no. 3 (Sep): 276-81.

Kushner, A., Do Wp West, and L. S. Pillarisetty. 2020. "Virchow Triad." In *Statpearls*. Treasure Island (FL).

Landgren, B. M., A. L. Unden, and E. Diczfalusy. 1980. "Hormonal Profile of the Cycle in 68 Normally Menstruating Women." *Acta Endocrinol (Copenh)* 94, no. 1 (May): 89-98. http://dx.doi.org/10.1530/acta.0.0940089.

Larivee, N., et al. 2017. "Drospirenone-Containing Oral Contraceptive Pills and the Risk of Venous Thromboembolism: A Systematic Review of Observational Studies." *BJOG* 124, no. 10 (Sep): 1490-1499. http://dx.doi.org/10.1111/1471-0528.14623.

Lete, I., et al. 2015. "Haemostatic and Metabolic Impact of Estradiol Pills and Drospirenone-Containing Ethinylestradiol Pills Vs. Levonorgestrel-Containing Ethinylestradiol Pills: A Literature Review." *Eur J Contracept Reprod Health Care* 20, no. 5: 329-43. http://dx.doi.org/10.3109/13625187.2015.1050091.

Levin, E. R. 2009. "Plasma Membrane Estrogen Receptors." *Trends Endocrinol Metab* 20, no. 10 (Dec): 477-82. http://dx.doi.org/10.1016/j.tem.2009.06.009.

Li, J., J. Ren, and W. Sun. 2017. "A Comparative Systematic Review of Yasmin (Drospirenone Pill) Versus Standard Treatment Options for Symptoms of Polycystic Ovary Syndrome." *Eur J Obstet Gynecol Reprod Biol* 210 (Mar): 13-21. http://dx.doi.org/10.1016/j.ejogrb.2016.11.013.

Lidegaard, O. 2018. "Severely Biased Review of Studies Assessing the Risk of Venous Thrombosis in Users of Drospirenone-Containing Oral Contraceptives." *BJOG* 125, no. 8 (Jul): 929-931. http://dx.doi.org/10.1111/1471-0528.15211.

Lidegaard, O., et al. 2012. "Thrombotic Stroke and Myocardial Infarction with Hormonal Contraception." *N Engl J Med* 366, no. 24 (Jun 14): 2257-66. http://dx.doi.org/10.1056/NEJMoa1111840.

---. 2009. "Hormonal Contraception and Risk of Venous Thromboembolism: National Follow-up Study." *BMJ* 339 (Aug 13): b2890. http://dx.doi.org/10.1136/bmj.b2890.

---. 2011. "Risk of Venous Thromboembolism from Use of Oral Contraceptives Containing Different Progestogens and Oestrogen Doses: Danish Cohort Study, 2001-9." *BMJ* 343 (Oct 25): d6423. http://dx.doi.org/10.1136/bmj.d6423.

Lindberg, U. B., et al. 1989. "A Comparison between Effects of Estradiol Valerate and Low Dose Ethinyl Estradiol on Haemostasis Parameters." *Thromb Haemost* 61, no. 1 (Feb): 65-9.

Lindh, I., A. A. Ellstrom, and I. Milsom. 2011. "The Long-Term Influence of Combined Oral Contraceptives on Body Weight." *Hum Reprod* 26, no. 7 (Jul): 1917-24. http://dx.doi.org/10.1093/humrep/der094.

Mac Bride, M. B., D. J. Rhodes, and L. T. Shuster. 2010. "Vulvovaginal Atrophy." *Mayo Clin Proc* 85, no. 1 (Jan): 87-94. http://dx.doi.org/10.4065/mcp.2009.0413.

Madden, J. D., et al. 1976. "The Pattern and Rates of Metabolism of Maternal Plasma Dehydroisoandrosterone Sulfate in Human Pregnancy." *Am J Obstet Gynecol* 125, no. 7 (Aug 1): 915-20. http://dx.doi.org/10.1016/0002-9378(76)90488-9.

Makepeace, AW, GL Weinstein, and MH Friedman. 1937. "The Effects of Progestin and Progesterone on Ovulation in the Rabbit." *American Journal of Physiology* 119, no. 512.

Maloney, J. M., et al. 2009. "A Randomized Controlled Trial of a Low-Dose Combined Oral Contraceptive Containing 3 Mg Drospirenone Plus 20 Microg Ethinylestradiol in the Treatment of Acne Vulgaris: Lesion Counts, Investigator Ratings and Subject Self-Assessment." *J Drugs Dermatol* 8, no. 9 (Sep): 837-44.

Mancuso, S., et al. 1968. "Studies on the Metabolism of C-19 Steroids in the Human Foeto-Placental Unit. 4. Aromatisation and Hydroxylation Products Formed by Previable Foetuses Perfused Withandrostenedione and Testosterone." *Acta Endocrinol (Copenh)* 57, no. 2 (Feb): 208-27. http://dx.doi.org/10.1530/acta.0.0570208.

Mannucci, P. M., and M. Franchini. 2015. "Classic Thrombophilic Gene Variants." *Thromb Haemost* 114, no. 5 (Nov): 885-9. http://dx.doi.org/10.1160/TH15-02-0141.

Mansour, D., et al. 2011. "Efficacy and Tolerability of a Monophasic Combined Oral Contraceptive Containing Nomegestrol Acetate and 17beta-Oestradiol in a 24/4 Regimen, in Comparison to an Oral Contraceptive Containing Ethinylestradiol and Drospirenone in a 21/7 Regimen." *Eur J Contracept Reprod Health Care* 16, no. 6 (Dec): 430-43. http://dx.doi.org/10.3109/13625187.2011.614029.

Marino, M., P. Galluzzo, and P. Ascenzi. 2006. "Estrogen Signaling Multiple Pathways to Impact Gene Transcription." *Curr Genomics* 7, no. 8: 497-508. http://dx.doi.org/10.2174/138920206779315737.

Micevych, P. E., A. M. Wong, and M. A. Mittelman-Smith. 2015. "Estradiol Membrane-Initiated Signaling and Female Reproduction." *Compr Physiol* 5, no. 3 (Jul 1): 1211-22. http://dx.doi.org/10.1002/cphy.c140056.

Mishell, D. R., Jr., et al. 2007. "Combined Hormonal Contraceptive Trials: Variable Data Collection and Bleeding Assessment Methodologies Influence Study Outcomes and Physician Perception." *Contraception* 75, no. 1 (Jan): 4-10. http://dx.doi.org/10.1016/j.contraception.2006.08.008.

Mithra. 2019. *Mithra Receives Orphan Drug Designation from Fda for E4 in Neonatal Encephalopathy Treatment*.

---. 2020. *E4 Paves the Road Towards a Revolutionary Era of Environmental Friendly Medicines*.

Mittelman-Smith, M. A., et al. 2012. "Arcuate Kisspeptin/Neurokinin B/Dynorphin (Kndy) Neurons Mediate the Estrogen Suppression of Gonadotropin Secretion and Body Weight." *Endocrinology* 153, no. 6 (Jun): 2800-12. http://dx.doi.org/10.1210/en.2012-1045.

Monis, C. N., and M. Tetrokalashvili. 2020. "Menstrual Cycle Proliferative and Follicular Phase." In *Statpearls*. Treasure Island (FL).

Montt-Guevara, M. M., et al. 2015. "Estetrol Modulates Endothelial Nitric Oxide Synthesis in Human Endothelial Cells." *Front Endocrinol (Lausanne)* 6: 111. http://dx.doi.org/10.3389/fendo.2015.00111.

Morayma Reyes, Gil. 2019. "Overview of the Coagulation System." In *Transfusion Medecine and Hemostasis (Third Edition)*, 559-564.

Morimont, L., et al. 2020. "Proof of Concept of a New Scale for the Harmonization and the Standardization of the Etp-Based Apc Resistance." *J Thromb Haemost* 18, no. 4 (Apr): 895-904. http://dx.doi.org/10.1111/jth.14745.

Mueck, A. O., and R. Sitruk-Ware. 2011. "Nomegestrol Acetate, a Novel Progestogen for Oral Contraception." *Steroids* 76, no. 6 (May): 531-9. http://dx.doi.org/10.1016/j.steroids.2011.02.002.

Nappi, C., et al. 2005. "Effects of an Oral Contraceptive Containing Drospirenone on Bone Turnover and Bone Mineral Density." *Obstet Gynecol* 105, no. 1 (Jan): 53-60. http://dx.doi.org/10.1097/01.AOG.0000148344.26475.fc.

Nath, A., and R. Sitruk-Ware. 2009. "Different Cardiovascular Effects of Progestins According to Structure and Activity." *Climacteric* 12 Suppl 1: 96-101. http://dx.doi.org/10.1080/13697130902905757.

Nelson, A, C Cwiak, and W Cates. 2011. "Combined Oral Contraceptives (Cocs)." *Contraceptive technology*

Nikolchev, Alexandra. 2010. "A Brief History of the Birth Control Pill." *http://www.pbs.org/wnet/need-to-know/health/a-brief-history-of-the-birth-control-pill/480/*.

Oakley, A. E., D. K. Clifton, and R. A. Steiner. 2009. "Kisspeptin Signaling in the Brain." *Endocr Rev* 30, no. 6 (Oct): 713-43. http://dx.doi.org/10.1210/er.2009-0005.

Odlind, V., et al. 2002. "Can Changes in Sex Hormone Binding Globulin Predict the Risk of Venous Thromboembolism with Combined Oral Contraceptive Pills?" *Acta Obstet Gynecol Scand* 81, no. 6 (Jun): 482-90.

Oelkers, W. 2002. "Antimineralocorticoid Activity of a Novel Oral Contraceptive Containing Drospirenone, a Unique Progestogen Resembling Natural Progesterone." *Eur J Contracept Reprod Health Care* 7 Suppl 3 (Dec): 19-26; discussion 42-3.

Oelkers, W. H. 2005. "Drospirenone in Combination with Estrogens: For Contraception and Hormone Replacement Therapy." *Climacteric* 8 Suppl 3 (Oct): 19-27. http://dx.doi.org/10.1080/13697130500330341.

Palacios, S., et al. 2010. "Efficacy and Safety of a Novel Oral Contraceptive Based on Oestradiol (Oestradiol Valerate/Dienogest): A Phase Iii Trial." *Eur J Obstet Gynecol Reprod Biol* 149, no. 1 (Mar): 57-62. http://dx.doi.org/10.1016/j.ejogrb.2009.11.001.

Parkin, L., et al. 2011. "Risk of Venous Thromboembolism in Users of Oral Contraceptives Containing Drospirenone or Levonorgestrel: Nested Case-Control Study Based on Uk General Practice Research Database." *BMJ* 342 (Apr 21): d2139. http://dx.doi.org/10.1136/bmj.d2139.

Pearl, Raymond. 1933. "Factors in Human Fertility and Their Statistical Evaluation." *The Lancet* 222, no. 5741: 607-611.

Pearlstein, T. B., et al. 2005. "Treatment of Premenstrual Dysphoric Disorder with a New Drospirenone-Containing Oral Contraceptive Formulation." *Contraception* 72, no. 6 (Dec): 414-21. http://dx.doi.org/10.1016/j.contraception.2005.08.021.

Perez-Gomez, F., and R. Bover. 2007. "[the New Coagulation Cascade and Its Possible Influence on the Delicate Balance between Thrombosis and Hemorrhage]." *Rev Esp Cardiol* 60, no. 12 (Dec): 1217-9. http://dx.doi.org/10.1157/13113924.

Pettersson, K., F. Delaunay, and J. A. Gustafsson. 2000. "Estrogen Receptor Beta Acts as a Dominant Regulator of Estrogen Signaling." *Oncogene* 19, no. 43 (Oct 12): 4970-8. http://dx.doi.org/10.1038/sj.onc.1203828.

Pincus, G. 1958. "The Hormonal Control of Ovulation and Early Development." *Postgrad Med* 24, no. 6 (Dec): 654-60. http://dx.doi.org/10.1080/00325481.1958.11692305.

Piper, J. M., and D. L. Kennedy. 1987. "Oral Contraceptives in the United States: Trends in Content and Potency." *Int J Epidemiol* 16, no. 2 (Jun): 215-21. http://dx.doi.org/10.1093/ije/16.2.215.

Practical-Haemostasis. 2020. "Resistance to Activated Protein C [Apcr] Assays." Accessed 26 JUL 2020, 2020. https://www.practical-haemostasis.com/Thromobophilia/apcr_assays.html.

Purcell, C., M. Tennant, and J. McGeachie. 1997. "Neo-Intimal Hyperplasia in Vascular Grafts and Its Implications for Autologous Arterial Grafting." *Ann R Coll Surg Engl* 79, no. 3 (May): 164-8.

Rad, M., et al. 2011. "Metabolic Profile of a Continuous Versus a Cyclic Low-Dose Combined Oral Contraceptive after One Year of Use." *Eur J Contracept Reprod Health Care* 16, no. 2 (Apr): 85-94. http://dx.doi.org/10.3109/13625187.2011.556761.

Regidor, P. A., E. Colli, and A. E. Schindler. 2016. "Drospirenone as Estrogen-Free Pill and Hemostasis: Coagulatory Study Results Comparing a Novel 4 Mg Formulation in a 24 + 4 Cycle with Desogestrel 75 Mug Per Day." *Gynecol Endocrinol* 32, no. 9 (Sep): 749-751. http://dx.doi.org/10.3109/09513590.2016.1161743.

Roach, R. E., et al. 2015. "Combined Oral Contraceptives: The Risk of Myocardial Infarction and Ischemic Stroke." *Cochrane Database Syst Rev*, no. 8 (Aug 27): CD011054. http://dx.doi.org/10.1002/14651858.CD011054.pub2.

Rosenberg, M. J., A. Meyers, and V. Roy. 1999. "Efficacy, Cycle Control, and Side Effects of Low- and Lower-Dose Oral Contraceptives: A Randomized Trial of 20 Micrograms and 35 Micrograms Estrogen Preparations." *Contraception* 60, no. 6 (Dec): 321-9. http://dx.doi.org/10.1016/s0010-7824(99)00109-2.

Rosenfield, Robert. "**Definition, Clinical Features, and Differential Diagnosis of Polycystic Ovary Syndrome in Adolescents**." Waltham, MA: UpToDate.

Rossouw, J. E., et al. 2002. "Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women: Principal Results from the Women's Health Initiative Randomized Controlled Trial." *JAMA* 288, no. 3 (Jul 17): 321-33. http://dx.doi.org/10.1001/jama.288.3.321.

Russo, J., and I. H. Russo. 2006. "The Role of Estrogen in the Initiation of Breast Cancer." *J Steroid Biochem Mol Biol* 102, no. 1-5 (Dec): 89-96. http://dx.doi.org/10.1016/j.jsbmb.2006.09.004.

Sangthawan, M., and S. Taneepanichskul. 2005. "A Comparative Study of Monophasic Oral Contraceptives Containing Either Drospirenone 3 Mg or Levonorgestrel 150 Microg on Premenstrual Symptoms." *Contraception* 71, no. 1 (Jan): 1-7. http://dx.doi.org/10.1016/j.contraception.2004.07.010.

Santos, P. C., J. E. Krieger, and A. C. Pereira. 2012. "Renin-Angiotensin System, Hypertension, and Chronic Kidney Disease: Pharmacogenetic Implications." *J Pharmacol Sci* 120, no. 2: 77-88. http://dx.doi.org/10.1254/jphs.12r03cr.

Schwers, J., G. Eriksson, and E. Diczfalusy. 1965. "Metabolism of Oestrone and Oestradiol in the Human Foeto-Placental Unit at Midpregnancy." *Acta Endocrinol (Copenh)* 49 (May): 65-82. http://dx.doi.org/10.1530/acta.0.0490065.

Schwers, J., et al. 1965. "Metabolism of Oestrone Sulphate by the Previable Human Foetus." *Acta Endocrinol (Copenh)* 50, no. 4 (Dec): 597-610. http://dx.doi.org/10.1530/acta.0.0500597.

---. 1967. "Urinary Metabolites of Estradiol and Extriol Administered Intra-Amniotically." *J Clin Endocrinol Metab* 27, no. 10 (Oct): 1403-8. http://dx.doi.org/10.1210/jcem-27-10-1403.

Shankaran, S. 2002. "The Postnatal Management of the Asphyxiated Term Infant." *Clin Perinatol* 29, no. 4 (Dec): 675-92. http://dx.doi.org/10.1016/s0095-5108(02)00056-8.

Shankaran, S., et al. 2005. "Whole-Body Hypothermia for Neonates with Hypoxic-Ischemic Encephalopathy." *N Engl J Med* 353, no. 15 (Oct 13): 1574-84. http://dx.doi.org/10.1056/NEJMcps050929.

Sher, A., and M. A. Rahman. 2000. "Enterohepatic Recycling of Estrogen and Its Relevance with Female Fertility." *Arch Pharm Res* 23, no. 5 (Oct): 513-7. http://dx.doi.org/10.1007/bf02976582.

Sidney, S., et al. 2013. "Recent Combined Hormonal Contraceptives (Chcs) and the Risk of Thromboembolism and Other Cardiovascular Events in New Users." *Contraception* 87, no. 1 (Jan): 93-100. http://dx.doi.org/10.1016/j.contraception.2012.09.015.

Singer, C. F., et al. 2014. "Antiestrogenic Effects of the Fetal Estrogen Estetrol in Women with Estrogen-Receptor Positive Early Breast Cancer." *Carcinogenesis* 35, no. 11 (Nov): 2447-51. http://dx.doi.org/10.1093/carcin/bgu144.

Sitruk-Ware, R. 2004. "Pharmacological Profile of Progestins." *Maturitas* 47, no. 4 (Apr 15): 277-83. http://dx.doi.org/10.1016/j.maturitas.2004.01.001.

Smirnova, N. F., et al. 2015. "The Activation Function-1 of Estrogen Receptor Alpha Prevents Arterial Neointima Development through a Direct Effect on Smooth Muscle Cells." *Circ Res* 117, no. 9 (Oct 9): 770-8. http://dx.doi.org/10.1161/CIRCRESAHA.115.306416.

Smith, R. 2001. *The Endocrinology of Parturition: Basic Science and Clinical Application*.

Southmayd, E. A., and M. J. De Souza. 2017. "A Summary of the Influence of Exogenous Estrogen Administration across the Lifespan on the Gh/Igf-1 Axis and Implications for Bone Health." *Growth Horm IGF Res* 32 (Feb): 2-13. http://dx.doi.org/10.1016/j.ghir.2016.09.001.

Stanczyk, F. Z., D. F. Archer, and B. R. Bhavnani. 2013. "Ethinyl Estradiol and 17beta-Estradiol in Combined Oral Contraceptives: Pharmacokinetics, Pharmacodynamics and Risk Assessment." *Contraception* 87, no. 6 (Jun): 706-27. http://dx.doi.org/10.1016/j.contraception.2012.12.011.

Stola, A., and J. Perlman. 2008. "Post-Resuscitation Strategies to Avoid Ongoing Injury Following Intrapartum Hypoxia-Ischemia." *Semin Fetal Neonatal Med* 13, no. 6 (Dec): 424-31. http://dx.doi.org/10.1016/j.siny.2008.04.011.

Sulak, P., et al. 2007. "Headaches and Oral Contraceptives: Impact of Eliminating the Standard 7-Day Placebo Interval." *Headache* 47, no. 1 (Jan): 27-37. http://dx.doi.org/10.1111/j.1526-4610.2007.00650.x.

Toro-Urrego, N., et al. 2019. "Neuroprotective Role of Hypothermia in Hypoxic-Ischemic Brain Injury: Combined Therapies Using Estrogen." *Curr Neuropharmacol* 17, no. 9: 874-890. http://dx.doi.org/10.2174/1570159X17666181206101314.

Trussell, J. 1995. "Contraceptive Efficacy." *Arch Dermatol* 131, no. 9 (Sep): 1064-8.

Trussell, J., et al. 1990. "A Guide to Interpreting Contraceptive Efficacy Studies." *Obstet Gynecol* 76, no. 3 Pt 2 (Sep): 558-67.

Tskitishvili, E., et al. 2014. "Estetrol Attenuates Neonatal Hypoxic-Ischemic Brain Injury." *Exp Neurol* 261 (Nov): 298-307. http://dx.doi.org/10.1016/j.expneurol.2014.07.015.

---. 2016. "Use of Estetrol with Other Steroids for Attenuation of Neonatal Hypoxic-Ischemic Brain Injury: To Combine or Not to Combine?" *Oncotarget* 7, no. 23 (Jun 7): 33722-43. http://dx.doi.org/10.18632/oncotarget.9591.

Tulchinsky, D., et al. 1975. "Plasma Estetrol as an Index of Fetal Well-Being." *J Clin Endocrinol Metab* 40, no. 4 (Apr): 560-7. http://dx.doi.org/10.1210/jcem-40-4-560.

UnitedNations. 2015. "Trends in Contraceptive Use Worldwide." *https://www.un.org/en/development/desa/population/publications/pdf/family/trendsContraceptiveUse2015Report.pdf*.

van Hylckama Vlieg, A., et al. 2009. "The Venous Thrombotic Risk of Oral Contraceptives, Effects of Oestrogen Dose and Progestogen Type: Results of the Mega Case-Control Study." *BMJ* 339 (Aug 13): b2921. http://dx.doi.org/10.1136/bmj.b2921.

van Rooijen, M., et al. 2004. "Sex Hormone--Binding Globulin--a Surrogate Marker for the Prothrombotic Effects of Combined Oral Contraceptives." *Am J Obstet Gynecol* 190, no. 2 (Feb): 332-7. http://dx.doi.org/10.1016/s0002-9378(03)00950-5.

van Vlijmen, E. F., et al. 2016. "Clinical Profile and Recurrence Rate in Women with Venous Thromboembolism During Combined Hormonal Contraceptive Use: A Prospective Cohort Study." *Br J Haematol* 172, no. 4 (Feb): 636-8. http://dx.doi.org/10.1111/bjh.13534.

Vinel, A., et al. 2016. "Role of Eralphamiss in the Effect of Estradiol on Cancellous and Cortical Femoral Bone in Growing Female Mice." *Endocrinology* 157, no. 6 (Jun): 2533-44. http://dx.doi.org/10.1210/en.2015-1994.

Vinogradova, Y., C. Coupland, and J. Hippisley-Cox. 2015. "Use of Combined Oral Contraceptives and Risk of Venous Thromboembolism: Nested Case-Control Studies Using the Qresearch and Cprd Databases." *BMJ* 350 (May 26): h2135. http://dx.doi.org/10.1136/bmj.h2135.

Visser, M., J. M. Foidart, and H. J. Coelingh Bennink. 2008. "In Vitro Effects of Estetrol on Receptor Binding, Drug Targets and Human Liver Cell Metabolism." *Climacteric* 11 Suppl 1: 64-8. http://dx.doi.org/10.1080/13697130802050340.

Visser, M., C. F. Holinka, and H. J. Coelingh Bennink. 2008. "First Human Exposure to Exogenous Single-Dose Oral Estetrol in Early Postmenopausal Women." *Climacteric* 11 Suppl 1: 31-40. http://dx.doi.org/10.1080/13697130802056511.

Visser, M., H. J. Kloosterboer, and H. J. Bennink. 2012. "Estetrol Prevents and Suppresses Mammary Tumors Induced by Dmba in a Rat Model." *Horm Mol Biol Clin Investig* 9, no. 1 (Apr 1): 95-103. http://dx.doi.org/10.1515/hmbci-2012-0015.

Wahab, F., et al. 2016. "Kisspeptin Signalling in the Physiology and Pathophysiology of the Urogenital System." *Nat Rev Urol* 13, no. 1 (Jan): 21-32. http://dx.doi.org/10.1038/nrurol.2015.277.

Westhoff, C., et al. 2012. "Efficacy, Safety, and Tolerability of a Monophasic Oral Contraceptive Containing Nomegestrol Acetate and 17beta-Estradiol: A Randomized Controlled Trial." *Obstet Gynecol* 119, no. 5 (May): 989-99. http://dx.doi.org/10.1097/AOG.0b013e318250c3a0.

Women Health Initiative, studies. 2020. "Main Trial Findings." Accessed 21/04/2020, https://www.whi.org/about/SitePages/WHI%20Findings.aspx.

Wu, J. P., M. H. Moniz, and A. N. Ursu. 2018. "Long-Acting Reversible Contraception-Highly Efficacious, Safe, and Underutilized." *JAMA* 320, no. 4 (07): 397-398. http://dx.doi.org/10.1001/jama.2018.8877.

Wynn, V., et al. 1979. "Comparison of Effects of Different Combined Oral-Contraceptive Formulations on Carbohydrate and Lipid Metabolism." *Lancet* 1, no. 8125 (May): 1045-9. http://dx.doi.org/10.1016/s0140-6736(79)92949-0.

Yonkers, K. A., et al. 2005. "Efficacy of a New Low-Dose Oral Contraceptive with Drospirenone in Premenstrual Dysphoric Disorder." *Obstet Gynecol* 106, no. 3 (Sep): 492-501. http://dx.doi.org/10.1097/01.AOG.0000175834.77215.2e.

Ziller, M., et al. 2014. "Risk of Venous Thrombosis in Users of Hormonal Contraceptives in German Gynaecological Practices: A Patient Database Analysis." *Arch Gynecol Obstet* 289, no. 2 (Feb): 413-9. http://dx.doi.org/10.1007/s00404-013-2983-9.

Zorbas, K. A., K. P. Economopoulos, and N. F. Vlahos. 2015. "Continuous Versus Cyclic Oral Contraceptives for the Treatment of Endometriosis: A Systematic Review." *Arch Gynecol Obstet* 292, no. 1 (Jul): 37-43. http://dx.doi.org/10.1007/s00404-015-3641-1.

Zucconi, G., et al. 1967. "Isolation of 15-Alpha-Hydroxy-Oestriol from Pregnancy Urine and from the Urine of Newborn Infants." *Acta Endocrinol (Copenh)* 56, no. 3 (Nov): 413-23. http://dx.doi.org/10.1530/acta.0.0560413.