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**PROGESTERONE RECEPTOR MODULATORS IN
CONTRACEPTION - CLINICAL IMPLICATIONS OF
OVARIAN AND ENDOMETRIAL EFFECTS**

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Progesterone Receptor Modulators in Contraception -clinical implications of ovarian and endometrial effects

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“ There is no tool for development more effective than the empowerment of women ”

Kofi Annan

“I think a woman is powerless if she cannot freely claim the right to her reproductive capacity”

Jamaica Kincaid

To Mom ♥

*“Okay, Ladies, now let’s get in formation ...
Always stay gracious, best revenge is your paper”*

Beyoncé Knowles

ABSTRACT

Background

Emergency contraception (EC) offers a last chance to prevent an unwanted pregnancy after unprotected sexual intercourse (UPSI) but before pregnancy occurs, for example after contraceptive failure, non-use of contraception or after rape. Progesterone receptor modulators (PRMs) exert their effect through acting as partial antagonists and partial agonists of the progesterone receptor (PR) and have proven effective for EC in low doses by postponing or inhibiting ovulation if it was taken pre-ovulatory in the menstrual cycle. They further affect the endometrium in a dose-dependent manner and high doses inhibit endometrial development and affect embryo implantation. There are concerns regarding thickening of the endometrium and the specific PRM associated endometrial changes (PAEC) that long-term PRM treatment can cause. However, there is no knowledge of the underlying molecular basis of this condition. It is further not known if the PRM ulipristal acetate (UPA) used as EC could interfere with the contraceptive effect of a regular combined oral contraceptive pill (COCP) initiated immediately after UPA intake or if UPA in the dose used for EC affects endometrial receptivity and embryo implantation. Low doses of mifepristone have not been explored regarding its effect on the embryo implantation process.

Aim

The overall aim of this thesis was to explore the effects of PRMs mifepristone and UPA when used for EC and further develop them for contraceptive purposes and to provide women with evidence-based information and advice on mechanisms of action of PRMs. The specific objectives were to investigate the clinical implications of their effects on ovaries and endometrium. This included short-term effects on endometrial receptivity and the embryo implantation process as well as long-term endometrial effects and further possible interaction with regular hormonal contraception.

Materials, Methods and Results

Study I was a multicenter, double-blind, randomized, placebo-controlled trial in which 76 healthy female volunteers between the ages of 18 and 35 with regular menstrual cycles were randomized to receive either 30 mg UPA or placebo at mid-cycle when the dominant ovarian follicle was ≥ 13 mm, followed by intake of a common COCP during 21 subsequent days. Hormonal measurements and transvaginal ultrasonography were performed regularly to assess ovarian activity. Ovarian quiescence was achieved after 7 days of COCP intake for most women, however in some women it took up to 14 days, irrespective of UPA administration. The proportion of women who ovulated in the study was approximately 30%, similar in both groups, which could be explained by follicle size at inclusion.

Study II and III were exploratory studies on the human embryo implantation process and endometrial receptivity markers after treatment with UPA in an EC dose (Study II) and mifepristone in two different low concentrations (Study III) using a 3D human endometrial *in vitro* cell culture model. Endometrium was collected from proven fertile volunteers with regular menstrual cycles at cycle day LH+4 and endometrial stromal and epithelial cells were isolated and 3D constructs developed. Viable human blastocyst stage embryos, donated from couples that underwent IVF, were randomly allocated to the different treatment groups (UPA 200ng/ml $\approx 0.4\mu\text{M}$, mifepristone 0.05 μM , mifepristone 0.5 μM and control). When the epithelial cells had grown into a confluent layer, the embryos were placed on top of the culture and further co-cultured for 5 days. The embryo attachment rates in different groups were: 5/10 in UPA, 4/10 in mifepristone 0.05 μM , 0/8 in mifepristone 0.5 μM and 7/10 in the control group. Some of the known endometrial receptivity markers in Study II were

altered in the UPA group but most were unaffected. On the other hand, most of the receptivity markers examined in Study III were altered, with both the concentrations of mifepristone. However, they exerted functionally different effects on embryo implantation in a dose-dependent manner.

Study IV was an exploratory study in which endometrial samples had been collected from fourteen healthy, pre-menopausal women, randomized to receive mifepristone during 3 months prior to surgical intervention of uterine leiomyoma in a previous placebo controlled trial. Endometrial biopsies obtained during surgery after the treatment period were assessed for the occurrence of PAEC and further explored regarding gene and protein expression in endometrium with PAEC compared to non-PAEC after mifepristone treatment. Methods used included histological evaluation, microarray analysis, Ingenuity Pathway Analysis, real-time PCR, protein extraction and mass spectrometry. Three genes relevant to functions and diseases of the uterus were upregulated in endometrium with PAEC; ADAM12, THY1 and TN-C and 25 different proteins were upregulated and five downregulated in endometrium with PAEC.

Conclusion

UPA-EC does not interfere with the ability of a COCP to induce ovarian quiescence when treatment is quickstarted immediately after EC intake and the treatment is safe and tolerable. Further, UPA in a concentration corresponding to EC dose does not inhibit embryo implantation, nor does a very low mifepristone concentration of 0.05 μ M, whereas mifepristone in a concentration of 0.5 μ M effectively inhibited attachment of the embryo to the endometrial construct *in vitro*. The specific morphological features of PAEC displayed after 3 months of mifepristone treatment may be explained by the altered expression of molecules affecting tissue architecture and extracellular matrix, however these molecules were not involved in endometrial cancer-signaling pathways based on IPA knowledge base.

LIST OF SCIENTIFIC PAPERS

- I. **The effects on ovarian activity of ulipristal acetate when ‘quickstarting’ a combined oral contraceptive pill: a prospective, randomized, double-blind, parallel-arm, placebo-controlled study**
Cameron ST, Berger C, Michie L, Klipping C, Gemzell-Danielsson K
Human Reproduction, 2015, 30, 1566-1572
- II. **Effects of ulipristal acetate on human embryo attachment and endometrial cell gene expression in an in vitro co-culture system**
Berger C*, Boggavarapu NR*, Menezes J, Lalitkumar PGL, Gemzell-Danielsson K
Human Reproduction, 2015, 30, 800-811
- III. **Effects of low doses of mifepristone on human embryo implantation process in a three-dimensional human endometrial in vitro co-culture system**
Boggavarapu NR*, Berger C*, von Grothusen C, Menezes J, Gemzell-Danielsson K, Lalitkumar PGL
Contraception, 2016, 94, 143-151
- IV. **Molecular Characterization of PRM Associated Endometrial Changes, PAEC, following Mifepristone Treatment**
Berger C, Boggavarapu NR, Norlin E, Hörnaeus K, Falk A, Engman M, Ramström M, Lalitkumar PGL, Gemzell Danielsson K
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* *joint first authorship*

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LIST OF ABBREVIATIONS

AR	Androgen Receptor
ART	Assisted Reproductive Technology
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
cRNA	Complementary Ribonucleic Acid
CI	Confidence Interval
COCP	Combined Oral Contraceptive Pill
Cu-IUD	Copper Intrauterine Device
DES	Diethylstilbestrol
DNA	Deoxyribonucleic Acid
DSG	Desogestrel
EC	Emergency Contraception
ECM	Extracellular Matrix
ECP	Emergency Contraception Pill
EE	Ethinyl Estradiol
EMA	European Medicines Agency
EML	Essential Medicines List
ER	Estrogen Receptor
ERA	Endometrial Receptivity Array
EVT	Extravillous Trophoblast
FAS	Full Analysis Population
FC	Fold Change
FDR	False Discovery Rate
FFPE	Formalin Fixed Paraffin Embedded

FSH	Follicle Stimulating Hormone
GBV	Gender Based Violence
GPCR	G-protein Coupled Receptors
GR	Glucocorticoid Receptor
HCD	Higher Energy Collision Dissociation
HMB	Heavy Menstrual Bleeding
HPA	Hypothalamic Pituitary Ovarian Axis
ICEC	International Consortium for Emergency Contraception
ICM	Inner cell mass
ICPD	International Conference on Population and Development
ICSI	Intracytoplasmic Sperm Injection
IPA	Ingenuity Pathway Analysis
ITT	Intention to Treat
IUD	Intrauterine Device
IUFD	Intrauterine Fetal Death
IVF	<i>In vitro</i> Fertilization
LARC	Long Acting Reversible Contraception
LCM	Laser Capture Micro-dissection
LFQ	Label Free Quantification
LH	Luteinizing Hormone
LNG	Levonorgestrel
LNG IUS	Levonorgestrel Intrauterine System
LUF	Luteinized Unruptured Follicle
mRNA	Messenger Ribonucleic Acid
MAPR	Membrane Associated Progesterone Receptor
MDG	Millenium Development Goals
MEC	Medical Eligibility Criteria

MMP	Matrix Metalloproteinases
NSAID	Non-Steroidal Anti-Inflammatory Drug
OR	Odds Ratio
OTC	Over-the-Counter
PAEC	PRM Associated Endometrial Changes
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
PGR	Progesterone Receptor (mRNA)
PGRMC1	Progesterone Receptor Membrane Component 1
PMS	Premenstrual Syndrome
POP	Progestogen Only Pill
PP	Per Protocol
PR	Progesterone Receptor
PRA	Progesterone Receptor isoform A
PRB	Progesterone Receptor isoform B
PRC	Progesterone Receptor isoform C
PRM	Progesterone Receptor Modulator
PM-GCBG	Perfect Match GC Composition Based Background Correction
PTEN	Phosphatase and Tensin Homolog
RCT	Randomized Controlled Trial
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RQ	Relative Quantification
SAM	Significance Analysis of Microarrays
SD	Standard Deviation
SRH	Sexual and Reproductive Health

SRHR	Sexual and Reproductive Health and Rights
TIMP	Tissue Inhibitor of Metalloproteinases
TGF- β	Transforming Growth Factor- β
TVU	Transvaginal Ultrasonography
UN	United Nations
uNK	Uterine Natural Killer
UPA	Ulipristal Acetate
UPSI	Unprotected Sexual Intercourse
WHO	World Health Organization
WOI	Window of Implantation

1 INTRODUCTION AND RATIONALE

Since the ovarian steroid hormone progesterone is essential for human reproduction, progesterone receptor modulators (PRMs), with their intrinsic antifertility properties, have been explored and developed for contraceptive purposes. PRMs are currently in use for emergency contraception (EC), as daily treatment of symptomatic uterine leiomyoma, in combination with a prostaglandin analogue for termination of early pregnancy, for cervical ripening prior to surgical abortion and induction of labor at intrauterine fetal death (IUFD).

Emergency contraception provides women with a last chance to prevent an unwanted pregnancy following unprotected sexual intercourse (UPSI), contraceptive failure or after rape. The most recently developed dedicated EC method is the selective PRM ulipristal acetate (UPA) in a low single oral dose, taken within 120 hours after the event of an UPSI.

The mechanism of action of UPA when given pre-ovulatory in the menstrual cycle is to inhibit or postpone ovulation beyond the life span of sperm, consequently preventing pregnancy. UPA has proven to be more effective than levonorgestrel (LNG), the other dedicated hormonal EC pill (ECP) on the market, which has raised a debate whether or not it also exerts postfertilizing effects. In addition, UPA is similar in molecular structure to the PRM mifepristone, which in a high single dose in combination with misoprostol is an established method for induced medical abortion.

Mifepristone in a low dose can be used as EC and together with UPA further has potential for development as regular estrogen-free contraception. However, implications of the frequently occurring PRM associated endometrial changes, PAEC, as seen during long-term leiomyoma treatment, are not known.

The cellular and molecular events that mediate human embryo implantation process are largely unknown and there are few in vitro models for studying early events in human pregnancies and regulating factors. Endometrial studies are of importance to identify biological markers of endometrial receptivity, which can be used for development of more effective EC methods and safe and effective long-term contraception.

When the research project started there were no data available on interaction between UPA-EC and regular hormonal contraceptives. Since UPA binds to the progesterone receptor (PR), it could theoretically interfere with progestogen-containing products and the manufacturers current advice to women was therefore to use barrier methods or abstinence after UPA intake until the next menses. However, effective regular contraception should preferably be initiated or continued as soon as possible after EC use in order to ensure ongoing contraception and prevent a future unintended pregnancy, also as further acts of UPSI in the same cycle as EC intake increases the risk of pregnancy.

The rationale for this project was the urgent need of data on the potential interaction between UPA-EC and a common combined oral contraceptive pill (COCP) when practicing ‘quickstart’ of a regular contraception after UPA intake, to enable evidence-based advice to women regarding additional contraceptive measures needed to prevent pregnancy before they can rely on their routine contraception. Likewise, it felt urgent to determine mechanisms of action of PRMs when used for EC, with regards to effects on the endometrium and the embryo implantation process. Women around the world are frequently withheld access to the most effective products due to beliefs of postfertilizing effects and a large number of women are thereby put at risk of becoming unwanted pregnant and further at risk of maternal morbidity and mortality. It is of great importance to provide women with evidence-based information in regards to UPA-EC effect on embryo implantation.

Knowledge about endometrial receptivity can apart from the development of novel agents for contraceptive purposes be used to understand and improve fertility for women who suffer from infertility. For safety reasons however, before developing PRMs as ongoing contraception or extending leiomyoma treatment, implications of PAEC need to be elucidated.

The overall purpose with the studies was to add more understanding to the field of female reproductive physiology in relation to the mechanisms of action of PRMs when used for prevention of unintended pregnancies and ultimately contribute to women’s reproductive health and wellbeing.

2 BACKGROUND

2.1 UNINTENDED PREGNANCIES AND UNSAFE ABORTIONS

Approximately 45% of pregnancies were unintended in the US in 2011 [1]. This rate is similar to the 85 million unintended pregnancies out of estimated 213 million pregnancies that occurred worldwide in 2012 [2]. An incomprehensibly large number of women and girls suffer and die everyday from preventable conditions related to reproduction around the world. Unsafe abortions of unwanted pregnancies substantially contribute to maternal morbidity and mortality and further almost exclusively take place in the developing world [3, 4].

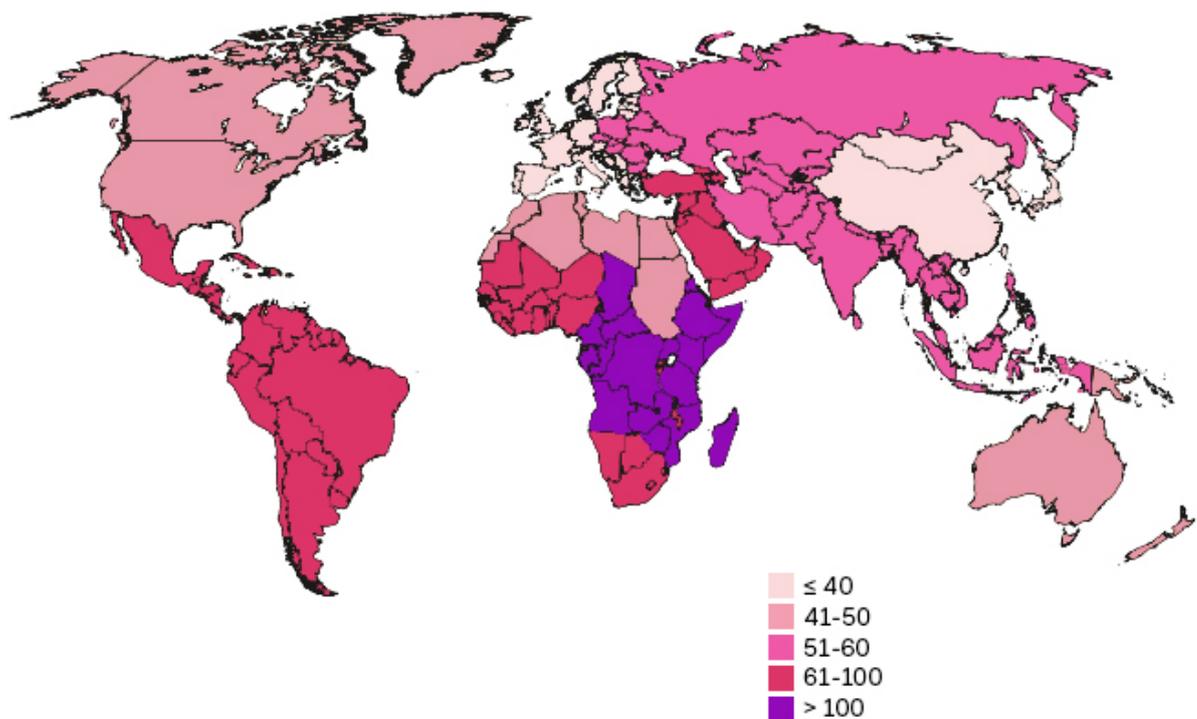


Figure 1. World map 2012 Unintended pregnancies per 1,000 women aged 15-44
Modified from Sedgh *et al* 2014, *Studies in Family Planning* 2014; 45(3): 301-314

Based on estimates for 2008, around 22 million unsafe abortions occurred worldwide, which was nearly half of all induced abortions that year [5]. More recent calculations on abortion incidence and levels worldwide estimated that 56 million abortions took place globally each year between 2010 and 2014 and thus one in four pregnancies ended in an induced abortion [6]. Sedgh *et al* further established that although abortion rates have declined significantly in the developed world since 1990, they in contrast increased in the developing world and concluded that increased investments and expanded access to family planning programs are needed [6]. In addition, it is important to consider the rapidly growing population and increasing number of women of reproductive age in areas where women are less able to

control their fertility due to restrictive abortion laws or where easy access to safe procedures is lacking. Irrespective of existing laws and religious beliefs, women will continue to have the need for abortions and to seek assistance for it. An abortion performed in accordance with recommended WHO guidelines is a very safe medical procedure with few complications. It is considered unsafe however if performed by a person lacking the appropriate skills or in a setting not conforming to minimal medical standards, or both [5]. Other risks, such as the legal status and social context are moreover important factors or barriers to consider when assessing safety of abortions. One aspect of safety regards the increasing informal use of misoprostol for medical induction of abortions, which could be performed safely even in settings where abortion is illegal. The concept of safety in this matter is complex and has lately been discussed and suggested to be assessed as a risk continuum rather instead of a binary measure [7]. In 2012, an estimated 7 million women were treated for abortion related complications in health facilities in the developing countries, however the total morbidity burden for these complications in the population naturally being much greater [8]. There is an uneven distribution of pregnancy-related complications globally and 62% of deaths related to unsafe abortions occur in Africa [5].

Unintended pregnancy is a very common condition that can be life threatening and in addition imply health consequences that are not fatal, thus constituting a public health problem. Most complications from unsafe abortions are unnecessary and avoidable with increased access to safe abortion and post-abortion care. Moreover and most important, unintended pregnancies are preventable with access to safe and effective contraception.

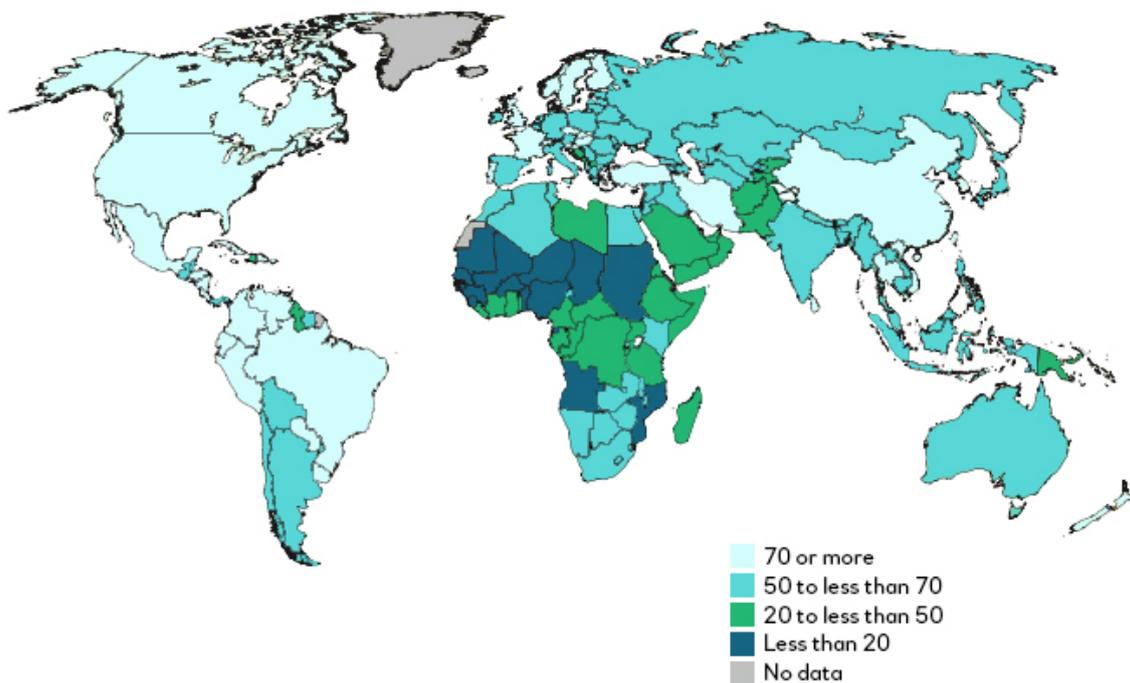


Figure 2. Percentage of women aged 15-49 (married or in a union) using any method of contraception 2015
 Modified from United Nations, Department of Economic and Social Affairs, Population Division (2015a)

2.2 UNMET CONTRACEPTIVE NEED

Despite an overall increase in contraceptive use globally, the unmet need for contraception remains high and the demand is increasing due to growing populations and simultaneous increased preference to have smaller families. The unmet need for contraception most often refers to sexually active women of reproductive age who do not wish to become pregnant but who are not using a modern method of contraception [9]. However, most calculations of the unmet need for contraception only consider women who are reported to be married or in a union, thus not taking into account all the other sexually active women in the world, why the figures arguably would be underestimated [2]. On the other hand, the results from studies estimating the unmet contraceptive need in all women might be less reliable and more difficult to compare from time to time or in between countries, due to difficulties in many places to obtain information about unmarried or adolescent women's engagement in sexual activities and thereby need for contraception.

Estimates for 2014 from the Guttmacher Institute in New York, US, showed that as many as 225 million women globally have an unmet need for modern contraception and that if the unmet contraceptive needs were to be fulfilled, an estimated 52 million unintended pregnancies and 24 million abortions would have been averted that year [10]. In addition, Ahmed *et al* earlier concluded that maternal mortality would be reduced by nearly a third if the unmet contraceptive need worldwide would be fulfilled [11].

There are unacceptable differences in reproductive health and inequalities in provision and access to SRH services between developing and developed countries as well as variations within the countries. The need for contraception is greatest in the developing countries where also nearly all unsafe abortions take place and the risks of maternal morbidity and mortality are highest. The most vulnerable women are disproportionately affected, consequently being the poorest and the youngest [12]. Considering the 23 million adolescent women with an unmet need for contraception worldwide [13] and whose lives and wellbeing are at risk, it is crucial to improve SRH services where needed to secure their reproductive future. This includes ensuring access to safe abortion services as well as providing information about and improve uptake of modern, effective and affordable contraceptive methods. It is further of great importance to understand barriers to contraceptive use and women's reasons for non-use where contraception is available in order to improve quality of interventional programs.

In the campaign Safe Motherhood Initiative from 1987, family planning was identified as one of four cornerstones to reduce maternal mortality. Later on in 1994, at another landmark event of sexual and reproductive health and rights (SRHR); the International Conference on Population and Development (ICPD), not only the definition of SRHR was articulated but further the importance to advance these rights, which included a consensus of achieving universal access to SRH services by 2015. Building further on the United Nations (UN) declaration of health-related Millenium Development Goals (MDG) from 2000, the commitment of ensuring universal access to SRH services was once again announced, together with the agenda to end preventable newborn, child and maternal deaths at the more

recent 2030 Agenda for Sustainable Development in 2015 [14]. Since socioeconomic inequities including low educational level and living in rural areas are associated with increased risk of experiencing complications following unintended pregnancies [12] these global initiatives also address poverty and empowerment of women as essential targets for improving reproductive health.

The freedom to choose if and when to become pregnant in order to achieve their fertility goals is not only beneficial for the woman and her autonomy to control spacing, timing and number of children, but likewise for her family and society. Positive correlations have also been demonstrated in high-income countries. In a Swedish study investigating the effect of subsidizing oral contraceptives, it was concluded that increased access to contraception results in a significantly higher socio-economic status later in life for the women and further in better health and performance in school for their offspring [15]. Thus, assuring the fundamental human rights of SRH also holds public health benefits for women and men.

2.3 EMERGENCY CONTRACEPTION

Emergency contraception (EC) is a postcoital contraceptive method that provides women with a last chance to avoid an unwanted pregnancy after the event of unprotected sexual intercourse (UPSI) but before pregnancy occurs [16], for example in situations of non-use of contraception or several missed pills, contraceptive failure such as condom breakage or after rape.

EC is often referred to as the “the morning after pill” in lay language. This is however somewhat misleading, both regarding the time window of effect of treatment and the route of administration. The current available categories of EC are intake of an oral hormonal EC-pill (ECP) as soon as possible within 3-5 days after UPSI or insertion of a copper-intrauterine device (Cu-IUD) within 5 days. A substantial percentage of expected pregnancies can be prevented by EC, reported between 57-99% depending on method and when used in the menstrual cycle [17-19]. Mechanisms of action of EC include preventing fertilization of the egg cell or preventing implantation of the fertilized egg in the uterus.

The increase in EC availability has been quite dramatic globally during the past two decades with development of new safe and effective dedicated ECPs and increased general knowledge of the method. Currently, EC is available in over 140 countries, among which in more than 60 possible to purchase over the counter (OTC). The term “dedicated ECP” refers to medication specially labeled and packaged for use as EC. Regular COCP on the contrary are not specifically developed for this purpose, although possible too use as EC if no other option is available. Despite a range of regular effective contraceptives available on the market, the unmet contraceptive need is large, where EC access and use might provide a bridge to start with a regular contraceptive method. Further, women who use regular contraception will in addition undoubtedly at times have a need for EC as inconsistent use or failure to use a contraceptive method correctly frequently occurs.

Due to difficulty in assessing the time of ovulation, EC treatment is recommended for women who do not wish to become pregnant after an UPSI regardless of when in the menstrual cycle it took place. Seemingly, the fertile window would be rather easy to predict for women with regular menstrual cycles as this period occurs in accordance with the time of ovulation. However, ovulation has shown to be highly variable and sporadically occurring outside the expected time, also for women stating that their cycles are regular, which in addition thus results in a range of unpredictable fertile days [20]. Moreover, the menstrual cycle day reported by women often does not coincide with objective hormonal measures [21] and UPSI outside the fertile period may also result in pregnancy [20].

Studies have further suggested that sexual intercourse and ovulation do not occur independently. The reason for this is the observation that even women who do not try to conceive are more likely to have sexual intercourse during the fertile days in the cycle with reported increasing frequency during the follicular phase up to a peak near ovulation and thereafter a rather sharp decrease [22, 23]. Several hypotheses regarding the reason for this pattern have been proposed, including cyclic increase in the woman's libido and sexual attractiveness, or even that ovulation is accelerated or triggered by sexual intercourse as seen in certain other species [23]. Altogether, EC should be offered to women who request it since it is not possible to objectively determine if the UPSI took place during the woman's fertile period and since the risk for pregnancy when a woman seeks EC is not negligible.

EC is further a critical treatment in post-rape care that substantially could reduce pregnancy following rape [24] and is recommended by the WHO to all women in all settings [25]. Although EC is important after sexual violence and should routinely be provided rape victims to prevent pregnancy following rape, provision of EC varies substantially worldwide. Many guidelines do not include offering EC to rape-survivors despite that pregnancies after rape are not uncommon and further risks additional traumatic consequences for the woman (<http://www.cecinfo.org>) (accessed April 2017). In addition, EC access is crucial in crisis-affected regions where wars, conflicts and natural disasters put millions of displaced girls and women at risk of sexual exploitation, rape and other forms of gender-based violence (GBV). Women in conflict settings most often do not have access to basic SRH services, including methods for prevention or treatment of unwanted pregnancies, nor to undergo a safe pregnancy and childbirth. These conditions underscore the importance of EC availability and for increased awareness and knowledge among health workers in refugee settings. Further, counseling and access to EC is also recommended in response to epidemic challenges such as the Zika virus [25].

Undoubtedly, safe and effective contraception, including EC, is essential in preventive medicine. Consequently, the World Health Organization (WHO) endorses EC and has it included on the Model Essential Medicines List (EML) [26]. EC is included on the EML in 60 countries in the world (<http://www.cecinfo.org>) (accessed April 2017).

2.4 HISTORICAL PERSPECTIVES ON EC

2.4.1 Emergency Contraception in ancient times

Various methods to prevent pregnancy with instructions on how to use medicinal plants with antifertility properties have been described since the beginning of recorded history. From Egyptian papyrus 1850 BC and classical texts from East Asia and India through ancient Greek and Roman documents, methods for abortion as well as contraception including methods for postcoital contraception are reported.

The Greek physician Soranus of Ephesus practiced gynecology in Alexandria and Rome approximately AD 98-138 and was the author of several medical textbooks. In his 4 volume textbook on Gynecology he describes a postcoital method to prevent pregnancy: “ ... *one must consequently be aware of having sexual intercourse at those periods which we said were suitable for conception. And during the sexual act, at the critical moment of coitus when the man is about to discharge the seed, the woman must hold her breath and draw herself away a little, so that the seed may not be hurled too deep into the cavity of the uterus. And getting up immediately and squatting down, she should induce sneezing and carefully wipe the vagina all round; she might even drink something cold. It also aids in preventing conception to smear the orifice of the uterus all over before with old olive oil or honey or cedar resin or juice of the balsam tree, alone or together with white lead; or with a moist cerate containing myrtle oil and white lead...* ” [27].

Although most recommendations are not efficacious, the antifertility activity of several medicinal products mentioned in ancient literature has been further explored in modern time and a few are undergoing clinical trials after reassuring animal studies. One of the plants used for abortion and contraception since ancient times, in addition described for postcoital use, is the wild carrot Queen Anne’s Lace (*Daucus carota*), which still is supposedly used traditionally in India. Extract from its seeds is in fact also possible to purchase nowadays via mail order, where advertised as “a natural contraceptive” with instructions to use daily from one day prior to ovulation and for one week ahead to avoid implantation after UPSI or when using a traditional method such as withdrawal. The mechanism is claimed to be hindering the conceptus to implant in the uterus. This particular compound has shown some anti-ovulatory properties in rabbit [28], variable and contradicting effects on inhibition of implantation in rat, and effective inhibition of implantation when administered postcoitally in mouse [29]. The mechanisms suggested for the postfertilizing effects are interference with progesterone synthesis by terpenoids in the seeds [30].

2.5 EMERGENCY CONTRACEPTION IN MODERN TIMES

2.5.1 High-dose Estrogens

After the discovery that estrogens could prevent pregnancy in mammals in the 1920s, veterinarians started to administer estrogen to horses and dogs that had mated out of owner control [31, 32]. In the late 1960s, postcoital treatment with high-dose estrogens like

diethylstilbestrol (DES) or EE within 72 h after UPSI was proved effective and introduced also for women in order to prevent pregnancy. Due to risk of vaginal cancer and anomalies in the reproductive tract of the offspring with DES treatment, the most common regimen subsequently consisted of 5 mg EE daily for 5 days. Endometrial biopsies revealed a retarded pattern after treatment. There were further considerable side effects with the treatment of mainly nausea and vomiting. The androgenic progestogen Danazol in a two-dose regimen starting within 72 h from an UPSI has also been used for EC, however due to higher efficacy with other compounds developed, this treatment was rather short-lived [33].

2.5.2 The Yuzpe regimen

The successful Yuzpe method was first described by Canadian physician Dr Albert Yuzpe in the 1970s and became a widely spread regimen that outrivalled and replaced previous high-dose estrogen methods. The initial hormonal combination consisted of a single dose of the progestin dl-norgestrel and EE [34]. Further development resulted in the regimen 200 µg EE and 1 mg LNG, divided into 2 doses with 12 h apart, the first taken within 72 h from UPSI. Several studies have concluded its efficacy, although side effects such as nausea, vomiting and breast tenderness were commonly reported [35, 36]. An advantage of this method was that the active ingredients were the same as in many types of regular COCP, which made it quite easy for women to get hold of the correct treatment and subsequently take an adequate number of tablets. The side effects were similar to that of high-dose estrogen, however they occurred to a lesser degree. The main mechanism of action of the Yuzpe regimen has been shown to be through an effect on follicular development and ovulation and not on endometrial function [37]. The Yuzpe regimen was the standard ECP method up until the 1990s. Only one previous study investigated the effect of a COCP containing another progestin than LNG for use as EC, namely norethindrone, and seemingly that regimen is safe and effective as EC as well [38].

2.6 CURRENT EC METHODS

Mono-treatment with mifepristone or LNG, thus without estrogen, was introduced in the 1990s and demonstrated higher efficacy and lesser side effects than the Yuzpe treatment and thus replaced this regimen. It consequently became the gold standard [35, 39]. The Yuzpe regimen is however still being used in countries lacking more effective EC options, considered as a dedicated ECP, and remains on the WHO EML. If no dedicated EC method is available, it is possible for a woman to use a specified number of regular COCPs containing EE and LNG as EC (as Yuzpe). The treatment includes intake of tablets in 2 doses with 12 h apart and the number of tablets needed depends on the hormonal content of that particular combination, ranging between 4-6 tablets for the most common preparations [40]. LNG containing regular Progestin-only Pills (POP) can also be used as EC and similarly the number of tablets needed vary depending on hormonal content.

Current dedicated EC methods include insertion of a Cu-IUD or intake of a hormonal ECP in a single oral dose, either LNG or one of the PRMs mifepristone or UPA, although

mifepristone is available for this purpose only in a very limited number of countries. With almost no medical contraindications, the current dedicated ECPs are safe and in addition perceived tolerable by women.

2.6.1 Copper Intrauterine Device

The most effective EC option is undoubtedly insertion of a copper intrauterine device (Cu-IUD) within 5 days after ovulation, which has an extremely low failure rate of 0.09% [19]. Since ovulation is difficult to assess, current recommendation is insertion within 5 days after UPSI. Turok *et al* however demonstrated that placement of a Cu-IUD for EC at any time in the menstrual cycle is highly effective, provided a negative urine pregnancy test prior to insertion [41]. The propensity to function as a long-term contraceptive for several years after placement is an additional advantage for this EC method.

The main mechanism of action when a Cu-IUD is used for regular contraception is the toxic effect on spermatozoa, interference with fertilization and early embryo development and in addition to render the endometrium unreceptive for an implanting embryo in the unlikely event that it would reach the uterus [42, 43]. When used continuously, it does not interfere with the normal cycling characteristic development of the endometrium [44], however it can alter certain receptivity markers [45].

The contraceptive mechanism when used for EC depends on when in the menstrual cycle the Cu-IUD is inserted. If pre-ovulatory, it may prevent fertilization in a similar way as when used for routine contraception, while postovulatory insertion but before implantation most probably inhibits implantation. These postfertilizing effects are not that of an abortifacient, but consist of preventing implantation at the endometrial level, which makes insertion of a Cu-IUD a superior method of EC [46]. Unfortunately, health care providers frequently fail to inform about this EC option and women are generally unaware of the effectiveness of the method. Furthermore, many women prefer hormonal ECPs as oral intake often is conceived as more convenient and in addition does not require access to insertion by a health care professional. There are currently ongoing clinical trials on the use of the LNG-releasing intrauterine system (LNG-IUS) for EC, with and without administration of LNG-EC orally prior to insertion. Such studies are welcome since hormonal IUD is increasingly preferred as a method for ongoing contraception due to its beneficial profile regarding improvement of menstrual related symptoms.

2.6.2 Levonorgestrel

Levonorgestrel-EC (LNG) is the most widely available ECP worldwide. Several studies have demonstrated LNG to be significantly more effective than the Yuzpe regimen and further with less side effects compared to the Yuzpe regimen [35, 47, 48]. Initially, LNG-only ECPs were administered in a two-dose regimen with intake of 0.75 mg within 72 hours from UPSI and a repeated dose of 0.75 mg 12 hours later. Several studies thereafter could demonstrate that a single LNG dose of 1.5 mg was equally effective so the recommended regimen switched, proving beneficial in terms of compliance [49, 50].

The mechanism of action of LNG when used for EC is delaying or inhibiting the Luteinizing hormone (LH) surge, which in turn delays or inhibits ovulation [51, 52]. Should LNG be administered after onset of the LH surge however, it has no ability to interfere with the ovulatory process and thus has no preventive contraceptive effect [52, 53]. Interestingly however, is that addition of the non-steroid anti-inflammatory drug (NSAID) meloxicam to LNG significantly prevented more follicles from rupturing than with LNG alone at an advanced stage [53, 54].

An effect of LNG on cervical and uterine mucus, which normally contributes to the contraceptive effect when used as regular contraception, is not likely contributing to the EC effect when postcoitally administered, and viable spermatozoa have been observed in the genital tract 24-48 h after LNG intake [55]. LNG-EC administered pre- or post-ovulatory does not significantly alter endometrial morphology or receptivity markers [51, 56]. Neither does it have an adverse effect on embryo viability or inhibit the process of human embryo implantation, as demonstrated *in vitro* [57]. A prospective cohort study of pregnancies after LNG-EC failure found no negative association between pregnancy outcome and LNG exposure [58]. The effectiveness of LNG decreases as the time between UPSI and treatment increases when used for up to 120 h after UPSI, with significantly higher failure rates after 72 h [59]. Thus it is recommended for use within 72 h. However, where no other dedicated option is available it is being used for up to 120 h after UPSI.

2.6.3 Progesterone Receptor Modulators mifepristone and UPA

2.6.3.1 Mifepristone

The first generation PRM mifepristone, moreover the most well known, is a synthetic steroid hormone with approximately two times greater affinity for the PR than progesterone in humans [60]. Mifepristone administered in a single high dose of 200 mg in combination with a prostaglandin analogue is a well-established regimen for medical induced abortion [61].

The effect of mifepristone depends on the dose and stage of menstrual cycle when administered due to the concentrations of mifepristone and progesterone, respectively, as well as the expression of PRs in the target tissue at that specific time [62]. A single high pre-ovulatory mifepristone dose of 200-600 mg will inhibit follicular development and ovulation, and ovulation will not resume until a new dominant follicle has been recruited [63, 64]. On average it takes approximately 3 weeks for ovulation to be resumed after early medically induced abortion, however for some women ovulation resumes as early as 8 days following intake of 200 mg mifepristone [65].

Initially high doses of mifepristone were tested and proved effective for EC [36], however lower doses demonstrated equal efficacy with the advantage of less delay in subsequent menstruation compared to a higher dose [66]. Low pre-ovulatory doses of 10-25 mg disrupt follicular development and delay or block ovulation in a dose-dependent manner, which makes such a regimen effective and suitable for EC [51, 59, 67]. However, presumably due to the association with abortion and thus for political reasons, mifepristone for EC is currently

available in a limited numbers of countries, namely China, Moldova, Russia, Ukraine and Vietnam [40].

2.6.3.2 *Ulipristal Acetate*

The so-called second generation PRM UPA is the most recent contribution to hormonal EC, for which it was specifically developed. It is administered as a single dose of 30 mg within 120 h after UPSI and was approved by the European Medicines Agency (EMA) 2009 and further authorized to be accessible from pharmacies without prescription from a doctor in all European states 2015.

Clinical trials comparing the failure rate of different ECPs have concluded that UPA is superior to the worldwide gold standard LNG in preventing unwanted pregnancies and should be recommended as first choice where available [18, 68, 69]. A meta-analysis with combined data from past clinical randomized controlled trials showed a significant reduction in pregnancy rates after UPSI for women who were treated with UPA compared to those who received LNG. If UPA was administered within 72 hours after UPSI the risk was halved and when administered within 24 hours, UPA lowered the risk by almost two thirds compared with LNG [18, 70].

In a series of pharmacodynamic studies comparing different oral EC regimens, Brache et al demonstrated that UPA was more effective than LNG in preventing follicular rupture at a late follicular stage when the risk of conception is high [52, 53, 71]. UPA administered before the onset of the LH surge inhibited 100% of follicular ruptures. When administered after the onset of the LH surge, but before the LH peak, UPA inhibited 79% of follicular ruptures, and even at a very late pre-ovulatory state with a mean follicle diameter of 18 mm, ovulation was delayed by UPA for at least 5 days in 59% of women [71]. The higher efficacy is thus seemingly due to a wider time window of effect, where UPA can inhibit follicular rupture and delay ovulation beyond the life span of sperm. This effect is maintained even when administered at an advanced phase of follicular development, after the onset of the LH surge, where LNG is no more effective than placebo. However, when the risk of conception is at its highest around the time of ovulation, at or after the LH peak, none of currently available ECPs can prevent follicle rupture.

2.7 ENDOMETRIAL EFFECTS OF MIFEPRISTONE AND UPA

2.7.1 Mifepristone

High doses of mifepristone affect implantation due to inhibition of endometrial development [62, 72, 73] and administration of a single high dose of mifepristone 200 mg once monthly on menstrual cycle day LH+2 (LH peak = 0) has been demonstrated to be an effective method of contraception without altering the menstrual cycle and for most part maintaining monthly ovulations [72, 74]. The regimen of a once-a-month pill is unfortunately not very feasible in clinical practice as timing of treatment is essential.

The effect of a high concentration of mifepristone (10 μ M) on human embryo implantation process has further been studied more closely *in vitro*, in a 3D endometrial co-culture system. None of the embryos in the mifepristone group attached to the construct, whereas embryos in the LNG treated cultures could attach to the cultures [57]. Mifepristone in a single post-ovulatory dose of 10 mg rendered the endometrium slightly out of phase and to a limited extent affected studied endometrial receptivity markers [51]. This dose was consequently not sufficient to prevent pregnancy when given once weekly and almost half of the women had monthly ovulations with this regimen [75]. In contrast, clinical trials with mifepristone given weekly in doses of 25 or 50 mg have been successful in preventing pregnancy [76] although inconsistent in inhibiting ovulation [77]. The preventive effect is presumably due mainly to endometrial impact. Very low doses that do not impair ovulation cause asynchronous endometrial changes when administered orally daily or weekly, however are not very effective to inhibit endometrial receptivity and implantation as evidenced by high pregnancy rates in clinical studies [75, 78, 79]. The threshold for continuous inhibition of ovulation has been estimated at a daily dose of 2 to 5 mg, which in a majority of women also causes amenorrhea [80-85]. Whether or not the pronounced endometrial changes caused by these regimens would permit embryo implantation had not been studied for the human embryo implantation process.

2.7.2 Ulipristal Acetate

Since UPA is a similar compound to mifepristone and more effective than LNG in preventing pregnancies without decrease in effect if taken within the 5 days following an UPSI, questions have been raised as to whether the mechanism of action of UPA involves postfertilizing effects on the endometrium and embryo implantation [86]. UPA has, similar to mifepristone, a dose-dependent effect on the endometrium and high or repeated doses affect endometrial histology with reduced endometrial thickness and inhibition of down-regulation of PRs. In low doses, such as those relevant for EC, UPA does not significantly delay maturation compared to placebo, although minor endometrial changes have been observed [87, 88].

UPA affects human spermatozoa to some degree, such as progesterone-induced acrosome reaction, intracellular calcium concentration and hyperactivation. However, the effect is not believed to be of any significance for EC since several other qualities of importance (such as viability, motility and capacitation) are not affected [89]. Consequently, a recent study could demonstrate that UPA in a concentration corresponding to EC dose did not have any effect on fertilizing abilities of human sperm [90]. Li *et al* have showed that UPA could effect tubal function to some extent [91], however speculated that this should not possess a post-ovulatory effect on sperm since they can be retrieved in the fallopian tubes 5-10 mins after UPSI [92, 93].

There are still few reported pregnancies after intake of UPA for EC and the manufacturer of UPA-EC has established a pregnancy registry in order to collect info on treatment failure (pregnancies occurring despite EC use) or pregnancies unrecognized at intake. A report from

postmarketing surveillance of UPA showed no evidence of teratogenic effects after treatment with EC. However, the number of exposed pregnancies was small, suggested due to unwanted pregnancies being terminated or non-reported if uncomplicated [94]. The direct effect of UPA on human embryo implantation had not been studied when this project started.

2.8 THE FERTILE WINDOW AND PREGNANCY RISK

An UPSI can result in pregnancy during a limited time in the menstrual cycle, ranging from approximately 5 days before to 1 day after ovulation, due to the survival length of spermatozoa in the female reproductive tract (120 h) as well as the life span of the oocyte after ovulation (12-24 h). The fertile window varies in accordance with the time of ovulation and the highest rates of conception have been observed within two days prior to ovulation [95, 96].

2.8.1 Probability of conception

The number of expected pregnancies was initially estimated based on conception probabilities by cycle day of UPSI relative to the day of ovulation, however since it is difficult to assess ovulation and assessments become imprecise it is most often estimated based on conception probabilities by cycle day of UPSI relative to the first day of bleeding. These estimates show that there are only two days of the menstrual cycle in which a woman faces zero risk of pregnancy and that is the two first days of her cycle [97].

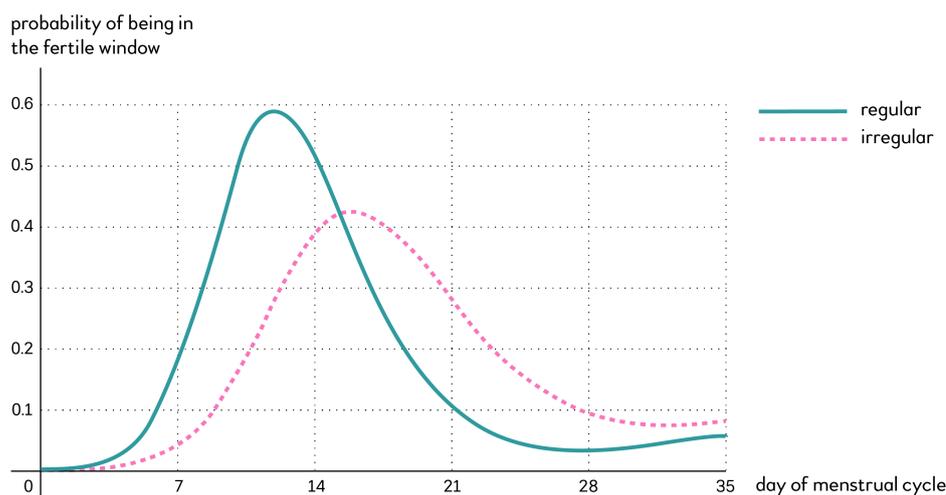


Figure 3. Probability of being in the fertile window for women with regular or irregular cycles. Modified from Wilcox, Dunson and Baird, BMJ 2000

2.9 EC EFFECTIVENESS

Clinical studies of EC efficacy are for ethical reasons most often designed as comparison trials between different EC methods instead of randomized placebo controlled trials. It is

therefore difficult to assess the actual effectiveness for each method to reduce the risk of pregnancy. The estimated efficacy is instead calculated based upon assumptions of the theoretical baseline risk for the particular woman depending on when in the menstrual cycle the act of UPSI took place, and how long before EC intake. These rates are further compared with the actual number of pregnancies after EC administration in clinical EC trials [40].

Currently available EC options have been estimated to prevent 57-99% of pregnancies, depending on method and when used in the menstrual cycle [18, 19, 35, 49, 98]. As Trussell *et al* correctly point out; accuracy in estimating EC efficacy depends on how accurate data is of menstrual cycle day and timing of UPSI [40]. Among women seeking EC in one study, over 30% inaccurately dated their cycles as being in the fertile period when they were not [99]. An even greater proportion with nearly half of women reporting a menstrual cycle day incompatible with hormonal measures had in addition been seen previously [100]. These observations have rendered arguments that EC effectiveness in fact might be overstated. On the other hand, 60% of women in the above study had engaged in more than one act of sexual intercourse when requesting EC and although for the most part reporting use of a barrier method at that occasion [99], a possible pregnancy due to a previous intercourse risks to doom EC treatment as having failed. Further, Li *et al* concluded that EC might be more effective than estimated when they reanalyzed data from previous studies on the probability of conception after the act of UPSI and in addition considered that it does not occur independently of ovulation [22].

Ultimately, calculating EC efficacy by comparing failure rates with different methods in clinical trials might be the most accurate approach. As argued in a recent review article with the example taken of combined data from comparison trials with the Yuzpe regimen and LNG [47, 48, 101]; as the number of pregnancies in women who received LNG was approximately half of those who received Yuzpe (RR 0.51), the LNG regimen should at least have an efficacy of 49% if Yuzpe was completely inefficacious. Additional efficacy points of the Yuzpe regimen would thus add further points of efficacy to the LNG regimen [40].

2.9.1 Risk factors for EC failure

2.9.1.1 Cycle day of Unprotected Sexual Intercourse

Risk factors associated with EC failure have been identified after compilation of data from conducted clinical studies and as expected, the cycle day of UPSI was significantly associated with pregnancy with the highest risk at the time of ovulation, independent of ECP type [102].

2.9.1.2 Treatment delay

With increasing treatment delay after UPSI, the risk of nearing an ovulation is greater and thus supposedly EC failure. Regarding effectiveness of treatment, several studies have shown that LNG is more effective if taken closer to the UPSI, with decreasing effect as the interval between UPSI and EC increases, markedly after 72 h [17]. Similarly, even when adjusted for cycle day when UPSI took place, treatment delay has demonstrated to decrease efficacy

[103]. In contrast, a combined analysis from pooled data of four WHO trials concluded that not until on day 5, after 96 h, was a decline in efficacy observed with LNG [104]. There is no decline in efficacy for the PRMs mifepristone and UPA [18, 59, 105].

2.9.1.3 Further Acts of Unprotected Sexual Intercourse

Since follicular development and ovulation usually resume within a week after intake of an ECP, women are at risk of becoming pregnant if further acts of UPSI take place in the same cycle due to timing of a postponed follicular rupture and ovulation, regardless of which ECP method is used. Glasier *et al* demonstrated that women who had further acts of UPSI after ECP use were four times more likely to get pregnant than were those who did not report such an event, with no difference in between the groups treated with UPA or LNG [102]. This finding is consistent with the meta-analysis in a Cochrane review which concluded that the pregnancy risk was approximately 3 times higher for women who had further acts of UPSI in the same cycle as ECP treatment, than for women who did not [17].

2.9.1.4 Body Mass Index

The variable Body Mass Index (BMI) had a significant impact on pregnancy risk with a 3 times greater risk for a woman with BMI above 30, compared with women who had BMI below 25. This increased risk was more pronounced for women treated with LNG compared to UPA, where LNG was no more effective than no treatment in women with BMI above 26, while loss of effectiveness with UPA was calculated to BMI 35. These trials were however not initially designed to study these relationships and the number of overweight and obese women was limited, as were the number of pregnancies [102]. The debate over what influence BMI and weight prepossess on the effect of EC has been vivid as it in some instances caused that women were denied treatment due to their weight.

Pooled analysis from four large clinical WHO trials showed an increased pregnancy rate in obese women. However, when excluding one site in the analysis, the impact of obesity did no longer have an apparent impact on LNG efficacy, with the reservation that few obese women in the latter analysis were included [106]. Current recommendations are that women of all weights should be provided ECP when needed since the benefits outweigh the risks of decrease in effectiveness. However, it has been shown that obesity has a negative impact of LNG-EC pharmacokinetics and that doubling the dose adjusts for reduced concentration in serum to levels seen in women with normal weight [107]. If this approach is correct and should lead to updated guidelines for effective treatment with LNG-EC in an overweight population remains to be elucidated.

2.10 QUICKSTART OF REGULAR CONTRACEPTION

Except for being the most effective EC method, with no reduction in effectiveness over time, including should further acts of UPSI take place or have occurred at the time of ovulation, or in regards to BMI, insertion of a Cu-IUD also has the additional propensity of serving as an effective ongoing and long-term contraception if left in place [108, 109]. In the view of

reducing pregnancy risk after ECP treatment, it is of great importance to take the opportunity to initiate a more effective regular contraceptive method when a woman seeks EC, as she most likely is motivated to do so at this time. In this capacity, EC could serve as a gateway to bridging over to long-term regular contraception. In a recent pharmacy-based intervention study in the UK, a higher proportion of women who were provided with a packet of progestogen only pill (POP) for temporary contraception when purchasing LNG-EC, were using a regular method after two months compared to those who only received the ECP [110].

There is an ongoing discussion regarding “quick-starting” contraception after ECP treatment, a term which refers to initiating a regular contraceptive method immediately after ECP use. The perspective is that the sooner a woman starts with a regular method, the sooner she will be protected from an unwanted pregnancy. It is recommended for women to use a barrier method for contraception or abstain from sexual intercourse for the first 7 days of treatment with a regular oral contraceptive pill following LNG-EC intake, which is the time expected until the regular contraception can be completely relied on for contraception, as otherwise when starting such treatment mid-cycle [16].

Regarding UPA, there are concerns that quick-starting contraception after EC intake could potentially jeopardize contraception for a longer time initially, due to possible interference of UPA with progestin-containing contraceptives at the PR site. This could alter the effectiveness of hormonal contraception and vice versa. When this project started, current advice to women who quickstarted an oral hormonal contraceptive pill after UPA treatment, was to abstain from sexual intercourse or to use a barrier method until the next menses before the regular contraception could be fully relied upon for contraception [111].

The information on the possible interaction between UPA and a regular oral hormonal contraceptive pill is necessary so that women can be provided with evidence-based advice on the most effective ways to reduce risk of pregnancy following EC use.

2.10.1 Barriers to use of regular contraception and EC

Efforts made have increased availability of EC worldwide and the method is well recognized nowadays, however access is still limited and unequally spread, as well as lacking in many settings (<http://www.cecinfo.org>) (accessed April 2017). Limited access in countries where EC is registered is due to various factors, such as cost, whether accessible only by prescription, behind-the-counter or OTC, as well as the woman’s age or marital status, where consequently young women are denied access. Unintended pregnancies could be avoided if barriers to the use of effective contraceptive methods would be reduced, however knowledge of EC as a possible method to prevent pregnancy is also unequally spread around the world [112].

Many factors contribute to the limited access and availability of EC for women globally. There are political and legislative, as well as religious and moral actions in many countries around the world in opposition against EC. Some of the myths and misconceptions

surrounding EC concern mechanisms of action of these methods where EC is often confused with induced abortion. There are further concerns that postcoital contraception possesses postfertilizing effects on the endometrial function and embryo implantation, should ovulation and fertilization have taken place before EC action. In the name of the catholic doctrine, the state of Vatican City opposes the use of EC and in a statement inaccurately declares that use of EC is a chemically induced abortion and furthermore that women seeking EC as well as EC providers wish to terminate a pregnancy in progress

(http://www.vatican.va/roman_curia/pontifical_academies/acdlife/documents/rc_pa_acdlife_doc_20001031_pillola-giorno-dopo_en.html) (accessed April 2017).

Lack of knowledge of mechanisms of action for EC is not only limited to women and the general public, but also exists among health care providers treating them. Although several reports have concluded that EC availability does not increase the rates of UPSI or sexually transmitted infections, nor adversely affect the use of regular contraception [113-116], these concerns are still regularly expressed.

Sedgh and Hussain examined reported reasons for not using regular contraception among married women with an unmet need in developing countries between 2006 and 2013. The reasons were diverse and included not having access to contraceptive services, opposition to contraception due to the woman's or partners religious or personal beliefs and breastfeeding among others. The most common reasons were however fear of side effects and health risks and infrequent sex. Nonetheless, at least half of the women who reported that the reason for not using contraception was due to infrequent sexual activity actually had had sex within the three recent months. The authors concluded that women with an unmet need of contraception would benefit from having access to contraceptive services where they could seek counseling and information, which includes help to select an appropriate method among a range of methods possible to choose from [117].

2.11 THE FEMALE REPRODUCTIVE SYSTEM

The female reproductive organs are located in the pelvis and include the uterus with cervix, vagina, ovaries and fallopian tubes. The pear-shaped uterus consists of three anatomical layers; the thin outer layer serosa, the thick middle smooth muscle layer myometrium and the inner mucosal layer endometrium (Figure 4). The uterus is highly responsive to ovarian hormones where the endometrium in a cyclic manner monthly prepares to receive a fertilized egg and if so with ability to maintain a pregnancy, or in absence of implantation, shed with subsequent self-renewal.

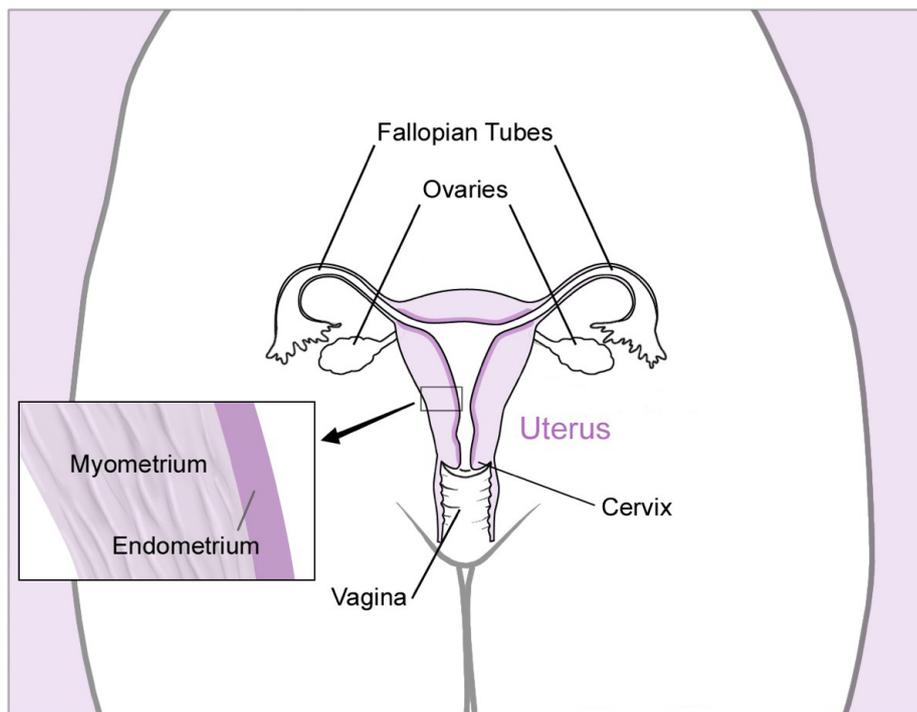


Figure 4. The female reproductive system
 Source: National Cancer Institute, via Wikimedia Commons

2.11.1 The Endometrium and endometrial cells

The endometrium consists of two layers; the basal layer adjacent to the myometrium, and the upper functional layer outwards the uterine lumen with the surface epithelium on top. The functional layer is highly sensitive and responsive to ovarian hormonal fluctuations and displays cyclic proliferative, secretory and degenerative changes, where it ultimately sheds during menstruation. The basal layer however is retained after menstruation and is the source for endometrial regeneration in future cycles (Figure 5). The endometrium is composed of several cell types, the two major being endometrial epithelial cells (both luminal and glandular) and endometrial stromal cells. In addition, the endometrium contains endothelial cells, stem cells and fluctuating levels of immune cells [118].

2.11.1.1 Epithelial and Stromal cells

The endometrial epithelial cells make up a single layer of columnar cells, lining the luminal surface and the glands. The luminal epithelial cells are normally covered with repellent protective glycocalyx molecules, not allowing opposing uterine or embryonic cells to adhere. At the window of implantation (WOI) however, the epithelial cells acquire a state of receptivity where loss of inhibitory components and upregulation of cell-adhesion molecules permits an embryo to attach. The glandular epithelial cells secrete autocrine and paracrine factors of importance for endometrial maturation as well as for embryo implantation.

The stromal cells are located in the connective tissue-like stromal compartment, which predominantly consists of proteoglycans and collagen. These fibroblast-like cells produce extracellular matrix (ECM) including a range of proteins such as matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) as well as growth factors and cytokines. In further preparation for implantation, edema increases throughout the stromal compartment and the stromal cells become less densely packed [119]. Stromal cells are furthermore located adjacent of endometrial spiral arteries where they are important for angiogenesis during the menstrual cycle and pregnancy. At the time of ovulation, mitosis of the stromal cells reaches a maximum and they are further increased nearing the menstrual phase, however in the peri-implantation period mitosis decreases markedly [120].

2.11.1.2 Leukocytes

In addition to epithelial and stromal cells, the endometrium contains a substantial leukocyte population that varies during the normal menstrual cycle, as well as during pregnancy. The regulating mechanisms for this dynamic process are not fully understood but are believed to involve interactions between steroid hormones and factors that are produced by the endometrial cells, as well as from trophoblast cells during pregnancy. The endometrial leukocytes are mainly located within the stroma and the main leukocyte cell types are uterine natural killer (uNK) cells, macrophages and T lymphocytes [121]. Uterine NK (uNK) cells are the major leukocyte population in non-pregnant secretory endometrium as well as in decidua during early pregnancy. uNK cells proliferate and differentiate in the uterus but their origin is debated. They probably originate from early NK progenitors recruited from blood. However several pathways are possible [122].

In the normal cycling endometrium, uNK cells proliferate and accumulate in the stroma postovulatory, with a dramatic increase from the mid-secretory phase as response to [121] circulating progesterone and through early pregnancy if such occurs. The influence from progesterone is indirect since uNK cells themselves lack the PR [121]. In studies exploring these mechanisms through treatment with antiprogestins, the IL-15 pathway has been identified as a key network in uNK development and function, where IL-15 mediates the differentiation of immature to mature uNK cells. IL-15 expression in endometrium correlates with circulating progesterone levels as well as with uNK cells and decreases in response to treatment with antiprogestins. Treatment with the antiprogestin Asoprisnil dramatically reduces the number of uNK cells in the endometrium and histological examination of tissue after exposure demonstrates altered vascular architecture [123].

2.11.1.3 Stem cells

The basal layer of the endometrium contains stem cells with the intrinsic capacity of regenerating the functional layer and surface epithelium every month during the reproductive years [124]. This knowledge has opened up for the exciting research field of endometrial stem cells, which have been shown to also shed during menstruation. Thus, menstrual blood provides a source of stem cells that can be collected non-invasively. Hopefully in the future

this may lead to novel treatment possibilities for women suffering from Ascherman's syndrome or infertility disorder due to endometriosis.

2.11.1.4 Endothelial cells

Endothelial cells in the endometrial vasculature are regulated by changes in levels of estrogen and progesterone during the menstrual cycle. Angiogenesis has a central role in the menstrual cycle during the dynamic events of endometrial regeneration and maturation. It is initiated to repair the endometrial vasculature during menstruation and new vessels are further generated from pre-existing ones during the proliferative phase. Angiogenesis moreover continues during the secretory phase when spiral arteries grow and coil and in the development of the subepithelial capillary plexus [125]. Thus, the endometrium becomes richly vascularized in time for implantation.

The endothelial cells express ER β and are stimulated to grow in response to estrogen [126]. However, they seemingly do not express PRs, although demonstrated to respond to progesterone withdrawal, which is believed to be due to paracrine factors mediated by stromal cells close to the vessels [127]. Helmestam et al demonstrated that mifepristone significantly reduced tube formation in endothelial cells when epithelial cells and stromal cells were co-cultured compared to mono-cultured epithelial cells, why the authors concluded that the stromal cells probably influenced the effect of mifepristone through paracrine mechanisms [128].

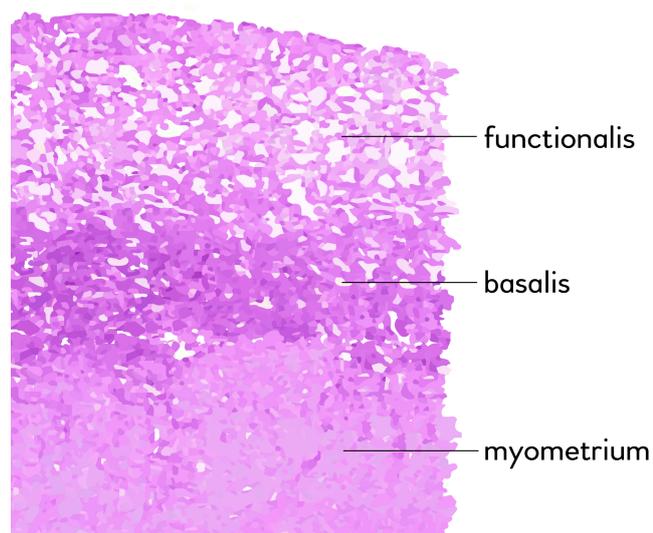


Figure 5. Endometrial morphology with Basalis and Functionalis layers

2.11.2 Hormonal regulation of the endometrial cycle

Each menstrual cycle begins with the first day of menstruation, cycle day one. Ovulation occurs 14 days before onset of the subsequent menstruation, approximately on cycle day 14 in an average menstrual cycle of 28 days. During a woman's reproductive life in the

developed world, these continuous cyclic events of regeneration and shedding take place more than 400 times [118].

2.11.2.1 Ovarian cycle

Pulsatile secretion of GnRH from the hypothalamus regulates production of gonadotropin hormones, which are secreted from the pituitary gland in a similar pulsatile manner. The gonadotropin hormones follicle stimulating hormone (FSH) and LH control the ovarian function by inducing sex steroid secretion and follicular growth. [129]. FSH stimulates the development of ovarian follicles by acting on granulosa cells in preantral and antral follicles leading to conversion from androgens to estrogens with help from aromatase enzyme. The granulosa cells surrounding the oocyte proliferate under FSH influence and FSH and estrogen further stimulate the production of follicular fluid.

The theca cells in the larger follicles express LH receptors and in response to LH produce androgens, which are transported to the neighbouring granulosa cells and subsequently converted into estrogens as described. The pulsatile frequency and amplitude of gonadotropin hormones determine the amount of steroid hormones to be produced and further varies due to feedback mechanism from hormones produced [129]. In addition, the preovulatory follicle synthesizes progesterone, which slows down follicular growth and suppresses apoptosis and mitosis through the progesterone receptor membrane component-1 (PGRMC1) in the granulosa cells.

The LH surge initiates angiogenesis of the ovulatory follicle through angiogenic factors in granulosa cells, including production of the key regulator prostaglandin E2 (PGE2) [130, 131]. PR signaling further induces expression of gene products necessary for follicular rupture [132]. Ovulation occurs approximately 36 hours after the LH peak and around 14 days before the subsequent menstruation. The former dominant follicle thereafter transforms into the corpus luteum with cells luteinized by LH. Angiogenic factors increase the blood supply, which permits the precursor cholesterol to reach the cells of the corpus luteum and be converted to progesterone. Postovulatory progesterone levels increase to above 10 ng/ml, with a peak during the mid-luteal phase (Filicori 1984). If conception and implantation does not take place, regression of the corpus luteum occurs rapidly 9-11 days postovulation.

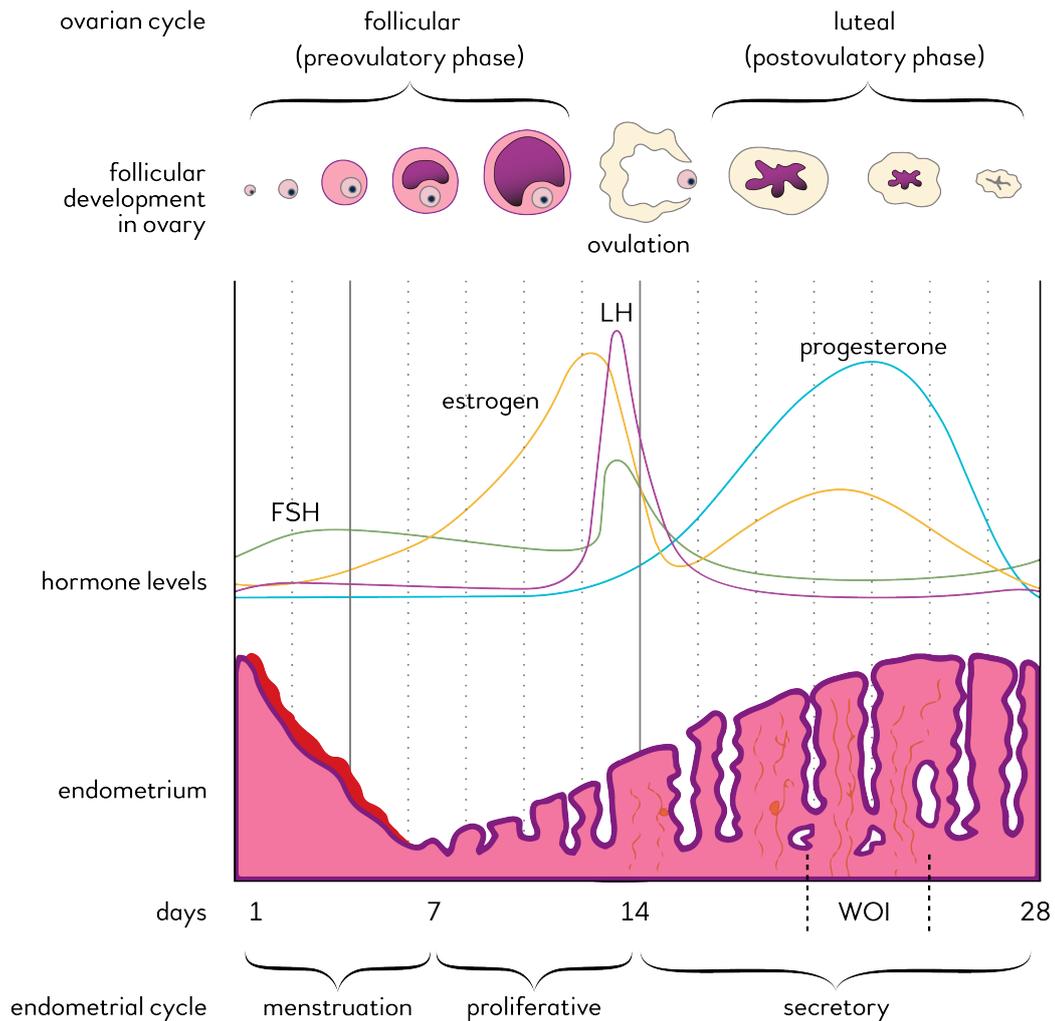


Figure 6. The menstrual cycle. Modified from Wikimedia Commons and Encyclopedia Britannica 2008.

2.11.2.2 Endometrial cycle

During the endometrial cycle, the functional layer of the endometrium undergoes morphological and functional changes in a cyclic manner in response to ovarian steroid hormones. The endometrial cycle is divided into three phases, each with typical histological features; the menstrual phase, the proliferative phase and the secretory phase, the latter can in turn further be divided into early secretory and mid to late secretory phases. The menstrual and the proliferative phases occur during the ovarian follicular phase and the secretory phase during the ovarian luteal phase (Figure 6) [118]. Although continuously in change, the endometrial alterations follow a specific, regular, sequenced pattern, which makes it possible to date the endometrium histologically from endometrial biopsies. The classical morphological criteria for evaluating endometrial development, established by Noyes' *et al* in the 1950s is still used and referred to [119]. However it has also been questioned whether it is

suitable for clinical guidance regarding evaluation of a receptive endometrium [133] or if it is a reliable tool for menstrual cycle day assessment [134].

Estrogen and progesterone receptor expression in the endometrial cells change dynamically during the menstrual cycle. Estrogen receptors are predominantly expressed in stromal cells during the proliferative phase, however the concentration falls drastically under the influence of progesterone in the luteal phase. Progesterone facilitates the action of estradiol in inducing the LH surge that triggers ovulation [135]. Progesterone is essential in initiation and maintenance of pregnancy and stimulates the proliferation and differentiation of stromal cells. PR expression in endometrial glandular epithelium peaks in the late follicular and early luteal phase with a decline at the mid-secretory phase, whereas in stromal cells PR expression shows only minor fluctuations [136, 137].

Estrogen secreted during the follicular phase exerts mitogenic effects on the endometrial tissue, inducing growth as well as angiogenesis of spiral arteries of the endometrium. Estrogen increases PR expression, which permits the endometrium to respond to progesterone that is produced during the following postovulatory luteal phase. The endometrium is regulated by both estrogen and progesterone during the early secretory phase, however characterized by progesterone dominance during the mid-secretory phase and progesterone withdrawal in the late secretory phase. Progesterone shifts the endometrium into a highly secretory state by inducing differentiation in the endometrial glandular epithelium as well as in the stromal compartment. The epithelial glands become increasingly tortuous with increase in secretory activity and produce a number of cytokines and growth factors with the purpose to facilitate implantation should fertilization occur. The stromal cells likewise respond to progesterone and differentiate into decidual cells during the secretory phase.

2.11.2.3 Decidualization

The stromal cells are elongated fibroblast-like cells that in response to progesterone transform into larger rounded specialized secretory decidual cells during the secretory phase. The decidualization process starts with the stromal cells close to the spiral arteries via endocrine influence and thereafter spread throughout the endometrial tissue through paracrine and autocrine signals. Decidualization involves remodeling of the ECM and vasculature along with the infiltration and/or proliferation of specific immune cells. The decidualization process in humans is independent of signals from a blastocyst, thus occurring in response to progesterone during the luteal phase in every normal cycle whether or not pregnancy occurs. The decidualized endometrial tissue is dependent on continuous progesterone stimulation in order to maintain its integrity [138].

In the absence of fertilization and implantation, the estrogen and progesterone levels rapidly decrease due to regression of the transient corpus luteum. The abrupt hormonal withdrawal induces spasm of spiral arteries, followed by ischemia, an increase of proteolytic enzymes and apoptosis and results in destruction of the functional layer, which ends with shedding and thus onset of menstruation.

2.11.3 Window of implantation

It is only during a short period of time in the menstrual cycle, in the mid-secretory phase, that the uterus will accept and accommodate the blastocyst. The receptive phase, or WOI refers to the time during the endometrial cycle when the endometrium under the critical influence by progesterone has transformed and attained receptivity necessary for successful embryo implantation. The WOI occurs during menstrual cycle days 20-24 when the postovulatory progesterone levels peak in serum in a regular cycle (Fig 6) [139].

The receptive endometrium is characterized by a glandular epithelium that is differentiated into a secretory state and produces a repertoire of molecules, such as a variety of cytokines, growth factors and modulatory proteins that enables implantation. Following a coordinated dialogue between the competent blastocyst and the receptive endometrium, the embryo attaches to the epithelial cells and invades the endometrial stroma. The orchestrated process of embryo implantation is highly dynamic and includes a cascade of molecular signaling. Under the influence of ovarian hormones and paracrine secretions, the different uterine cells and extracellular matrix (ECM) interact in a spatiotemporally complex network to accommodate the embryo. The synchronous development of the endometrium and the implanting blastocyst is essential for adequate reciprocal communication between them and consequently for successful implantation [140]. In many mammals, the demise of preimplantation embryos is commonly occurring, suggested to be part of a natural endometrial selection process ensuring correct implantation of high quality embryos only [141]. In humans, the estimated implantation rate in natural cycles is estimated to less than 30% [142].

2.12 EMBRYO IMPLANTATION

The fallopian tube catches the released oocyte at ovulation and if there are spermatozoa present, fertilization can occur. The fertilized oocyte thereafter migrates through the fallopian tube with help from beating motion of cilia and smooth muscular contractions, while undergoing regulated mitotic cleavage, and enters the uterus in the morula stage on cycle day 17-18 [143]. After having floated freely in the cavity, it hatches out from the zona pellucida as a blastocyst stage embryo and arrives at the luminal epithelium 5-6 days postovulation. The blastocyst consists of an inner cell mass (ICM), which eventually develops into the embryo, and the outer trophoblast layer, which eventually develops into the embryonic placenta. The blastocyst begins to implant day 20-21 in dialogue with the receptive endometrium [140].

Embryonic trophoblast cells adhere to the endometrial epithelium and thereafter penetrate between the epithelial cells and invade through the underlying basal lamina, where some of the trophoblast cells fuse to form syncytial trophoblast (Figure 7). These cytotrophoblast cells become invasive extravillous trophoblast (EVT) and invade the maternal decidua as well as the inner third of the myometrium [144]. EVTs possess the ability to invade the decidua by producing large amounts of MMPs that degrade the ECM [145]. In order to ensure provision

of placental blood supply they further penetrate and remodel maternal blood vessels, thereby establishing contact between the embryo and the maternal blood supply.

The endometrium has traditionally been considered as a passive tissue in the process of embryo invasion during implantation. However, more recent studies have shown that decidualized cells are highly active and migrate towards the implanting embryo, encapsulating it as well as providing a favorable matrix for trophoblast expansion. These migratory and invasive capacities are further enhanced by close contact with trophoblast cells. It is the dialogue between the decidua and blastocyst that orchestrates the dynamic process by which the blastocyst becomes completely embedded in the endometrium [146].

The embryo-endometrial crosstalk requires a range of molecules, secreted from both the endometrium and the implanting blastocyst itself. Such factors include modulating proteins, such as growth factors and cytokines with corresponding receptors, cell-adhesion molecules, proteolytic enzymes and inhibitors among others. Interaction between these molecules forms cascades of molecular signaling into the endometrium [140, 147].

Several factors can repress or increase trophoblast invasion; autocrine factors from the trophoblast cells themselves or paracrine factors secreted from cells in the endometrium [148]. Decidualized cells produce a variety of cytokines and growth factors that are either stimulatory or inhibitory of invasion by regulating processes such as protease activity or expression of integrins. Pro-invasive factors including IL-1 β , IL-6 and IL-15 mainly function by increasing MMPs and thereby invasion and migration of trophoblasts. In order to regulate trophoblast invasion, the decidua can also control and limit the trophoblast invasion by producing protease inhibitors and/or control the direction of invasion through chemoattractant substances. IL-10 holds anti-inflammatory and immunosuppressive properties and inhibits invasion of trophoblast cells but the underlying signaling mechanisms are not fully understood. Several chemokines promote trophoblast migration and adhesion on fibronectin and collagen through their chemotactic activities, which contributes with increased invasion into the decidua [144].

Chemokines and their receptors are central in the process of embryo implantation as they are involved in the recruitment of different immune cells necessary to generate the fetal-maternal interface. Trophoblast cells actively recruit immune cells, such as uNK cells, uterine macrophages and dendritic cells, through chemokine production. Trophoblast cells can also modulate different leukocyte subsets towards an immune tolerant microenvironment, including induction of regulatory T cells, to sustain homeostasis and successful invasion and acceptance from maternal tissue [149].

The switch from pro-inflammatory to an anti-inflammatory microenvironment in the uterus in the post-implantation stage is essential for successful establishment of pregnancy. For this reason, a functional redundancy of different mechanisms (many yet to be elucidated) that trigger immunomodulation from trophoblast cells has evolved. One of the demonstrated mechanisms include epigenetic silencing with methylation of T cell-attracting inflammatory

chemokines genes in decidualized stromal cells, thereby modulating these cells capacity of recruiting T cells [149].

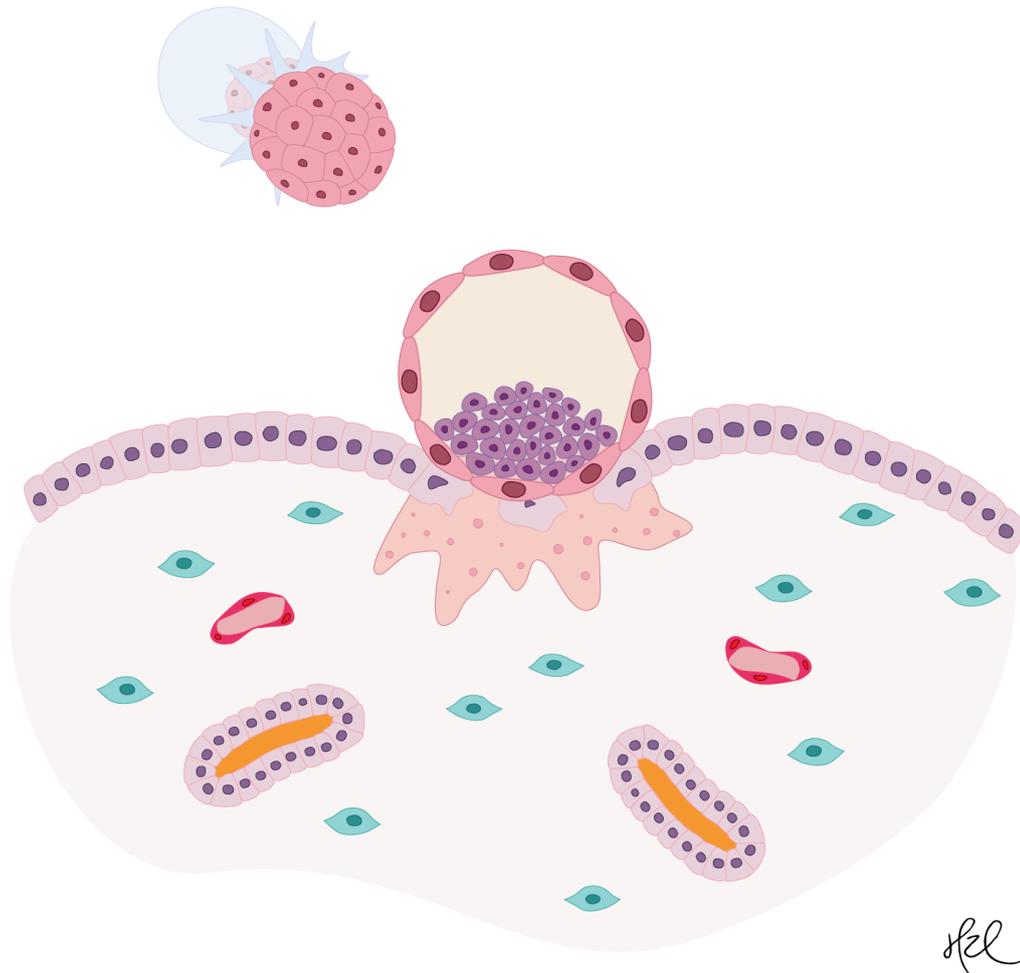


Figure 7. Early embryo implantation. The blastocyst hatches out of the Zona pellucida, attaches to the surface epithelium of the endometrium and penetrates between the epithelial cells to invade the endometrial stroma

2.12.1 Molecular markers of endometrial receptivity

The uterus needs to become shifted towards a functionally receptive state where possible to perceive the blastocysts signals in order for it to implant in the uterine wall. Downstream target genes from the activated PR regulate uterine differentiation and a range of molecules in the hormonally primed endometrium is needed for allowing the blastocyst to implant. Several molecules of importance for embryo implantation during the WOI have been identified and studied. These markers of endometrial receptivity belong to different functional groups such as cell adhesion molecules, cytokines, transcription factors and decidualization factors.

With the aim to understand endometrial receptivity and presumably in addition to find a key marker, scientists have explored the molecular signature of the receptive endometrium

through several approaches. No single suitable biomarker defining endometrial receptivity has been established but many potential markers are suggested. The list of candidates is also growing due to the rapid expansion of endometrial studies, including global gene expression studies [150-154]. Such studies have provided a vast amount of data, however often with low reproducibility and with variable results [155]. At the same time, the transcriptomic signature of the receptive endometrium has been identified through microarray and the more recent development of epigenetics and proteomics has further expanded knowledge [156]. In addition, development of the diagnostic tool Endometrial Receptivity Array (ERA) has made it possible to assess endometrial receptivity in biological samples, knowledge that could be used in the context of assisted reproductive technology (ART) when the reason for infertility is due to endometrial dysfunction [157].

2.12.2 Models to study embryo implantation

In vivo studies of human embryo implantation are difficult to perform for technical and ethical reasons and proper models for studying this complex molecular process are lacking. Most of the information on the human embryo implantation process has derived from different animal or in vitro models. Advantages of using experimental animals include high fertility and predictable patterns of physiological responses that are easy to affect. Animal models have generated valuable information on certain phases of early embryo implantation. Investigations on genetically engineered mice have further yielded important knowledge about factors of importance for the implantation process [158, 159]. However, rodent or rabbit models have considerable limitations as the reproductive physiology such as endometrial function and type of implantation process is significantly different from humans.

In contrast, the hormonal regulation and cyclic endometrial histological changes are similar to that of humans in a few non-human primates such as Rhesus monkeys (*Macaca mulatta*) and Cynomolgus monkeys (*Macaca fascicularis*). Studies of the embryo implantation process are however not very practical to conduct in larger scale in these species due to fairly low fertility in captivity and high cost. On the other hand, non-human primate models have proven to be good for testing different contraceptive drugs. Altogether, both non-primate models and non-human primate models have contributed with data on the mechanism of action of UPA and mifepristone on the endometrium and embryo implantation [160-164].

Nevertheless, due to important differences between species and since the implantation process in humans undoubtedly is unique, several different in vitro cell culture systems using human endometrial cells and human trophoblast cells or embryos have evolved. Initially, in vitro studies of implantation were conducted using endometrial explants from rabbit [165]. Mid-secretory human endometrial explants have also been used and proven to successfully enable human embryo attachment [166]. Although this method perhaps simulated the state in vivo and in addition yielded important information, it conveyed challenges such as limited possible culturing length and difficulty to assess implantation due to toxic degeneration and necrosis of the tissue.

Advantages with two-dimensional *in vitro* cell culture models for implantation studies are that they generally are easier to carry out than more advanced, complex models [167]. However, they do not typically include several cell types and thereby do not consider the important network of interaction that occurs between endometrial cells in the *in vivo* tissue. The cells in these models are further grown in a monolayer on an artificial plastic surface with loss of important 3D features. Although having given valuable information on events during implantation [168, 169], 2D studies might be of limited predictive value of conditions *in vivo*.

2.12.3 3D *in vitro* endometrial co-culture model

In order to better imitate the architecture of physiological endometrium *in vivo* and to establish a uniform reliable and reproducible model for studies on the human embryo implantation process, an *in vitro* 3D endometrial co-culture system has been developed. The method is validated and has previously been demonstrated to structurally mimic endometrial tissue *in vivo*. When histologically evaluated under light microscopy, this 3D model has proven to mimic the *in vivo* endometrial structure, including a polarized epithelium with well developed inter-cellular junctions and stromal density similar to that seen in endometrial biopsies [170-172]. Further, immunostaining for the markers cyokeratin and vimentin in the 3D construct have confirmed a monolayer of epithelial cells on the surface and stromal cells embedded in the 3D collagen matrix respectively, which was also observed with Hematoxylin-Eosin staining [56].

This model has further proven to resemble *in vivo* conditions with regards to a relevant microenvironment and to mimic the receptive endometrium, including expression of progesterone regulated receptivity markers. Thus, the molecular profile of the *in vitro* 3D culture resembles receptive endometrium *in vivo*. The 3D endometrial construct has subsequently allowed for studies of the cellular and molecular events that occur during the first days of the human implantation process, including studies on markers of endometrial receptivity. It is now well established and has previously been used to study the impact of LNG and high dose mifepristone on human embryo implantation [56, 57, 173].

2.13 PROGESTERONE AND PROGESTERONE RECEPTORS

Steroid hormones derive from cholesterol in the biosynthetic pathway and have a common basic molecular four-ring structure made up from 17 carbon atoms. There are three groups of sex steroids depending on the total number of carbon atoms; estrogens (n=18), progestins (n=21) and androgens (n=19). Progesterone circulates in the blood bound to protein with a half-life of only a few minutes, metabolizes in the liver and metabolites are further excreted in the urine. When scientists in the 1950s replaced a methyl group with a hydrogen atom at position 19 on progesterone, it resulted in a more potent progestin, leading to the development of synthetic progestins [174].

Steroid receptors all have a similar structure with a DNA-binding domain (C), a hinge domain (D) and a ligand-binding domain (E). The steroid receptors are transcription factors

that regulate gene transcription in a multistep process. When the steroid receptors are activated by their ligand, they form homodimers or heterodimers and recruit coregulators and thereafter bind the specific DNA sequences called steroid response elements. At binding, the complex induces or represses the transcription of downstream target genes [147]. The expression of steroid receptors in the human endometrium varies both spatially and temporally across the menstrual cycle [136].

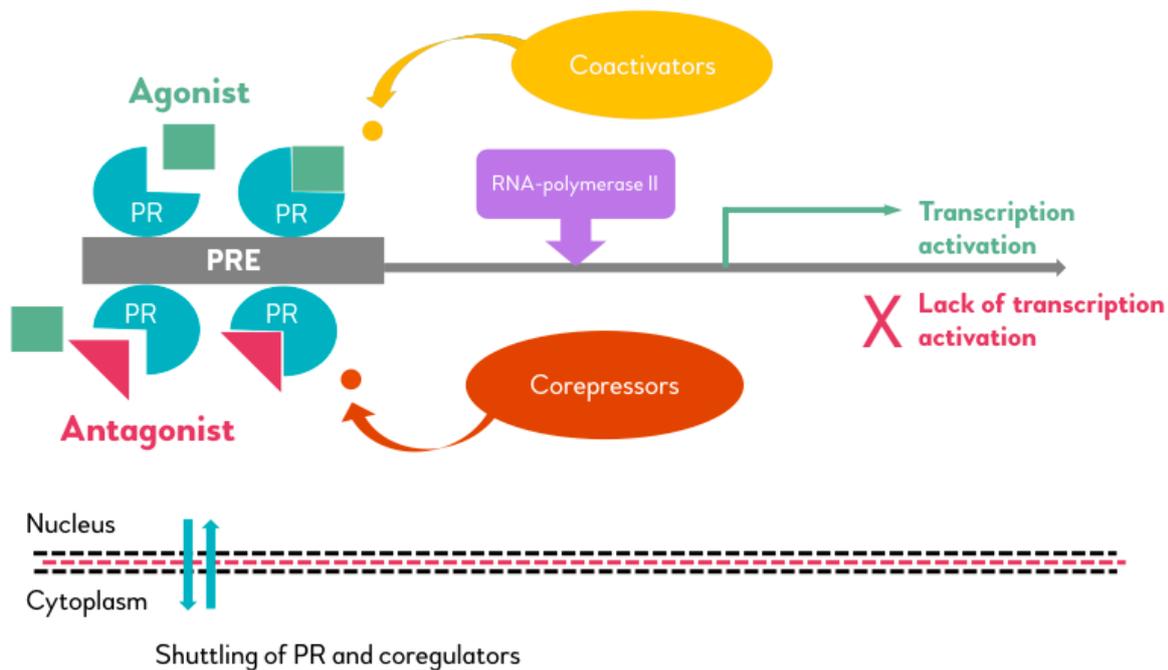


Figure 8. Activation of the progesterone receptor (PR) by its ligand. PRE=progesterone response element. Modified from Chabbert-Buffet, Hum Reprod Update 2005.

The genomic progesterone receptor (PR) belongs to the steroid receptor family and is a main regulator in the female reproductive tract. The PR is an intranuclear protein that specifically can bind progesterone and other progestins and through several steps induce transcriptional activity. Progesterone binds its receptor in the cytoplasm of cells, which causes a dimerization and conformational change that in addition attracts co-activators that bind and form a PR complex that subsequently translocates into the cell nucleus, binds the DNA and initiates transcription of the target gene. If a Progesterone agonist binds the receptor, co-activators are similarly bound and gene transcription is activated, whereas if an antagonist binds to the receptor, the induced conformational change will instead make it possible for co-repressors to bind, which will inhibit transcription. PR action takes minutes to hours. The human PR has two major isoforms; PR-A and PR-B and both isoforms bind to progesterone with identical affinity but with respective distinct functions in different cell types. They are structurally

alike although PR-B has an additional 164 amino acids in the N-terminal containing a third transcription domain, which PR-A lacks [62]. Both subtypes are expressed within the endometrium and are dramatically reduced in the epithelial cells during the secretory phase but persist in the upper functional zone of the endometrial stroma. PRA is the predominant type in the stroma during the secretory phase and early pregnancy, and the stromal cells near the uterine vasculature have the highest expression. Both the glands and stroma in the basal layer express PR throughout the cycle [175]. Progesterone receptor C (PRC) is another type of PR, which modulates the transcriptional activity of PRA and PRB [176]. High levels of PRC are expressed in the fundal region of the uterine myometrium during labor [177].

There further exists a non-genomic, membrane bound PR in human tissue, which has predominantly been studied in the ovary, however also is expressed in intestines, smooth muscle, spermatozoa, breast cancer cells and in certain cells that lack nuclei such as red blood cells and platelets. The signaling from these non-genomic G-protein-coupled receptors (GPCR) is immediate and rapid by the activation of downstream second messengers. The membrane associated PRs (MAPR) are a group of four proteins, the most known being PGRMC1 [178].

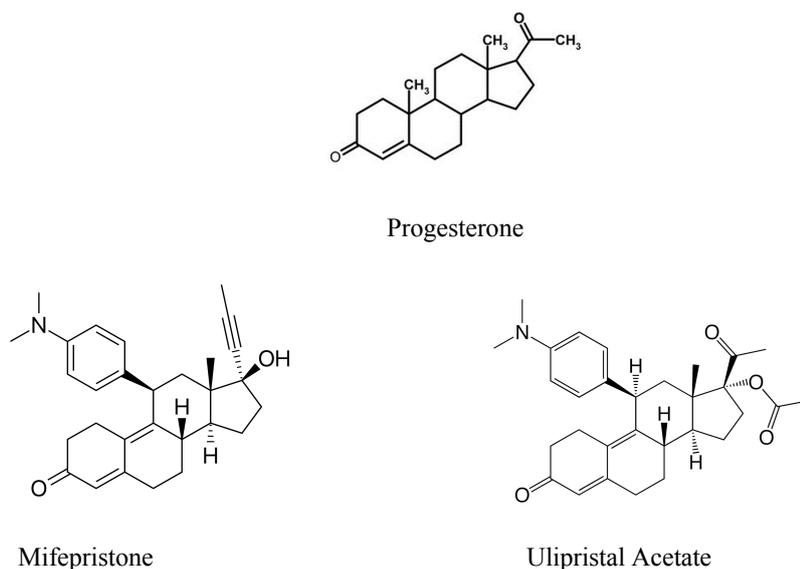


Figure 9. Molecular structure of Progesterone, Mifepristone and Ulipristal Acetate

2.14 PROGESTERONE RECEPTOR MODULATORS

Although belonging to the same class of compounds, different PRMs differ in activity on the PR, as well as in selectivity and potency of their metabolites and thus also in biological profile [179]. Differences in molecular structure of different PRMs influence their effect in the tissue. The relative proportion of co-regulators in different tissues will further determine PRM action and thus whether genes are being transcribed or downregulated [180]. PRMs bind both isoforms of PR with high affinity and can activate both co-activators and co-

repressors, thus producing a mixed agonist-antagonist effect. Due to differences between different PRMs, they exert somewhat variable effects on the endometrium [181].

Mifepristone exerts a more antagonistic effect than UPA.

Since PR signaling is essential for oocyte release at ovulation [182], it is not surprising that PRMs affect the follicular development and ovulation. PRMs have been shown to induce apoptosis in granulosa cells [183]. Both mifepristone and UPA further inhibit muscular contractions and ciliary beating frequency in the fallopian tubes [91].

Mifepristone, which is derived from 19-nortestosterone binds to the glucocorticoid receptor (GR) with high affinity and thus in addition to antagonistic effect on the PR elicits antiglucocorticoid activity. However low or single doses do not significantly affect cortisol levels [184]. UPA on the other hand is a derivate of 19-norprogesterone and *in vitro* studies show that UPA has less antiglucocorticoid activity than mifepristone, suggested to be due to decreased activity by their proximal supposed metabolites [179]. The antiproliferative effect of progestins on the endometrium is suggested to be due to upregulation of AR in the epithelium in presence of estrogen [185]. Mifepristone can also bind to the AR and further, mifepristone treatment increases the expression of AR in the endometrium [186]. AR expression is increased when progesterone levels drop and treatment with a PRM thus in line with this upregulates AR expression..

2.15 PRM ASSOCIATED ENDOMETRIAL CHANGES

The pharmacodynamic effects of PRMs are tissue- and species specific and vary depending on dose and duration of treatment, as well as presence of progesterone and PRs. A PRM alone can exhibit progestational, anti-proliferative and anti-estrogenic effects, while it has antagonistic properties in the presence of progesterone [62]. In the normal endometrium, the ratio of progesterone and estrogen, as well as their respective receptors determines histology. A prominent effect of long-term treatment with mifepristone or UPA is induction of amenorrhea and an increase in endometrial thickness detected by ultrasound. Since treatment with a PRM blocks the PR and the endometrium is left unopposed to estrogen, there are concerns that the described increased thickness is due to proliferative hyperplasia, perhaps with an intrinsic pre-malignant potential.

PRM treatment can cause a spectrum of specific non-physiological endometrial changes. The appearances are similar with different PRMs and are thus considered a class effect [180, 187]. Endometrial pathology studies have histologically described the observed morphological features after PRM exposure, which have been coined PRM associated endometrial changes (PAEC). Glands in endometrium with PAEC can have varying appearance with discordant features and secretory activity, displaying admixed influence. The most prominent and distinctive trademark of PAEC is extensive dilated cystic glandular formations with watery content and inactive glands where the epithelial lining exhibits low mitotic activity and apoptosis. The characteristic features of PAEC are varying, also in accordance to the PRM used. Dilated cysts are believed to appear approximately to the same extent with UPA treatment as with mifepristone, however UPA generates more of non-physiological secretory

effects compared with mifepristone [187-189]. Further features include vascular changes with most often thin, however partially thick walls and compaction of the stromal compartment, which often is not decidualized [190]. The endometrium displaying PAEC does not fit into the histological classification of hyperplasia and neither to regular classification since the feature is neither proliferative nor secretory [187, 190, 191]. According to several reports and expert pathologists, the endometrial changes caused by PRMs are benign with no signs of atypia and have been demonstrated to be reversible after a few weeks to months after cessation of treatment [192-195]. A few studies that included analysis of proliferation markers in endometrium after PRM treatment report decreased levels of these markers [184, 196].

2.15.1 PRMs for treatment of uterine leiomyoma

Several clinical studies of PRMs for the treatment of leiomyomas have been published the last years and this approach has emerged as an attractive new therapeutic alternative to surgery. PRMs can inhibit cell proliferation and induce apoptosis in leiomyoma tissue, confirming the role of progesterone in development of uterine leiomyoma [184, 197]. Thus, both mifepristone and UPA effectively reduce the volume of leiomyomas and alleviate associated symptoms, with the prompt reduced bleeding in a majority of patients being an appreciated effect [184, 193, 194, 198-200]. However, due to the endometrial effects described previously, current recommendations include intermittent treatment during a limited time, with breaks between treatment periods for endometrial shedding. UPA in a dose of 5 mg daily is approved for use in clinical practice for treatment of leiomyomas and can safely be administered intermittently for long-term treatment. Each treatment period lasts 3 months, with further shrinkage of leiomyoma with successive treatment periods.

In a study by Donnez et al, 11% of the women displayed non-physiological endometrial changes similar to PAEC at screening without any pharmacological treatment, whereas 25% of women displayed PAEC 6 weeks after the 4th treatment course [195]. In previous studies, PAEC was reported in approximately 60% of women when the endometrial biopsy was taken at the end of a treatment period [193, 194]. The use of mifepristone for leiomyoma treatment has been studied in daily doses of 2.5-25 mg [184, 199, 201]. The lowest dose displayed relief of symptoms similar to higher doses, although the reduction of leiomyoma volume was smaller. No signs of PAEC were observed with the lowest dose, however study subjects discontinued treatment to a higher extent with this dose, which in addition also had been seen in a similar trial [200].

At the time when we started our studies, the morphology of PAEC had been described but there were no reports of underlying molecular basis or cell proliferation pathways to understand future implications of these changes.

3 AIMS OF THE THESIS

The overall aim of the thesis was to explore the effects of PRMs UPA and mifepristone when used for emergency contraception and further developed for contraceptive purposes, in order to provide women with evidence-based information regarding mechanisms of action and implications of long-term use. Its main focus was on endometrial effects of these mechanisms, including possible impact on endometrial receptivity and embryo implantation after short-term treatment with UPA and low dose mifepristone as well as molecular exploration of PAEC after long-term treatment with mifepristone. Further, the possible interference of UPA used as EC on the ability of a regular COCP to achieve ovarian quiescence, when treatment is initiated immediately after UPA intake, was explored.

The specific objectives of each study were:

Study I

- To compare the effects on ovarian activity when quickstarting a COCP after intake of UPA versus placebo.
- To compare the effects on bleeding patterns and tolerability when quickstarting a COCP after intake of UPA versus placebo.

Study II

- To explore the effects of UPA on human embryo implantation process and known endometrial receptivity markers in an *in vitro* three-dimensional cell culture model.

Study III

- To explore the effects of two different low doses of mifepristone on known endometrial receptivity markers and human embryo implantation process in an *in vitro* three-dimensional endometrial cell culture model.

Study IV

- To characterize the differentially expressed genes and proteins in endometrium displaying PAEC compared with non-PAEC after long-term treatment with mifepristone.

4 MATERIALS AND METHODS

The studies included in this thesis were conducted at the WHO collaborating center in the Department of Women's and Children's Health at Karolinska University Hospital in Stockholm, Sweden. Study I was a multicenter trial with three study sites and participants in addition from Dinox in Groningen, Netherlands and Chalmers Sexual Health Clinic in Edinburgh, Scotland. Embryos used in study II and III were superannuated and donated by couples for research purpose and obtained from the IVF clinic Fertilitetscentrum in Stockholm, Sweden. Methods used in the studies are diverse and include a randomized controlled trial (RCT) and explorative molecular laboratory studies. More detailed description of the materials and methods is provided in the original articles (Study I-III) and manuscript (Study IV).

4.1 STUDY DESIGNS

4.1.1 Overview of studies

Study	Design and subjects	Outcome	Methods	Comparison groups	Statistical Analysis
I The effects on ovarian activity of UPA when 'quickstarting' a combined oral contraceptive pill	RCT A multicenter, randomized, double-blind, placebo-controlled clinical trial Non-inferiority design Healthy women 18-35 years old with regular menstrual cycles	Proportion of women reaching ovarian quiescence during 21 days of COCP after intake of UPA or placebo Bleeding pattern, safety	Transvaginal ultrasonography + Hormonal measurements in blood and urin Hoogland score (ovarian activity) Diary	UPA (n=39) Placebo (n=37)	Non-inferiority approach unachievable, abandoned Competing risk survival Analysis with and without covariates competing risk regression Cumulative incidence of ovarian quiescence (and ovulation) per treatment Chi-squared test (χ^2) for categorical outcome
II Effects of UPA on human embryo attachment and endometrial cell gene expression	Exploratory laboratory studies Endometrial biopsies LH+4 from healthy fertile women + blastocyst stage embryos	Blastocyst attachment rate Expression of endometrial receptivity markers	3D co-culture system from endometrial cells Light microscopy RNA extraction Real-time PCR	UPA 200ng/ml (n=10) Mifepristone 0.05 μ M (n=10) Mifepristone 0.5 μ M (n=8) Control (vehicle) (n=10)	Fisher's exact test Independent t-test (parametric) or Mann-Whitney U test (non-parametric)
III Effects of low doses of mifepristone on human embryo attachment and endometrial cell gene expression	Exploratory laboratory study Endometrial biopsies from pre-menopausal women randomized to mifepristone treatment	Gene and protein expression	Hematoxylin-Eosin staining RNA extraction Microarray Real-time PCR Ingenuity Pathway Analysis (IPA) Orbitrap Mass spectrometry	PAEC (n=7) Non-PAEC (n=6)	Independent t-test (parametric) or Mann-Whitney U test (non-parametric) (microarray, real-time PCR and proteomics) Right tailed Fisher's exact test (IPA)
IV Molecular characterization of PAEC after 3 months of mifepristone treatment	Exploratory laboratory study Endometrial biopsies from pre-menopausal women randomized to mifepristone treatment	Gene and protein expression	Hematoxylin-Eosin staining RNA extraction Microarray Real-time PCR Ingenuity Pathway Analysis (IPA) Orbitrap Mass spectrometry	PAEC (n=7) Non-PAEC (n=6)	Independent t-test (parametric) or Mann-Whitney U test (non-parametric) (microarray, real-time PCR and proteomics) Right tailed Fisher's exact test (IPA)

Table 1. Overview of study designs, methods and statistical analysis

4.2 STUDY SUBJECTS

4.2.1 Study I

Healthy, non-smoking women aged 18-35 years old with BMI < 30 kg/m², regular menstrual cycles (25-35 days) and no contraindications to using COCP according to WHO Medical Eligibility Criteria (MEC) 1 or 2 and not at risk of pregnancy nor currently breastfeeding, were eligible for the study. Further, the physical and gynecological examination had to be normal at recruitment. Exclusion criteria included any contraindications to the study medications or use of an intrauterine device or progestogen-only method of contraception three months prior to study enrolment, or 12 months for the injectable progestogen method. However, current use of a COCP at enrolment was permitted since it was considered clinically relevant to describe the effect of treatment in relation to women who have missed several COCPs when seeking EC.

4.2.2 Study II and III

Endometrial biopsies were obtained from healthy, non-smoking volunteers between the age of 22 and 40, with regular menstrual cycles (25-35 days) and proven fertility with at least one spontaneous pregnancy previously. The women had not used any intrauterine or hormonal contraception within 3 months prior to the biopsy, were confirmed non-pregnant and further recommended to use a barrier method for contraception in the cycle that the biopsy was taken, if not sterilized (themselves or their partner if male). A routine gynecological examination was performed prior to inclusion.

The blastocyst stage human embryos used in this study were donated by couples who previously had undergone *in vitro* fertilization (IVF) at the IVF clinic Fertilitetscentrum Stockholm in Sweden. The embryos were about to be discarded due to legislation as they had been either cryopreserved for 5 years or because the couple did not want to keep them stored at the IVF clinic any longer. The embryos were of good quality, suitable for transfer on thawing and graded by an experienced embryologist.

Gardner and Schoolcraft's classification	
Expansion grade	Blastocyst development and stage status
1	Blastocoel cavity less than half the volume of the embryo
2	Blastocoel cavity more than half the volume of the embryo
3	Full blastocyst, cavity completely filling the embryo
4	Expanded blastocyst, cavity larger than the embryo, with thinning of the shell
5	Hatching out of the shell
6	Hatched out of the shell
ICM grade	Inner cell mass quality
A	Many cells, tightly packed
B	Several cells, loosely grouped
C	Very few cells
TE grade	Trophectoderm quality
A	Many cells, forming a cohesive layer
B	Few cells, forming a loose epithelium
C	Very few large cells

Table 2. Gardner and Schoolcraft's blastocyst grading

4.2.3 Study IV

The endometrial biopsies used in this study were obtained from healthy, pre-menopausal women that had been treated with mifepristone during 3 months prior to surgical intervention due to symptomatic uterine leiomyoma in a randomized, placebo-controlled trial at Karolinska University Hospital, Stockholm, Sweden [184]. The women had not used any steroid hormonal compounds within 3 months prior to study start and were confirmed non-pregnant.

4.3 ETHICAL PERMITS AND CONSIDERATIONS

All the studies were approved by the Regional Ethical Review Board, Karolinska Institutet, Stockholm, Sweden. **Study I:** Dnr 2012-123-31/1, clinical trial reg no NCT01569113, **Study II and III:** Dnr 00-134, **Study IV:** Dnr 02-410, clinical trial reg no NCT00579475. All studies were conducted in accordance with the revised ethical standards of the original Helsinki Declaration of 1975. The procedures in **Study I and IV** followed guidelines for Good Clinical Practice (GCP) and the studies were approved by the Swedish Medical Products Agency, Uppsala, Sweden. All participating subjects and couples that donated embryos were provided oral and written information and signed informed consent prior to participation.

Study I included treatment with a COCP and the volunteering women were thoroughly examined and reviewed regarding their medical history prior to inclusion. The most common side effects from this well known COCP are headache, nausea, breast tenderness and abdominal pain, however the risk of severe adverse events such as thrombosis from treatment were extremely low due to thorough screening and strict exclusion criteria. UPA is also a safe and well-studied compound, currently in use on the market. Participation in this study required several visits to the study centers during approximately 2 months, when at most intense this included transvaginal ultrasonography and blood testing 3 times per week.

Since reproduction and the embryo implantation process significantly differ between species, the use of human endometrial cells and human embryos were considered necessary for **Study II and III**. The technique of collecting endometrial tissue is safe with few risks and is a standard procedure during gynecological examination. It can cause some discomfort and pain similar to insertion of an IUD but is of short duration and does not typically require anesthetics, although oral pain relievers were offered.

The embryos donated for this study were generated through IVF and either donated by couples as they had decided not to store them further or the embryos had been stored for 5 years and were supposed to be discarded as per Swedish legislation for storage of human embryos in IVF clinic. The embryos were handled by an experienced embryologist and cultured during 5 days, which falls well within national policy of permissible legal and regulatory duration for culturing human embryos *in vitro* (<http://www.socialstyrelsen.se/barnochfamilj/graviditet/assisteradbefruktning>) (accessed April 2017).

Study participants and endometrial donors who volunteered for **Study I-III** were compensated economically for their contribution but participation was otherwise not obviously beneficial to the women and neither to the couples who donated embryos. Participants in **Study IV** had been referred to the hospital and were scheduled for surgery due to symptomatic uterine leiomyoma. Side effects from mifepristone treatment are well known and considered mild including nausea, headache, hot flushes and more seldom elevated liver transaminases. The women who were randomized to treatment with mifepristone rather benefited from participation as the treatment had positive effects on their symptoms, most prominently bleeding, and several of the women wished to continue treatment and not pursue with the planned surgical intervention at the end of study.

All the blood- and urine samples in **Study I** were coded and sent to the laboratories as anonymous samples and endometrial biopsies and embryos used in **Studies II-IV** were also collected, coded and sent to the experimental laboratory as anonymous samples.

4.4 METHODS STUDY I

This was a multinational three-site, double-blind, randomized, placebo-controlled clinical trial conducted in Sweden, Scotland and the Netherlands. The women were randomized to receive either 30 mg of UPA (ellaOne®, Cenexi, France) or placebo when a lead follicle was

greater than 13 mm during a spontaneous cycle, followed by 21 consecutive days of COCP treatment. This follicle size was chosen as it corresponds to the mid- to late- follicular phase in the menstrual cycle, which is clinically relevant as EC intake at this stage possibly could delay the ovulation but a subsequent UPSI put the woman at risk of becoming pregnant if she would ovulate. During the pre-treatment period before randomization and inclusion, study visits were scheduled every 2-3 days for control of follicle growth, with a maximum of 6 visits.

4.4.1 UPA/placebo treatment and COCP period

At inclusion, the study medication, (manufacturer of ellaOne® and placebo: Cenexi, France, packaged by: Créapharm, Le Haillan, France) was dispensed in a double-blind fashion and the subjects swallowed the tablet in front of the study staff. The COCP containing 30 µg EE and 150 µg LNG (Microgynon®, Bayer plc, Newbury, UK) was dispensed in an open-label fashion, given to the women in a box containing the blister with tablets. Instructions were to take one pill daily at approximately the same time each day, starting the day after UPA or placebo intake.

During the COCP period, study visits continued every 2-3 days, where transvaginal ultrasonography and blood sampling for hormonal measurements were performed, producing a Hoogland score (Table 3), until the largest follicle was smaller than or equal to 13 mm, a maximum of 9 visits. When ovarian quiescence was reached (Hoogland score ≤ 3), study visits were reduced to once weekly. Some tablets from each blister were kept at the study site in sealed, marked envelopes for further drug accountability. The women made notes of tablet intake each day in their diary and were instructed to bring this and their box and blister to each scheduled visit during the study time. These were all further collected at the last visit. If sexually active with a male partner and not sterilized, the women were instructed not to rely on the COCP for contraception but to use condoms or abstain from sexual intercourse during participation in the trial. Condoms were handed out to study subjects throughout the study when applicable. The end of study visit was scheduled 9 ± 2 days after the COCP period.

4.4.2 Randomization

An independent statistician produced a computer-generated randomization schedule. This schedule included a link between assigned treatment numbers and treatment codes and was securely stored by an independent contract research organization until locked for analysis. Sealed, sequential randomization envelopes corresponding to each treatment number were allocated and kept at respective site.

4.4.3 Assessment of effect and safety

4.4.3.1 Transvaginal Ultrasonography (TVU)

Follicular growth and endometrial thickness were measured through TVUs at each study visit. The largest follicle in each ovary was measured in two axes and endometrial thickness measured at the thickest point between the anterior and the posterior uterine wall including

both endometrial layers. From the baseline visit at cycle day 5 ± 1 after onset of menses, or withdrawal bleeding for COCP users, TVUs were performed 3 times every week during the pre-treatment period, until the size of the dominant follicle was larger than 13 mm. At this point, as earlier described, the woman was included and thus randomized to receive the UPA or placebo. During the following COCP period, TVUs were also performed 3 times weekly until the dominant follicle was smaller than or equal to 13 mm at two consecutive visits but at the weekly visits thereafter TVUs were not performed until again at the end-of-study visit. If an ovarian cyst larger than 30 mm persisted at this visit, subsequent TVUs were performed monthly until it resolved.

4.4.3.2 Blood and urinary analysis

Blood sampling for analysis of progesterone, estradiol and FSH was performed at the inclusion visit and at each study visit during the COCP period. A central laboratory in France, (CEMO, Choisy le Roi) conducted the blood analyses. The women were provided with tubes for daily urine samples, which were then brought back to the research site continuously, and stored at -20°C . These aliquots were used as backups, as analyses of pregnanediol glucuronide and oestrone glucuronide, the urinary metabolites of progesterone and estradiol, could be performed later on to assess ovarian activity in case of missing data on hormonal parameters or TVUs. A hospital-based laboratory in Scotland conducted the urinary analyses (RIE, Edinburgh, UK).

4.4.3.3 Diary

The study subjects were provided with a diary, in which they made daily records of vaginal bleeding and any adverse events occurring during the trial. Information about the bleeding episodes included duration and volume. Any untoward medical occurrence that a study subject might experience was considered an adverse event, although not necessarily with a causal relationship with the study treatment.

4.4.4 Hoogland score

Hoogland score	Ovarian activity grade	Follicular diameter (mm)	Estradiol (nmol/l)	Progesterone (nmol/l)
1	No activity	≤ 10 mm	-	-
2	Potential activity	> 10 mm	-	-
3	Non-active follicle like structure	> 13 mm	≤ 0.1*	-
4	Active follicle like structure	> 13 mm	> 0.1*	≤ 5
5	Luteinized unruptured follicle	> 13 mm persisting	> 0.1*	> 5
6	Ovulation	> 13 mm ruptured	> 0.1*	> 5

* 0.1 nmol/L ≈ 27 pg/ml

Table 3. Grading of ovarian activity (Hoogland and Skouby 1993)

The Hoogland Score is a method to measure the suppressive effect of a COCP on ovarian activity, described by Hoogland and Skouby 1993. Here, the stage of ovarian activity is assessed with measurements of endogenous hormone levels in blood and follicle size by ultrasonography [202]. Several investigators have previously used this scoring system in research studies where ovarian suppression was assessed during use of COCP [203-205].

4.4.5 Statistical analysis

The hypothesis was that there would not be a significant difference in the number of women who would attain ovarian quiescence during COCP treatment in the UPA compared to the placebo group. Ovarian quiescence was considered as Hoogland score ≤ 3 and the assumption was that approximately 90 percent of women would reach this event. The study was designed as a non-inferiority trial, where the UPA arm was declared non-inferior to the placebo arm for the parameter percentage to attain ovarian quiescence. The non-inferiority margin was chosen at 20%, meaning that the difference between groups would not differ more than 20%, with an alpha risk of 2,5% (unilateral test). To test this hypothesis with sufficient power (80%), the sample size necessary was calculated to 72 subjects (36 women in each arm). In order to obtain this number, allowing for 10% dropouts, an estimated 80 subjects needed to be enrolled in the study.

In this study however, women were included and treated with the study medication with larger than anticipated leading follicles, which resulted in a larger than anticipated proportion of women who ovulated. The women who ovulated would never reach the primary efficacy criterion of attaining ovarian quiescence, but were instead doomed to be excluded in a non-inferiority analysis, why this goal was not judged achievable. Since it was not feasible to rescue the original non-inferiority approach, the statistical plan had to be altered. Considering ovulation as a competing risk to attaining ovarian quiescence made it possible to apply a competing risk survival analysis of quiescence model. The analyses thus included estimating the cumulative incidence in the presence of competing risk events for both treatment arms. The covariates included were follicle size at inclusion, use of COCP in the previous cycle and cycle day at treatment. The full Analysis population (FAS) was analyzed, which included all participants that had been randomized and treated and with at least one available assessment of Hoogland score. Comparison of groups was performed using the chi-squared test (χ^2) and the covariates were accounted for by the competing risk regression approach. The change in statistical plan was finalized before the database was unblinded and locked.

4.5 METHODS STUDY II AND III

In study II and III, the human embryo implantation process and markers of endometrial receptivity were studied after treatment with UPA and mifepristone respectively, using the 3D human endometrial *in vitro* cell culture model which previously had been demonstrated to mimic *in vivo* endometrium [56, 57, 173]. The method included collection of endometrial tissue from proven fertile women, isolation of endometrial stromal and epithelial cells, construction of the 3D co-culture with the endometrial cells and allocating blastocyst stage human embryos to the cultures for further observation and analyses with and without treatment.

4.5.1 Endometrial biopsies

The participating study subjects determined the urinary LH peak by using a rapid self-test twice daily from menstrual cycle day 10 (Clearplan, Searle Unipath Ltd, Bedford, UK). Endometrial biopsies were collected on cycle day LH + 4. The tissue was obtained using a Pipelle aspirator (Cooper surgicals, USA or Prodimed, Neuilly-en-Thelle, France) from the upper part of the uterine cavity without local anaesthesia or prior dilatation of the cervix. The fresh biopsies were transported to the laboratory in sterile tubes containing fresh transport medium, Ham F-10, for further cell isolation processing.

4.5.2 Endometrial cell isolation

Isolation of endometrial stromal and epithelial cells was performed immediately after the biopsy was taken. The fresh biopsies were minced with a scalpel into very small pieces, approximately 1 x 1 mm and thereafter incubated with pancreatin-trypsin EDTA (0.05g/ml Trypsin-EDTA solution, Sigma-Aldrich, Stockholm, Sweden). A mixture of collagenase IV and DNase (Worthington Biochemical, Lakewood, NJ, USA) was added and the supernatant removed after sedimentation. The treated cell mixture was washed with Ham F-10 and

thereafter filtered using a 40 micron mesh cell strainer (Falcon, BD Biosciences, Belgium), which allowed single stromal cells to pass through. The epithelial glands that were restrained in the filter were collected and treated with a collagenase III and DNase mixture (Worthington Biochemical) and subsequently filtered through a 40 micron mesh cell strainer, yielding single epithelial cells. Stromal and epithelial cells were further preserved and stored separately in liquid nitrogen until used for the 3D endometrial co-culture.

4.5.3 Embryos

Embryos used in study II and III were all viable blastocyst stage human embryos of good quality, suitable for transfer. The standard method of embryo grading according to Gardner and Schoolcraft's classification (Istanbul consensus 2011) was practiced and the embryos were graded between 3 and 4; AA/AB/BA by an experienced embryologist (Istanbul consensus 2011) (Table 2). The blastocysts derived from oocyte retrieval after ovarian stimulation, followed by standard IVF or intracytoplasmic sperm injection (ICSI) as per protocols of the IVF clinic. They had been individually cultured for 5 days; 2 days in droplets of G1 plus medium and under oil (Vitrolife, Gothenburg, Sweden) and then in blastocyst culture medium, G2 plus or CCM (Vitrolife) and thereafter vitrified on day 5 or 6, using RapidVit Blast (Vitrolife), placed in cryoloops and stored in liquid nitrogen. At thawing, the embryos were warmed in Thermoblast™ Blast kit (Nidacon, Mölndal, Sweden) according to protocol and equilibrated in complete alpha medium for at least 2 hours, allowing them to expand, before they were transferred to the 3D cultures. The blastocysts that survived thawing and clinically met the quality standards for transfer were eligible for use in the studies. An embryologist handled the blastocysts during the thawing procedure and made quality assessment.

4.5.4 Three-dimensional *in vitro* endometrial co-culture system

The endometrial 3D co-cultures used in the studies were prepared, with only minor modifications, as described by Lalitkumar *et al* [173]. This was a modified and standardized version of the originally described model by Bentin-Ley *et al* [170, 206]. The collagen gel solution used, composed of bovine collagen solution Type I (Purecol 3 mg/ml, Advanced Biomatrix, San Diego, CA, USA), 10X PBS and NaOH with adjusted pH to 7.4, was first prepared and 175 µl of the mixture portioned at the bottom and lower sides of the Millicell cell culture inserts (EMD Millipore, Darmstadt, Germany), which were placed in a culture dish. Thereafter, the thawed stromal cells and 200µl of the collagen gel solution were mixed and added into the inserts. Each insert contained approximately $0.5-1.0 \times 10^6$ stromal cells/ml. After formation of the stromal gel, a thin layer of basement membrane matrix (Matrigel®, BD Biosciences, Belgium) was coated on top. Epithelial cells were mixed with modified alpha medium and seeded on top of the basement membrane coating, covering approximately 2/3 of the surface, which would allow them to gradually spread and grow as a monolayer (Figure 10). The modified alpha medium had been supplemented with 4 ml Amniomax C100, 5 ml fetal calf serum, 2000 IU penicillin-streptomycin and 0.2 ml *L*-glutamine (200 mmol/L) (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5 g Bovine Serum Albumin (Sigma-

Aldrich). Complete alpha medium, which had an final estrogen concentration of 0.3 nmol/L and progesterone concentration of 902 nmol/L and the endometrial 3D constructs were thereafter cultured for 5 days at 37°C in an incubator, with 5% CO₂ in the air. The medium was changed every other day, when also the cultures were checked under a stereomicroscope (Zeiss, Oberkochen, Germany).

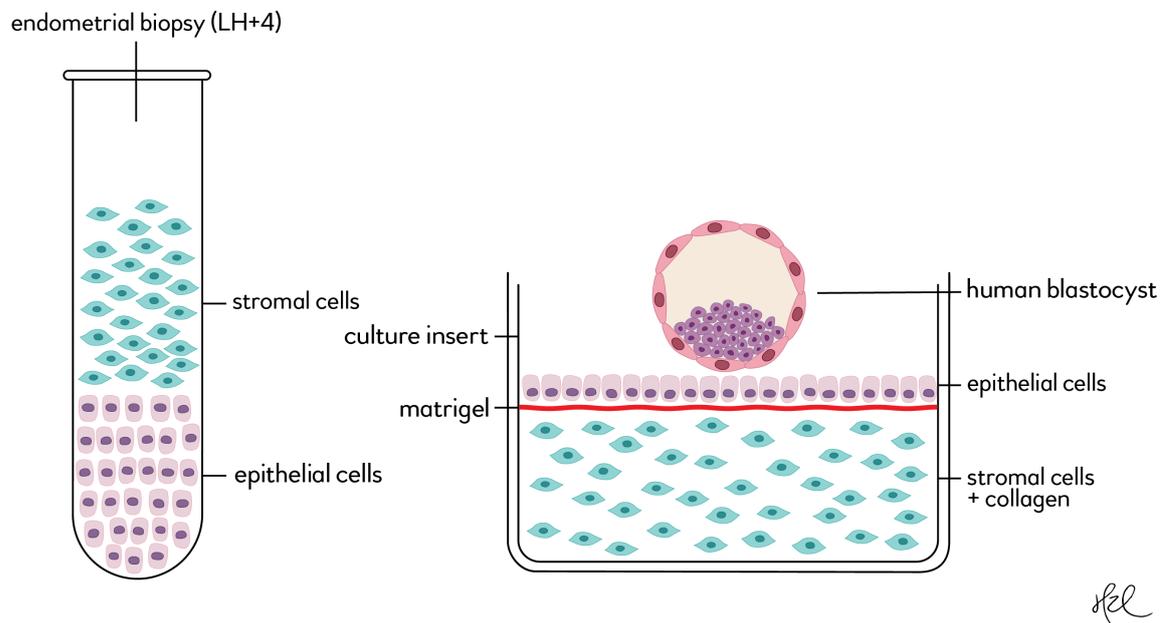


Figure 10. 3D *in vitro* endometrial co-culture model

4.5.5 PRM treatment

After 3-5 days of culture or when the epithelial layer was confluent, the endometrial co-cultures were treated with UPA or mifepristone in the treatment groups of respective study. In Study II, UPA in 5 µl of ethanol as vehicle (Medchem Express LLC, Princeton, NJ, USA) was added to 5 ml of media to reach a final concentration of 200 ng/ml (approx. 0.4 µM) [89], whereas in Study III, 5 µl of mifepristone (Sigma-Aldrich) in respective concentrations in ethanol as vehicle was added to 5 ml of media to obtain a final concentration of 0.05 or 0.5 µM of mifepristone. The control group of cultures was exposed with media containing 5 µl of ethanol as vehicle/5 ml of media. The thawed blastocysts were randomly allocated to the different treatment groups and placed on the surface of the cultures on the same day as treatment, one per endometrial culture construct. They were thereafter cultured during 5 days until ultimately terminated.

4.5.6 Assessment of embryo attachment and culture termination

The cultures were examined for embryo under stereomicroscope (Zeiss) every other day when medium was changed. On day 5 of embryo-endometrial culture, the embryos were checked and tested for attachment by shaking the cultures mechanically and washing twice with phosphate buffered saline (PBS) and attachment rates were documented. The blastocysts were washed away if not attached. The 3D constructs were thereafter removed from inserts, the embryo portion dissected out and the remaining construct dissolved in 1 ml of Trizol reagent (Thermo Fisher Scientific) and stored at -80°C.

4.5.7 RNA extraction and cDNA synthesis

Total RNA was extracted from the Trizol dissolved 3D constructs using the Arcturus PicoPure RNA isolation kit (Thermo Fisher Scientific) as per the manufacturer's protocol and the RNA concentration was measured using Nanodrop™1000 Spectrophotometer (Thermo Fisher Scientific). The samples were treated with DNase in order to eliminate contaminating DNA. RNA integrity was determined using RNA 6000 Pico Labchip® and Bioanalyzer 2100 Expert Software (Agilent Technologies, Santa Clara, CA, USA) was used to calculate the RNA Integrity Number (RIN) as per the manufacturer's protocol.

20 µl of first strand complementary DNA (cDNA) was synthesized from culture derived RNA using SuperScript VILO MasterMix (Thermo Fisher Scientific) and the Biorad Mycycler Thermal cycler was used for the thermal steps. A no template control and a reverse transcriptase negative control (RNase free water) were also prepared at the same time as controls in the real time polymerase chain reaction (PCR).

4.5.8 Real-time PCR

Real-time PCR analysis with quantification of mRNA levels was performed for PR and a number of genes of relevance for endometrial receptivity, decidualization and implantation (n = 17 in study II and n = 16 in study III) using Taqman primers (Thermo Fisher Scientific) (Table 4). 18S was chosen as the housekeeping reference gene on StepOne Plus instrument (Thermo Fisher Scientific) and amplification was performed in triplicates using Taqman Universal PCR MasterMix (Thermo Fisher Scientific) in a Microamp Fast Optical 96-well Reaction Plate (Thermo Fisher Scientific) with relative quantification (RQ) method. In accordance with the manufacturer's protocol, the following thermal cycling conditions were performed: 2 min at 50°C, 10 min at 95°C and 40 cycles for 15 seconds at 95°C and for 1 min at 60°C. Real-time PCR data was collected using StepOne Plus software (Thermo Fisher Scientific) and further analyses were performed in Excel (Microsoft, Redmond, WA, USA). The results were analyzed by comparative threshold cycles ($\Delta\Delta C_t$) method, where C_t is the first cycle number at which the fluorescence passes the threshold of detection. The difference in expression levels between the groups was expressed in terms of fold change $2^{-\Delta\Delta C_t}$, as the product amount doubles every cycle.

4.5.9 Statistical Analysis

Statistical analysis for the blastocyst attachment rate and real-time PCR data were performed using XLStat 2014 (AddinSoft SARL, Paris, France) and Graphpad Prism (Graphpad software, San Diego, CA, USA). Fisher's exact test was used to calculate the difference in blastocyst attachment rate between the control and treatment groups. Based on the observations of real-time PCR data distribution and homogeneity of variance, either parametric Independent *t*-Test or non-parametric Mann-Whitney *U* Test was performed for intragroup analysis. The results were presented as mean \pm standard deviation (SD) and a *p*-value of < 0.05 was considered as statistically significant.

4.6 METHODS STUDY IV

Fourteen healthy, pre-menopausal women had been randomized to receive mifepristone during 3 months prior to surgical intervention of symptomatic uterine leiomyoma in a clinical placebo-controlled trial conducted at Karolinska University Hospital between November 2004 and June 2007, investigating the impact of mifepristone treatment on uterine leiomyoma [184]. Endometrial biopsies obtained during surgery after the treatment period had been divided into two sections, one snap frozen and stored in liquid nitrogen and the other formalin-fixed, paraffin-embedded (FFPE), sectioned and stored at 4°C, awaiting further analyses.

This study aimed at exploring the gene and protein expression in endometrium that displays PAEC in comparison with endometrium that does not have these non-physiological changes after three months of mifepristone treatment. Methods used included histological evaluation, microarray analysis, real-time PCR, protein extraction and further liquid chromatography and mass spectrometry.

4.6.1 Mifepristone treatment

Eligible women in the earlier published study were randomized to receive either 50 mg mifepristone, as one quarter of a 200 mg tablet Mifegyne® (Exelgyn, Paris, France), or an inactive comparator as one quarter of a B-vitamin tablet TrioBe® (Recip, Stockholm, Sweden) that visually looked identical, every other day during 3 months. Sixteen women had been randomized to placebo treatment and 14 women to treatment with mifepristone.

4.6.2 Endometrial biopsies

Endometrial biopsies were collected from the upper part of the uterine cavity with a Randall®curette (Stille, Stockholm, Sweden) without local anaesthesia or prior dilatation of the cervix. The biopsies used in this study were collected during surgery after 3 months of mifepristone treatment.

4.6.3 Hematoxylin & Eosin Staining and histological evaluation

An experienced pathologist with expertise within endometrial pathology, blinded to treatment groups, had previously evaluated slides with Hematoxylin-Eosin stained endometrial samples

regarding occurrence of PAEC in the original study. The histological findings in the treatment group were reconfirmed with new Hematoxylin-Eosin staining of paraffin-embedded sectioned endometrial samples (6µm thickness), after initial deparaffinization. First, the sections were stained for 1 minute in Hematoxylin (Vector Laboratories, Inc. Burlingame, CA, USA) and then destained in HCl-EtOH, followed by Eosin staining (Sigma-Aldrich, St Louis, MO, USA) for 1 minute. Zeiss Axio Scope Microscope (Zeiss International) was used to image the sections, which subsequently were processed using Stereo Investigator Software (MBF Bioscience, Magdeburg, Germany).

4.6.4 RNA extraction

The endometrial tissue was homogenized in chambers at a shaking frequency of 30/second for 2 minutes with intermittent freezing in liquid nitrogen repetitively using a Retsch™ tissue mill (Retsch KG, Haan, Germany). Total RNA was extracted using TRIzol (Invitrogen®, Thermo Fisher Scientific) and the RNA concentration was measured using Nanodrop™1000 Spectrophotometer (Thermo Fisher Scientific). (All samples had an optical density 260/280 ratio > 1.8) The samples were treated with DNase in order to eliminate contaminating DNA. RNA integrity was determined using RNA 6000 Pico Labchip® and Bioanalyzer 2100 Expert Software (Agilent Technologies) was used to calculate the RNA Integrity Number (RIN) as per the manufacturer's protocol.

4.6.5 Microarray

100 ng of RNA per sample was labeled with biotin as per the protocol of SensationPlus™ FFPE Amplification and WT Labeling Kit (Affymetrix, Santa Clara, CA, USA). The labeled RNA was thereafter reverse transcribed to cDNA, which was further transcribed into labeled cRNA *in vitro*, and hybridized to GeneChip® Human Transcriptome Array 2.0 ST (HTA 2.0) (Affymetrix) at Bioinformatics and Expression Analysis core facility (Karolinska Institutet, Sweden). The raw data files of HTA 2.0 were processed using Affymetrix® Expression Console™ Software (Affymetrix) and estimation of gene signals through summarization was obtained with the algorithm iterative Probe Logarithmic Intensity Error Estimation (IterPLIER), with perfect match GC composition based background correction (PM-GCBG). Quantile sketch normalization was applied and further if probe sets were not annotated, they were discarded from analysis. In order to assess data quality including identifying outliers, hierarchical clustering, MA-plots and principal component analysis were produced using R software. Probe sets that had an expression intensity of < 40 in either of groups were removed for background correction. Analysis of the filtered data was then performed using Significance Analysis of Microarrays (SAM) in R software (<https://github.com/MikeJSeo/SAM>). The parameter fold-change was chosen at a minimum of two, either up- or downregulated for differentially regulated genes. The expressions of significant genes that were identified in microarray were reconfirmed by real-time PCR.

4.6.6 Real-time PCR

Total RNA in equal amounts was reverse transcribed to cDNA in accordance with the protocol of Ovation® Pico WTA System V2 protocol (NuGEN Technologies Inc., San Carlos, CA, USA). Real-time PCR was performed on cDNA from 7 PAEC and 4 non-PAEC samples (as the 5th non-PAEC sample did not yield enough RNA) using the selected TaqMan gene primers and the TaqMan Gene Expression Assays and 7300 Real Time PCR System detection system (Thermo Fisher Scientific) as per manufacturer's protocol. Nine target genes, identified by microarray analysis, were selected from the dataset and further analyzed with real-time PCR in order to determine the relative amount of each transcript in the two groups. 18S was chosen as the housekeeping reference gene as an endogenous control for data normalization and amplification was performed in triplicates using Taqman Universal PCR MasterMix (Life technologies, Thermo Fisher Scientific) in a Microamp Fast Optical 96-well Reaction Plate (Applied Biosystems, Thermo Fisher Scientific) with relative quantification (RQ) method. The thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C and 40 cycles for 15 seconds at 95°C and for 1 min at 60°C. Real-time PCR data was collected using StepOne Plus software (Applied Biosystems, Thermo Fisher Scientific) and further analyses were performed in Excel (Microsoft, Redmond, WA, USA). The results were analyzed by comparative threshold cycles ($\Delta\Delta C_t$) method. The difference in expression levels between the groups was expressed in terms of fold change $2^{-\Delta\Delta C_t}$.

4.6.7 Ingenuity Pathway Analysis

The web-based software application and bioinformatics tool Ingenuity Pathway Analysis (IPA®, QIAGEN, Redwood City, CA, USA), (www.ingenuity.com) was used for performance analysis, integration and interpretation of microarray data. IPA gives access to its manually curated content of ingenuity knowledge base, which derives from sources like Entrez gene, Gene Ontology, GWAS, OMIM and RefSeq. In order to determine the major differences between the two groups regarding association with major canonical pathways, function and disease, the genes were uploaded into IPA, provided the change in expression profile was more than two-fold and that the genes were significantly differentially regulated. Additional filters for the functional analysis included only annotated genes and source of data only from human primary cells and tissues.

4.6.8 Protein extraction

Protein extraction from formalin-fixed paraffin-embedded endometrial sections from PAEC (n = 6) and non-PAEC (n = 6) and mass spectrometry including quantitative and qualitative data analysis were conducted at BMC and Science for Life Laboratory in Uppsala, Sweden. The first step of sample preparation included pooling of six tissue sections from each subject into one new sample. These samples were deparaffinized as previously described by [207], with minor modifications, and 20 μ L of denaturation buffer thereafter added to each sample. The protein concentration was measured as per protocol using the Dot-it Spot-it® assay (<http://dot-it-spot-it.com>), with bovine serum albumin (BSA) as standard. The total volume was digested, unless the sample contained a higher protein concentration than 3.0 μ g/mL, in

which case 71 ng of the protein amount was taken out for digestion. The proteins were further reduced, alkylated and in-solution digested with trypsin and resulting peptides thereafter purified using ZipTip® Pipette Tips (Merck Millipore, Darmstadt, Germany). After drying and resolving in 15 µL 0.1% formic acid, the peptides were subsequently separated in reversed-phase using the EASY-nLC II system (Thermo Fisher Scientific) according to protocol.

4.6.9 Liquid Chromatography-Orbitrap Mass Spectrometry

5 µL from each sample was placed on the pre-column EASY-Column, 2 cm, with inner diameter 100µm, 5 µm, C18-A1 and the peptides further eluted on the EASY-Column of 10 cm with inner diameter 75 µm, 3 µm, C18-A2 (Thermo Fisher Scientific). The peptides were further separated using mobile phase A (Milli-Q water with 0.1% FA) and B (ACN with 0.1% FA) at a flow rate of 250 nL/min with applied stepwise gradient from 2% of B up to 100% of B during 90 min. Thereafter they were electrosprayed on-line to a Q Exactive™ Plus Mass Spectrometer (Thermo Finnigan, San Jose, CA, USA). The scan range of the survey scan was conducted between 400 Th and 1750 Th at a resolution of 70000 and fragmentation of the 10 most abundant peaks were performed with Higher-energy Collision Dissociation (HCD). The dynamic exclusion was set to 20s.

A search in the database SWISS-PROT (EMBL-EBI, Cambridge, UK) against human proteins and file processing using the quantitative proteomics software MaxQuant version 1.5.1.2 (Computational Systems Biochemistry, Martinsried, Germany) enabled protein identification and quantification. Search criteria for protein identification included at least two peptides matching, with 95% confidence level per protein. The false discovery rate (FDR) was estimated using a reverse database and a decoy database that included common contaminants.

4.6.10 Statistical analysis

Analysis of the filtered microarray data was performed using SAM in R software (<https://github.com/MikeJSeo/SAM>). Normalized data from microarray, real-time PCR and proteomic studies were subjected to either parametric Independent t Test or non-parametric Mann-Whitney U Test for intragroup analysis in order to find significance between groups. A p-value < 0.05 was considered as statistically significant.

In IPA, the most significant canonical pathways were calculated based on the right tailed Fisher's exact test, with a p-value < 0.05 considered significant. The generated p-value indicated the likelihood that the association between genes in our dataset and a given related pathway was due to random chance. A predicted activation or inhibition of a pathway was performed on IPA regulation Z-score algorithm (≤ 2 or ≥ 2 score).

For the proteomic studies, proteins were included in the statistical analysis only if they had been identified in both sample categories and in at least two out of three samples in each category. Due to large variation in the number of proteins identified between samples, normalization was performed within each sample, with the obtained label-free quantification

(LFQ) intensities against the LFQ intensity for Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). In the semi-quantitatively approach, proteins were reported as unique if detected in all samples from one category but not in any of samples in the other category. These proteins were considered upregulated in the group in which they were identified, however a fold change could not be calculated as those proteins were below detection limit in the other group.

5 RESULTS

5.1 STUDY I

5.1.1 Summary of main findings

5.1.1.1 *Study subjects and treatment allocation*

The study was conducted between March 2012 and August 2012. A total of 103 women were screened as potential participants at the three study sites and 76 women were enrolled in the study (Edinburgh, Scotland n = 18, Groningen, Netherlands n = 45, Stockholm, Sweden n = 13) and received UPA (n = 39) or placebo (n = 37). There was no significant difference in demographic characteristics of the women in the two groups.

All 76 subjects completed the study and were included in the FAS population. 5 subjects were excluded from the per protocol (PP) population due to 9 protocol deviations, all in the UPA group. These deviations included more than 3 days between visits before quiescence (n = 4), missed visits before outcome was reached (n = 2), intake of the first COCP on the same day as UPA (n = 2) and non-visualized dominant follicle at a visit (n = 1). Thus, 71 subjects were included in the PP population.

5.1.1.2 *Ovarian activity grades and cumulative proportions of quiescence and ovulation*

Among the 76 women treated, 47 (61.8%) reached ovarian quiescence (Hoogland score 1-3) and 25 (32.9%) ovulated (Hoogland score 6). There was no significant difference between the two groups in proportion of women reaching respective event (see Figure 2 and 3 in Paper I). The odds ratio (OR) for reaching ovarian quiescence or not did not significantly differ between UPA and placebo groups: 0.97 (95% CI: 0.39-2.46).

By day 7 of the COCP period, 17 women (70.8%) in the UPA group and 14 (60.9%) in the placebo group had reached ovarian quiescence. All women had reached ovarian quiescence by day 14 of COCP intake. The patterns of cumulative proportions of women who ovulated were comparable between the groups. 10 out of 13 women in the UPA group had ovulated by day 6 and all women had done so by day 11. In the placebo group, all 12 women had ovulated by day 6.

5.1.1.3 *Factors influencing rate of quiescence or ovulation*

A significant covariate for ovarian quiescence proved to be follicular size at inclusion as quiescence occurred faster with smaller follicles at treatment (UPA or placebo), hazard ratio for follicle size 0.721 (95% CI: 0.569-0.913), $p < 0.0066$. All three women in the study who received treatment at a follicular size ≥ 18 mm ovulated, one in the placebo group and two in the UPA group. Cycle day at treatment or method of contraception in the cycle preceding study participation had no significant effect on proportions of women who reached ovarian quiescence or ovulated.

5.1.1.4 Safety

Two women in the UPA group and one in the placebo group developed a luteinized unruptured follicle (LUF), and one woman in the placebo group a persistent asymptomatic follicular cyst of maximum diameter 44.5 mm, which had spontaneously resolved at a visit one month after end of treatment. 13 women altogether in the study reported unscheduled bleeding, with no difference in incidence between the two treatment groups. The most common adverse events that the study subjects experienced were headache, nausea, abdominal pain and nasopharyngitis. These were generally common although none considered serious, as 49 women reported at least one event during the course of the study (26 in the UPA group and 23 in the placebo group), however no woman discontinued the study due to an adverse event.

5.2 STUDY II AND III

5.2.1 Summary of main findings

5.2.1.1 Embryo attachment

The blastocyst stage embryos were randomly allocated to the different exposure groups; mifepristone 0.5 μM (n = 8), mifepristone 0.05 μM (n = 10), UPA 200 ng/ml (n = 10) and controls (n = 10). None of the blastocysts attached in the mifepristone 0.5 μM group whereas 4/10 in the mifepristone 0.05 μM , 5/10 in the UPA 200 ng/ml and 7/10 in the control group attached to the 3D endometrial construct. There was no significant difference in blastocyst attachment rate between the UPA group compared to the control group (p = 0.650) and neither between the group exposed to mifepristone 0.05 μM compared to controls (p = 0.370). In the mifepristone 0.5 μM group however there was a significant difference in attachment rate compared to the control group (p = 0.004).

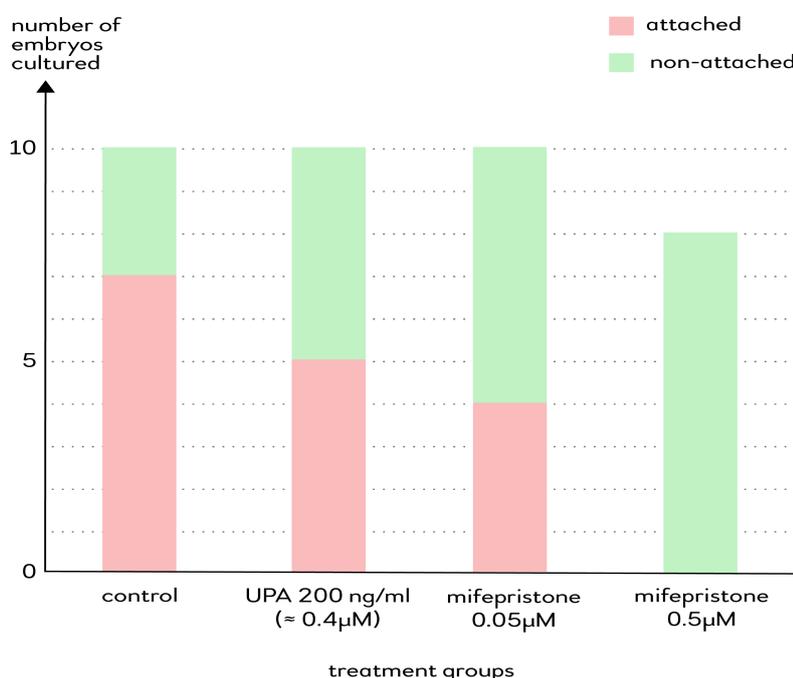


Figure 11. Blastocyst attachment rate in control and treatment group

5.2.1.2 Endometrial receptivity markers

Genes belonging to different functional groups such as cytokines, decidualization factors, growth factors, integrins and transcription factors, which are of relevance to embryo implantation and endometrial receptivity, were selected for real-time PCR in Study II (n = 17) and Study III (n = 16), including mRNA for the PGR. The results are expressed as fold change (FC) between the treatment groups and the control group. All the cultures expressed PGR, although significantly downregulated in all treatment groups compared with the control group (Table 4). In Study II, 6 out of 17 genes were differentially expressed in the endometrial construct after UPA exposure compared to the control group; CALCR, FGF2, HAND2, OPN, HBEGF, IL6. Eleven out of 17 genes were thus not significantly altered with treatment. In the mifepristone groups in Study III, all genes were differentially regulated in the same direction at both concentrations of mifepristone in comparison with the control group. Nine out of the 16 studied genes were significantly altered in both mifepristone groups compared to controls and an additional three genes were significantly altered in only the group with the lowest mifepristone concentration (0.05 μ M) in comparison with controls, namely IL6, IL1A and VEGFA. IL6 was the only gene that was significantly differentially expressed between the two different mifepristone groups (data not shown in table).

Gene	FC UPA 200 ng/ml (\approx 0.4 μ M)	p-value UPA 200 ng/ml (\approx 0.4 μ M)	FC mifepristone 0.05 μ M	p-value mifepristone 0.05 μ M	FC mifepristone 0.5 μ M	p-value mifepristone 0.5 μ M
Transcription factors						
COUP-TFII	-2.29	0.306	6.75	0.008*	7.88	0.0001*
FOXO1	-2.01	0.055	-7.56	0.0001*	-4.54	0.0001*
HAND2	-2.74	0.003*	-2.32	0.004*	-2.28	0.004*
HOXA10	-1.07	0.307	-7.41	0.003*	-7.94	0.0003*
Cytokines						
CSF1	-1.09	0.480	1.06	0.999	1.36	0.088
IL1A	1.26	0.065	-1.56	0.028*	-1.15	0.472
IL6	2.63	0.025*	3.51	0.003*	1.19	0.165
LIF	-1.39	0.107	-2.13	0.005*	-2.15	0.005*
OPN	-3.89	0.004*	-10.12	0.0001*	-2.96	0.005*
Growth factors						
FGF2	-2.06	0.023*	-13.21	0.0001*	-12.04	0.0001*
HB-EGF	2.48	0.009*	0.6	0.154	1.03	0.670
VEGFA	1.09	0.596	-3.77	0.0001*	-1.29	0.219
Decidualization factors						
IGFBP1	-1.34	0.653	-2.38	0.003*	-2.29	0.0002*
PRL	-1.47	0.427	-18.68	0.0001*	-8.79	0.0001*
Cell adhesion molecule						
MUC1	-1.44	0.391	1.43	0.428	1.7	0.154
Receptors						
CALCR	-2.52	0.016*	-	-	-	-
PGR	-4.89	0.001*	-1.85	0.01*	-2.14	0.001*

* p-value < 0.05

Table 4. Gene expression of receptivity markers studied by real-time PCR after exposure to UPA 200 ng/ml (\approx 0.4 μ M), mifepristone 0.05 μ M and mifepristone 0.5 μ M, measured as difference in fold change (FC) compared with controls.

5.3 STUDY IV

5.3.1 Summary of main findings

5.3.1.1 Endometrial histology

Hematoxylin-Eosined stained slides with sectioned endometrial tissue were reassessed regarding occurrence of PAEC. In 7/13 (53.8%) of collected endometrial samples, a varying degree of non-physiological features corresponding to PAEC were observed among the PAEC samples. These changes were in line with the previous analysis by expert endometrial pathologist. Changes observed included both inactive and extensively apoptotic glands as well as secretory active, mitotic glands and cyst formations. In addition, atrophy and apoptotic degeneration was observed. Vascular changes included thin “chicken wire pattern” and ectatic vessels with thick walls (Figure 12).

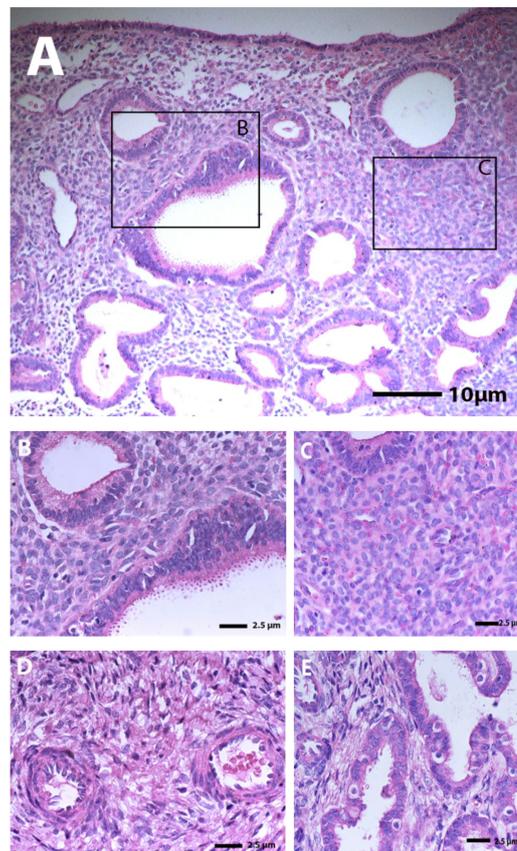


Figure 12. Hematoxylin-Eosin stained endometrial tissue following 3 months of mifepristone treatment showing non-physiological features agreeing with PAEC. Endometrial glands lined with single layers as well as incongruous, discordant epithelial type (A). Glands exhibit cyst formation and secretory activity (B). Chicken wire pattern (C) and thick-walled ectatic vessels (D). Glands further display apoptosis and accompanying apoptotic degeneration (E).

5.3.1.2 Microarray and IPA

142 genes were upregulated and 39 genes downregulated by a minimum of two-fold in PAEC samples compared to non-PAEC. These 181 differentially regulated genes were uploaded into IPA for canonical pathway and network analysis. The predicted top five canonical pathways were corticotropin releasing hormone signaling, tight junction signaling, thyroid hormone metabolism I, protein kinase A signaling and cAMP mediated signaling. The five top associated molecular networks for genes in our dataset in the category diseases and disorders were connective tissue disorders, reproductive system diseases, organismal injury and abnormalities, cancer and immunological diseases. The predicted activation state was investigated for cancer diseases and none of the cancer related pathways were significantly activated or inhibited.

5.3.1.3 Real-time PCR

Nine genes relevant to functions and diseases of the uterus were selected from the dataset for real-time PCR analysis. Among these, three were significantly upregulated in endometrium with PAEC compared to non-PAEC endometrium; ADAM12 ($p = 0.04$), THY ($p = 0.02$) and TN-C ($p = 0.04$). The proliferation marker MKi67 was not statistically significantly altered (Figure 13).

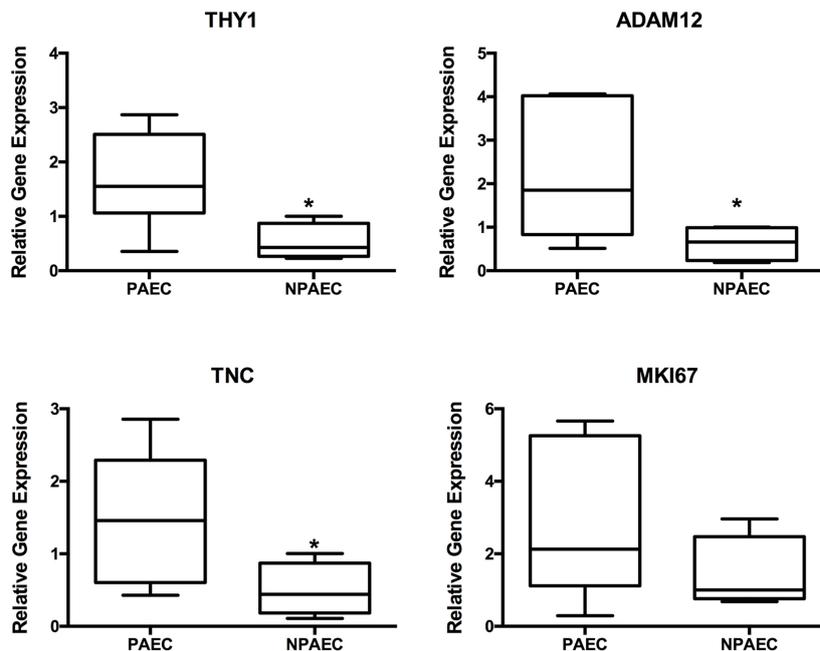


Figure 13. Tukey plots for significantly differentially regulated genes (THY1, ADAM12 and TN-C) and proliferation marker MKi67 analyzed by real time PCR. * $p < 0.05$

5.3.1.4 *Mass Spectrometry-based Proteomics Analysis*

From the qualitative data analysis, three endometrial samples with the highest number of proteins identified in each group were selected for quantitative analysis. A total number of 1772 proteins were identified across the six samples analyzed. Twenty-five proteins were upregulated and five downregulated in endometrium with PAEC compared with non-PAEC. Among them, 5 were significantly upregulated; TUBA4A ($p=0.002$), MYOF ($p=0.006$), TPM4 ($p=0.013$), RRBP1 ($p=0.021$) and DDOST ($p=0.041$) and 3 significantly downregulated; CSRP1 ($p=0.001$), DPYSL3 ($p=0.012$) and FMOD ($p=0.045$) at least two-fold in the PAEC group compared to the non-PAEC group. Twenty of the remaining 22 proteins were within detectable level only in endometrium with PAEC and two within detectable level only in non-PAEC endometrium. These proteins are considered highly differentially regulated in respective group since below detection level in the comparison group, even though a fold change and p-value cannot be calculated.

6 METHODOLOGICAL CONSIDERATIONS

The four experimental studies of the translational project that constitute this thesis comprise several methodological approaches. The different methods complement one another, all with aim at exploring different aspects of the effects of PRMs when used or to be developed for contraceptive purposes, including short-term impact on ovary and endometrium as EC and possible repercussion of long-term use on the endometrial tissue if used as regular contraception. Study I is a clinical randomized placebo-controlled multicenter trial and Study II-IV are exploratory molecular studies, each with individual weaknesses and strengths.

6.1 STUDY I

6.1.1 Strengths

The main strength of the study is that it was as a double-blind, randomized, placebo-controlled trial and as such provides the most compelling and highest level of evidence. Placebo was chosen as a control and the treatment was administered in a randomized manner in order to control for confounding factors and prevent bias from occurring, including strict inclusion criteria and thus avoiding systematic error. The effect of the COCP on ovarian activity was furthermore calculated by partially objective and subjective measures producing the validated Hoogland score. The double-blind randomization procedure reduced possible bias of the subjective measurement of follicle diameter to a minimum. The combination of EE and LNG used in the COCP studied is common worldwide and thus the findings in this study are also relevant to most country settings.

6.1.2 Limitations

The main limitation of this study is that the statistical analysis plan had to be changed due to the higher than expected proportion of women who ovulated.

It was previously demonstrated in a study that initiation of a COCP when the leading follicle was 10 ± 1 mm achieved ovarian quiescence in all study participants, whereas if the lead follicle size was 14 ± 1 mm at initiation, 64% of them reached ovarian quiescence (Baerwald 2006). Based on these results, the assumption for our study was that approximately 90% of women in the placebo group would attain ovarian quiescence during COCP treatment when this was initiated from follicle size > 13 mm. However, follicle size was measured at study visits every 2-3 days and not daily in our study, which rendered the lead follicle on average larger at intake of study medication than was expected at the planning stage.

Pursuing the strategy of the initial non-inferiority analysis would result in exclusion of a large number of participants who ovulated, since they would never reach the primary efficacy criterion of attaining ovarian quiescence. Had not the statistical plan been changed, the results would have lost validity due to the substantial loss of statistical power with exclusion of many participants. It was therefore considered essential with a new appropriate statistical plan. Another limitation is that it was not possible to establish the exact day of ovulation due

to the 2-3 day intervals between study visits. On the other hand, error in accuracy of determining day of ovulation or ovarian quiescence rather postponed assessment, leading towards slightly overrating pregnancy risk if UPSI during COCP period, rendering the following interpretation and advice to women overly precautionous. Moreover, ultrasonography measurements were performed at the three study sites by several investigators and an inter-observer variability cannot be ruled out. However, this weakness was controlled for by the double-blind design. Within the RCT setting, study subjects recruited are often well motivated, comply to the treatment regime and are closely followed through regular visits. To what extent the setting and study subjects are representative of the target population for which treatment is addressed and results generalizable to a larger population, nationally or internationally and thus considered clinically relevant is

Taken into account the strict inclusion - and exclusion criteria of such trials, this rather selected study population can limit the external validity of any RCT trial. However, in this case many exclusion criteria already apply when COCP treatment is initiated in settings other than a clinical trial, as use of WHO's MEC for contraceptive use is widely spread. The inclusion criteria of BMI < 30 in this study is probably the one parameter most likely to be renounced in the general setting. Population-based studies are needed in order to show that the impact of a treatment is true for a general population. Furthermore, the results from this study cannot be extrapolated to progestogen-only contraceptives or other methods of combined hormonal contraception. Neither does this study answer whether the COCP impacts the ability of UPA to postpone ovulation due to its study design.

6.2 STUDY II AND III

6.2.1 Strengths

There are no good animal models to understand human endometrial receptivity and the process of human embryo implantation. The 3D *in vitro* endometrial co-culture system used in these studies does to a large extent mimic the receptive human endometrium *in vivo*. Fresh human endometrial cells are collected, isolated and immediately cryopreserved until used in the primary cell cultures, which is preferable to using indefinitely dividing continuous cell lines in order to more closely reflect the physiology of cells *in vivo* endometrium. The model has repeatedly proven to express a number of endometrial receptivity markers and to be a suitable system for studies of progesterone regulated factors. Its 3D structure provides a physiologically adequate space, which enables endometrial stromal and epithelial cells in the culture to interact and communicate in a natural paracrine and endocrine manner. In addition, it provides an invaluable tool for studies of early stages of human embryo implantation process, for which it was specifically developed. The model is reliable and reproducible and now well established for such studies. It can further be used to investigate various fertility regulating substances, as well as for studies of molecular signaling between the embryo and the receptive endometrial construct.

6.2.2 Limitations

The 3D endometrial *in vitro* construct used in the studies contains both epithelial and stromal cells, which are the two main cells of the human endometrium. Although the co-culture model is complex, the endometrial tissue is far more so *in vivo*, with a high number of stromal and epithelial cells as well as other cell types, such as immune cells and endothelial cells, normally present in the cycling endometrium in a variable and dynamic manner. It is known that reciprocal interactions between all types of cells in the endometrium contribute to the endometrial milieu and formation of the transient receptive endometrium. Thus, further modifications of the model, incorporating other cell types and improving culture conditions could more accurately reflect *in vivo* endometrium. This would be a beneficial development of this model as a more in-depth understanding of the complex event of human embryo implantation and endometrial function could lead to improved contraceptive methods. Knowledge from such models further has the potential to enhance results from ART by developing therapies for women who suffer from infertility due to implantation failure.

During the study process the 3D model had to be modified regarding number of stromal cells used in the cultures by decreasing the number of cells in the collagen matrix as initially the gel tended to contract. In addition, the model was further improved by using unicellular epithelial cells on top of the cell cultures as seen in endometrium, instead of initially used epithelial glands that easily grew as irregular clumps.

Studies II and III are first time exploratory studies. Thus no power calculation based on previous study results could be performed. Research using advanced co-cultures with embryos and molecular studies with diverse techniques of exploring gene and protein expression in endometrial tissue and constructs is very resource intensive. Therefore, such studies are only possible to conduct in small scale. An important limitation of the implantation studies is the small number of cultures. This is due to the fact that human embryos are difficult to obtain for research purposes. The few number of cultures in our studies implies a risk of failing to demonstrate an effect of the PRM exposure, although one actually might be present, thus creating a false negative result. In statistical terms of hypothesis testing this represents a type II error.

To test a series of graded PRM concentrations would be beneficial in order to more closely study the dose-response effect of these compounds. However, the limited availability of embryos restricts the number of cultures and by necessity also the number of different PRM concentrations possible to study. The concentration of UPA used in the treatment group (200 ng/ml) was chosen to resemble the condition after UPA intake when used as oral EC, where a maximum mean serum concentration of 176 ± 89 ng/ml has been observed one hour after ingestion. Previous *in vitro* studies of UPA-EC have also used this concentration [89]. Regarding concentration of mifepristone, we chose to test two different low doses not previously studied regarding effect on embryo implantation or endometrial receptivity (0.5 μ M and 0.05 μ M) in order to determine the dose-response. Another consideration regarding the *in vitro* culture compared to *in vivo* concerns the difference in metabolic drug clearance,

which is taken care of by liver enzymes such as cytochrome P450 enzyme 3A4 *in vivo*, however lacking in the *in vitro* construct.

Analysis of the gene expression in our endometrial constructs gives information on the total expression emanating from the merged population of cells. If a technique to extract information regarding gene expression from individual cells in the endometrial construct were to be developed, such as laser capture micro-dissection (LCM) feasible in regular tissue, a detailed view of gene expression in compartments of each cell type could be performed. This would further deepen the knowledge of the substance studied as well as of dynamics within compartments during the endometrial implantation window.

6.3 STUDY IV

6.3.1 Strengths

For best accuracy regarding diagnostics of PAEC occurrence, an experienced pathologist with expertise in endometrial pathology examined and evaluated the endometrial slides. The pathologist was blinded to treatment groups in order to minimize bias. Strengths of this study further include its study design with endometrial mifepristone-exposed samples originating from a randomized placebo-controlled trial and additionally the various methods used for molecular analysis of both gene and protein expression in these samples. A successful method to extract proteins from the FFPE endometrial samples has been standardized and described. This will hopefully inspire to further proteomic studies of endometrial tissue in the future, considering the presumed quantity of endometrial tissue stored worldwide in biobanks and may well contribute to new important discoveries. For the first time to our knowledge, a list of upregulated proteins in endometrium with PAEC is presented, providing a ground for future functional studies of proteins and potentially also for finding a valid biomarker for PAEC detection. In this study, we did not add endometrium from women who were not treated with mifepristone. The reason is that endometrium with mifepristone treatment loses its normal physiological stage and ends up in amenorrhea. This state of endometrium does not correspond to any of the phases of cycling endometrium of healthy women. Thus, the addition of such group will not provide us with any meaningful information in understanding the physiology of endometrium with PAEC.

6.3.2 Limitations

The main limitation of this study is the small sample size of the clinical trial, resulting in small comparison groups in the molecular studies of mifepristone-exposed endometrium. Another general limiting factor is that assessment of PAEC is subjective with currently no existing standard marker for the condition. This limitation was controlled for by the blinded fashion in which assessment was performed and further as just one expert pathologist performed the evaluation of endometrial samples in attempt to ascertain best possible accuracy. Although similarities of mifepristone with UPA, the results from this study cannot unreserved be extrapolated to UPA induced non-physiological changes. Further, all treated women in the study had symptomatic leiomyoma, which theoretically might influence the

endometrial response to mifepristone. On the other hand this condition is currently the most common indication for long-term PRM treatment and thus implications of the treatment indeed of particular importance to study in this specific group of women. Whether the results apply also to endometrium in non-affected uterus is indeterminate. Another possible limitation lies in the fact that the features of PAEC can vary substantially within the tissue and thus not be representative in the sample taken and/or differ between the different sections of the divided sample, rendering slightly uncorrelated results between morphology, gene expression and protein expression. In addition, the amount of endometrial tissue was insufficient to reconfirm protein quantification findings with Western Blot. The results from bioinformatics studies have to be confirmed in future experimental studies.

7 DISCUSSION

7.1 SUMMARY OF MAIN FINDINGS AND POSSIBLE UNDERLYING MECHANISMS IN STUDY I

We have explored the effects of UPA-EC on ovarian activity when quickstarting treatment with a common COCP after UPA intake in a double-blind, randomized, placebo-controlled trial. Our study demonstrates that UPA does not interfere with the ability of an ongoing COCP to induce ovarian quiescence when treatment is initiated immediately after EC intake. Quickstart treatment of COCP after UPA-EC intake is further safe and tolerable and does not increase the incidence of breakthrough bleeding. Quiescence was achieved after 7 days of COCP intake for most women, however could take up to 14 days for some (irrespective of UPA). No woman among them who ovulated did so after day 11 of COCP intake. The proportion of women who ovulated in the study was approximately 30%, similar in both groups, which can be explained by follicle size at inclusion.

UPA binds the PR with high affinity but does not at the receptor site seem to interfere with the action of the subsequent daily dose of the progestin component LNG in the COCP studied. Although the contribution of EE in inhibition of ovulation is believed to be minor and EE alone requires much higher doses for a suppressive effect, a possible explanation for the maintained capacity of the COCP to induce ovarian quiescence might be that this effect is due to the component EE in the COCP, as it is undisturbed in exerting a suppressive effect on the hypothalamic pituitary ovarian axis (HPA) [208]. Another explanation is that although ovarian quiescence was studied, the trial was not designed to examine any impact of the COCP on the ability of UPA to delay ovulation. Following UPA-EC, the main effect to prevent fertilization seems to be a delay in ovulation rather than inhibition of ovulation [53]. The findings are consistent with previous reports of initiation of COCP at different follicular cycle stages where evidenced that the risk of ovulation increases with increased follicular size [209]. The reason for choosing a mid- to late-follicular phase in the ovarian cycle was the relevance to a clinical situation where a woman seeking EC would be in an obvious risk of unwanted pregnancy should further acts of UPSI take place and ovulation occur but where the ECP in addition could delay ovulation. However, as discussed in methodological considerations, due to the design where the women weren't examined daily, treatment was given when the leading follicles were generally larger than had been anticipated and thus obstructed the initial statistical plan.

7.2 THE RESULTS FROM STUDY I IN CONTEXT

The results from our study cannot be extrapolated to other PRMs as the ECP, or to quickstart with a POP as the ongoing contraceptive method. Neither can a negative effect of the COCP on the ability of UPA to delay ovulation be excluded due to our study design where we did not have a group that received placebo instead of COCP after UPA.

In a subsequent pharmacodynamic study by Brache *et al*, it was demonstrated that a POP containing the progestin desogestrel (DSG) initiated immediately after UPA-EC intake

significantly reduced the ovulation-delaying ability of UPA with 45% of women ovulating within 5 days after EC, compared to 3% among women only receiving the UPA-ECP. Similar to the results in our study using COCP as the ongoing contraceptive after EC, UPA did not have a negative effect on the ability of the POP to exert its contraceptive effect [210]. This information revealed that quickstart of a POP appears to counteract the main contraceptive effect of UPA, which is to delay ovulation beyond the life span of sperm. Therefore it is biologically plausible that quickstart of any progestin containing method after UPA-EC actually would mean that the intention to postpone a possible imminent ovulation and thereby prevent an unintended pregnancy with UPA could be jeopardized by the subsequent immediate start of a progestin containing oral contraceptive. Thus leaving the woman at the same risk of pregnancy as if she had not taken any contraceptives following UPSI. The study referred to is small in size, as is ours. However, based on these findings as well as findings in previous studies on interaction between PRMs and gestagens [211], it is not unlikely that this mechanism may apply broadly.

7.3 SUMMARY OF MAIN FINDINGS AND POSSIBLE UNDERLYING MECHANISMS IN STUDY II AND III

We investigated the effects of UPA and low doses of mifepristone on human embryo implantation and putative endometrial receptivity markers in a well-established 3D co-culture model. Although some of the endometrial receptivity markers in Study II were altered when exposed to UPA in an EC dose, most were unaffected. UPA did not significantly inhibit the early embryo implantation process, as there was no significant difference in attachment rate in the UPA group compared to controls. In study III, most receptivity markers were altered with the two different low concentrations, however they exerted functionally different effects on embryo implantation in a dose-dependent manner.

The concentration 0.5 μ M effectively inhibited embryo implantation whereas a tenth of that concentration; 0.05 μ M did not affect the implantation rate significantly. Both concentrations however correspond to oral doses much lower than when used for EC [60, 81, 212-215].

Metabolic and pharmacokinetic studies after oral intake of mifepristone have demonstrated a linear kinetic relationship between single doses of 2-25 mg and increasing concentrations accordingly in serum. The higher of our concentrations chosen would roughly correspond to an oral dose of 5 mg. Should we further assume a linear relationship with doses below 1 mg, the lowest chosen concentration in our study would roughly correspond to 0.8 mg orally.

Although without significant difference in our study, we observe that it seems to be a tendency towards less attachment to the construct even in the very low dose mifepristone group compared to the control group (40% vs 70%), which suggests a dose-dependent effect on the endometrium with mifepristone even at very low doses. On the other hand, the condition *in vitro* is different from *in vivo* tissue where the metabolic clearance of hormones occurs through the liver, whereas cultures in contrast are exposed in a continuous manner.

The PRM binds to the PR with high affinity and thereby alter transcription of the factors regulated by progesterone. Although structurally similar, there seems to be differences in the

action of mifepristone and UPA. Whether these are due to the difference in action on the PR, due to an antiglucocorticoid effect, are mainly dose dependent or depend on other factors remains to be explored. It is possible that mifepristone due to a more antagonistic profile has a more pronounced impact on altering studied endometrial receptivity markers compared to UPA. The dose-dependent difference in ability to attach to the mifepristone exposed constructs is believed due to effect on the endometrial construct and not to effect on embryo, as mifepristone in higher doses does not adversely affect embryo viability or its ability to implant [216].

7.4 THE RESULTS FROM STUDY II AND III IN CONTEXT

Our studies confirm the findings from previous endometrial studies and clinical trials showing that PRMs exert a dose-dependent effect on the endometrium [72, 73, 75, 79, 85, 87, 88, 217]. In a recent study by Lira-Albarrán et al, the gene expression in endometrium was explored through microarray after a single UPA-EC dose at mid-cycle compared to controls and further compared to ERA [218]. They concluded that UPA had an antiprogestational endometrial effect associated with a non-receptive endometrium and further that a key gene (PAEP) in trophoblast attachment was much downregulated in UPA treated endometrium. The post-ovulatory rise in progesterone was normal, however their results suggested that the decidualization process was affected. Contrastingly, the two well-known decidualization markers IGFBP-1 and PRL were not significantly altered by UPA in our study.

Moreover and most importantly, our functional study of embryo implantation showed that the blastocyst in fact could attach to the UPA exposed endometrial construct, although some of the studied receptivity markers were altered. There seems to be a functional redundancy within the threshold of receptive endometrium, where the observed changes in levels of certain receptivity markers are counteracted by other compensatory mechanisms in a complex molecular interplay in order to enable successful implantation.

There are obvious differences between *in vitro* and *in vivo* endometrial studies that can explain different findings in studies, apart from methodology used. The endometrial tissue *in vivo* is also influenced by factors from ovaries and from other cell types in the tissue, such as endothelial cells and leukocytes, which the *in vitro* system lacks.

There is currently no reliable diagnostic tool for endometrial receptivity or valid single receptivity marker and studies on endometrial receptivity therefore importantly have to be interpreted with caution. Global gene expression studies report that PRMs can affect markers of endometrial receptivity at the molecular level [123, 219-221] but not all markers altered are associated with endometrial receptivity or relevant for embryo implantation. Recently it was shown that a single dose of mifepristone significantly altered the transcript profile in the endometrium of fertile women during WOI, however only 37% of the genes were associated with endometrial receptivity. They concluded that not all genes in the endometrium that change during transition from pre-receptive (LH+2) to receptive phase (LH+7) are regulated by progesterone [219].

Low doses of mifepristone administered daily or weekly in humans do not affect ovulation

and fail to prevent pregnancy sufficiently although endometrial morphology and some of studied receptivity markers are affected [75, 79, 217]. Further, even well studied compounds such as LNG alter the gene expression in endometrium, although LNG does not adversely affect implantation [56, 57, 222, 223].

The results from our studies demonstrate the importance of functional evaluation of endometrial receptivity. The finding that the blastocysts could attach to the cultures exposed to UPA is in line with clinical trials of LNG- and UPA-EC. In a recent clinical study by Li *et al* it was further demonstrated that UPA intake before ovulation significantly prevented more pregnancies than when administered after ovulation compared to the number of expected pregnancies [92]. There is a marked increase in pregnancy risk for women who reported UPSI during days of highest probability of conception, as well as for those who had further acts of UPSI after ECP intake, regardless of method, which arguably indicates lack of significant postovulatory effects also for UPA [18, 59, 70, 102]. The observation of pregnancies in clinical trials after UPA intake thus supports the results in our *in vitro* study. The merged evidence is in line with the conclusion that the higher efficacy of UPA compared with LNG in clinical trials can be explained by the wider pre-ovulatory time window of effect of UPA, even after LH has started to rise when LNG has no effect [53, 71], and not through an anti-implantation effect.

7.5 SUMMARY OF MAIN FINDINGS AND POSSIBLE UNDERLYING MECHANISMS IN STUDY IV

We have characterized the altered expression of genes and proteins in endometrium displaying PAEC compared with non-PAEC after 3 months of mifepristone treatment in premenopausal women with uterine leiomyoma. The morphological features of PAEC samples observed coincided with previous descriptions and included disordered glands with variable degree of dilatation, lined with an incongruous epithelium as well as changes in vasculature and stroma. Three genes of relevance to function and diseases of the uterus were upregulated in endometrium with PAEC, as were 25 proteins, and 5 proteins were downregulated compared with non-PAEC endometrium.

ADAM12, which was one of the upregulated genes in PAEC samples, is a member of a family of proteolytic enzymes with gelatinase activity, involved in cell-cell and cell-ECM interactions and involved in tissue remodeling [224]. Overexpression of the adhesion-modulating glycoprotein TN-C has previously been observed in leiomyoma tissue [225], however we could not explain the upregulation in PAEC samples with correlation of submucosal location of leiomyoma in our study. The glycoprotein surface marker THY1 is involved in several cellular processes including wound healing and tumor suppression and of importance for fibroblast-ECM interactions. A decrease in the expression of THY1 is associated with malignant transformation in murine fibroblasts [226], however in our study it was upregulated in endometrium displaying PAEC compared to non-PAEC samples. The genes upregulated in our study are all associated with the cytokine transforming growth factor- β (TGF- β), and both TGF- β 1 and TGF- β 2 were upregulated in our study, however not

significantly or by two-fold or more respectively, which was some of the filters set in our microarray study. Mifepristone exposure has shown to increase levels of TGF- β in endometrium of Rhesus Monkey (Ghosh 1998) and possibly the mechanism by which the genes in our study were upregulated could involve TGF- β although this factor was not significantly increased here.

The differentiated genes and proteins in this study are engaged in ECM, cytoskeletal organization and tissue architecture, indicating impact on endometrial morphology. The altered expression of these molecules may explain the specific histological features that constitute PAEC. None of the differentially regulated genes in our dataset were however involved in the endometrial cancer-signaling pathway based on IPA knowledge base.

7.6 THE RESULTS FROM STUDY IV IN CONTEXT

The frequency of PAEC in our study was 53.8%, which is in line with several previous reports from studies of mifepristone treatment of uterine leiomyoma [200, 227, 228]. Most studies examining the effect of mifepristone on uterine leiomyoma and additional endometrial impact have used a lower dose (2.5-10 mg daily), however the specific non-physiological changes can be observed in the endometrium to a similar extent and sometimes to a higher extent than we report. In one study with the very low dose of 2.5 mg mifepristone daily, 86% of exposed endometrial samples displayed the characteristic features of large dilated cystic glands [192]. The non-physiological changes of PAEC do not increase with the number of treatment periods and are further reversible after discontinuation of treatment when the endometrium has been shed. Interestingly, PAEC can appear normally in endometrium that has not been exposed to a PRM, as diagnosed in approximately 10% of women in trials at screening [229].

Since development of PAEC during PRM treatment is a class effect of these compounds, the features are also seen in endometrium exposed to UPA and to a similar extent (\approx 60%). The features of dilated cyst formations have been observed in approximately 30% of UPA treated women [187, 229]. UPA in a daily dose of 5 mg for treatment of uterine leiomyoma has emerged as an attractive pre-operative treatment or therapeutic alternative to surgery, particularly important for women who wish to preserve their childbearing potential. Among women of reproductive age, uterine leiomyoma are the most common benign pelvic tumors, with a cumulative incidence of 70-80% at 50 years of age and a leading cause of hysterectomy worldwide [230]. In light of these facts, the implications of the commonly occurring features of PAEC during PRM treatment require in-depth exploration for evaluating the safety of long-term treatment. To our knowledge, when this study started, the molecular changes of PAEC had not previously been explored.

Recently on the other hand, Whitaker *et al* published a study in which they examined the histological and molecular expression in endometrium after pre-operative UPA treatment of leiomyoma during 9-12 weeks [231]. They established that all the nine endometrial samples collected after UPA treatment displayed PAEC features and further compared them with the

molecular expression of proliferative and secretory phase endometrium collected from healthy women. Due to methodology, the rate of PAEC and the different PRMs studied, it is not possible to compare the two studies. In order to understand the molecular changes associated with PAEC after treatment with mifepristone, we instead chose to compare endometrium that displays PAEC with non-PAEC endometrium. Evidently, such study was not possible since all UPA treated samples had dilated cystic glands in the study by Whitaker *et al.* The comparison in their study was instead done between UPA treated women who had lost their normal endometrial cycle with healthy women having normal endometrial function, collected from different phases of the menstrual cycle. Further, one third of the women in their study reported spotting, which may interfere with the gene expression studies, whereas bleeding had ceased in all women within two weeks of mifepristone treatment in our study [184].

One of the most distinctive and appreciated effects of PRM treatment, apart from reducing the volume of the leiomyoma, is that endometrial bleeding is rapidly suppressed. The mechanisms for PRMs reducing bleeding effect are not known but seemingly frequency of amenorrhea increases with higher doses of PRM [184]. ADAM12 was upregulated in our study and it has previously been demonstrated that progesterone upregulates ADAMTS1 whereas mifepristone inhibits the increase [232]. All of the samples in our study had been treated with mifepristone and thus gives the reason to speculate whether a more pronounced antiprogestosterone effect, as in a higher mifepristone dose, would in fact reduce PAEC, as with bleeding.

A question raised is further whether occurrence of PAEC simply is indicative of endometrium that for some reason has not been properly shed, since a significant minority of women has these non-physiological changes even without PRM treatment and since they disappear after cessation of treatment. Minor alterations in tissue hormonal levels could possibly add to the appearance. In our trial, mifepristone treatment decreased serum levels of estradiol and progesterone and there was a slight significant increase of free testosterone and androstenedione, although within the normal reference limit, compared with the control group [184]. Mifepristone is known to increase AR expression in the endometrium, which in addition may add to the mechanism of action by which it exerts its antiprogestational endometrial effect.

PAEC has previously been classified as benign with no evidence of hyperplasia or atypia, which is in line with the findings in our study where no such changes were seen. Reassuringly, the endometrial cancer-signaling pathway was further not affected in our analysis as studied by IPA.

8 CONCLUSIONS

- UPA when used for EC does not negatively affect the ability of a common COCP to induce ovarian quiescence when initiated after UPA intake.
- Most women reach ovarian quiescence by day 7 of COCP treatment but some women do not reach quiescence until day 14 of COCP.
- Exposure to UPA in a dose corresponding to the dose used for EC does not inhibit human embryo implantation process *in vitro*, though some of studied endometrial receptivity markers are significantly altered.
- Exposure to a low concentration (0.5 μM) of mifepristone during the receptive period successfully inhibits human embryo implantation process *in vitro*. Mifepristone in low doses exert a dose-dependent effect on endometrial receptivity at the functional level.
- The altered expression of molecules affecting ECM and tissue architecture in endometrium displaying PAEC demonstrated in this study may explain the specific non-physiological morphological features of PAEC.
- None of the significantly differentially regulated genes in endometrium with PAEC are involved in the endometrial cancer-signaling pathway based on IPA knowledge base.

9 CLINICAL AND SCIENTIFIC IMPLICATIONS

Based on the findings in study I, we concluded that quickstart of an ongoing COCP after intake of UPA-EC was safe and did not impair the COCPs effect on repressing ovarian activity. We pointed out however that the study was not designed to evaluate the possible impact that the COCP could have on the ability of UPA to exert its effect on delaying ovulation. After gathering evidence-based information from our trial as well as subsequent trials on the interaction of POP and UPA, EC guidelines have recently been changed. The current advice include to avoid quickstart of a regular contraception after UPA-EC intake and to delay initiation of hormonal contraception for at least 5 days after UPA. On the other hand, it is suggested in some guidelines that it may be more biologically correct to count the five days from the episode of UPSI rather than from UPA intake, as sperm after this time no longer can survive in the female reproductive tract

(http://americansocietyforec.org/uploads/3/4/5/6/34568220/asec_fact_sheet-hormonal_contraception_after_ec.pdf) (accessed April 2017).

In addition, women should not take UPA-EC within 7 days after use of progestin containing contraceptives, such as when women seek EC after UPSI when having missed pills from their regular POP or COCP. Further, a question raised from our study regarding whether or not the well-established advice of 7 days of COCP intake before it is reliable as contraception when initiated at mid-cycle or after LNG-EC really is sufficient, as it took up to 14 days to reach ovarian quiescence even for some women in the placebo group.

From Study II and III new insights on the mechanism of action of mifepristone and UPA when used for EC has been presented. Exposure to UPA in a concentration corresponding to EC dose did not compromise the capability of the blastocyst stage embryo to attach to the endometrial construct *in vitro*. Women are withheld EC around the world due to the apprehension that UPA has postfertilizing effect and thus is to be regarded as a method of abortion. Our results are in line with the previously described mechanism of UPA to delay ovulation and thereby function as an EC when administered pre-ovulatory, not exhibiting anti implantation effect. We can thereby provide women with evidence-based information on UPA effect on human embryo implantation. Since UPA does not inhibit embryo implantation at the endometrial level, an unwanted pregnancy can occur if UPA was taken just before or at the time of ovulation. For an EC to be near perfect in preventing pregnancy after UPSI it is necessary for it to be effective during the whole cycle, postovulatory but before implantation, as is the case with the Cu-IUD.

Interestingly, even a low concentration of mifepristone 0.5 μM , corresponding to approximately 5 mg orally, could effectively inhibit embryo implantation in our *in vitro* model, whereas a tenth of that concentration could not, displaying the known dose-dependent effect of mifepristone on the endometrium. Low doses of mifepristone had not previously been studied on the embryo implantation process, only a much higher concentration of 10 μM , which also effectively inhibited implantation. From our results we conclude that low

dose mifepristone has great potential to be developed for contraceptive purposes, possibly as EC or on demand, which thus would provide a new type of contraceptive measure.

Study IV generated information on the molecular profile of endometrium displaying PAEC after 3 months of mifepristone treatment due to symptomatic leiomyoma. In regards to the increasing use of PRMs for this indication, it is of great importance to closely evaluate the safety and understand the repercussions of this treatment, as the endometrial changes are prominent and commonly occurring. The results from this small study are reassuring as no molecules altered were involved in the endometrial cancer-signaling pathway, however mainly in the structural architecture of tissue, which may explain the morphological features of PAEC. A method of protein extraction from FFPE treated endometrial samples has been standardized and thus we present a list of proteins significantly altered in endometrium with PAEC, which could enable for future studies aiming to find a biomarker for detection of PAEC and further provides groundwork for studies on the functional role of altered proteins.

10 FUTURE PERSPECTIVES

Insertion of a Cu-IUD is undoubtedly the most effective EC method and should be further promoted since underused and many remain unaware of this option. However, a Cu-IUD is not always suitable or available and thus the development of more effective methods for EC is clearly needed. Adding a COX-2 inhibitor to LNG-EC proved higher efficacy in postponing ovulation than LNG alone and future studies with this or similar compounds in combinations with different ECPs should be further explored. The advantage of a LNG based method is that LNG is widely available and doesn't affect the efficacy of hormonal contraception in case of missed pills or quickstart of contraception after EC use.

However, in order to address the wish of an ECP with efficacy in line with insertion of a Cu-IUD, it is not enough to lean on methods solely affecting the ovulatory process, instead a more pronounced effect on endometrial receptivity is essential. An additional preventive effect after fertilization but before implantation is a determinant of near perfect prevention of unwanted pregnancy, since effectiveness of a method is closely correlated with its mechanism of action. New contraceptive strategies should therefore include manipulation of factors critical for endometrial receptivity in addition to affecting follicular development and ovulation. The most effective approach would address protection against pregnancy if further acts of UPSI occurred in the cycle, not risk interaction with hormonal contraceptive methods or be affected by body weight.

An option to use contraception on demand would be a welcome contribution to the arsenal of possibilities in preventing unwanted pregnancy. Based on previous trials and observations from our studies, a PRM such as mifepristone has great potential to be developed for peri-coital use. A suggestion would be to further study mifepristone in a single medium dose weekly or on demand (25-50 mg) and also in low daily short-term doses for EC following UPSI (for example 5 mg daily for 5 days). The suggested regimens are believed to affect the endometrium, making it non-receptive to the fertilized ovum, should UPSI have taken place during the fertile period and thus would increase effectiveness to ensure best possible prevention of unwanted pregnancy. Any such trial should include studies of bleeding patterns during and after the treatment as such disturbances might occur.

PRMs like UPA and mifepristone further both have the potential to be developed as ongoing contraception. Studies for developing a daily pill or intrauterine method containing UPA are currently ongoing. The contribution of a PRM to the arsenal of contraceptive methods is believed to be an important and attractive option. When it comes to oral hormonal contraceptives, many women with contraindications to estrogen for medical reasons are confined to POP only if they do not wish to use an IUD or implant. The POP is not optional for some women due to bleeding irregularities and necessity of strict timing of pill intake and thus a new estrogen-free alternative would be most welcome. A PRM further could provide other added health benefits and appreciated side effects such as amenorrhea, thus a possible treatment for heavy menstrual bleeding (HMB) and related anemia. Of great importance to

follow through, should trials with PRMs as ongoing contraceptive methods be conducted, is the effect of the drug on breast, as mifepristone has been demonstrated to have an antiproliferative effect in breast cells [233] and thus speculatively PRM contraception have the added benefit of protection against breast cancer. Furthermore, trials are ongoing exploring PRMs for treatment of premenstrual syndrome (PMS).

11 POPULÄRVETENSKAPLIG SAMMANFATTNING

11.1 BAKGRUND TILL STUDIerna

Osäkert utförda aborter bidrar stort till graviditetsrelaterad sjuklighet och dödlighet i världen och sker nästan uteslutande i låginkomstländer. Det uppskattas att var fjärde av de ca 213 miljoner graviditeter som uppkommer årligen i världen avslutas med abort och att ungefär hälften av dessa är osäkert utförda. Under 2012 behandlades ca 7 miljoner kvinnor för abortrelaterade komplikationer på vårdinrättningar i låginkomstländer men mörkertalet i sjuklighet är förstås stort. Trots att en abort utförd i enlighet med WHO-riktlinjer är en väldigt säker medicinsk procedur med få komplikationer dör ungefär 23 000 kvinnor årligen i världen till följd av osäkert utförda aborter. De mest drabbade är de yngsta och fattigaste kvinnorna och fördelningen i världen är mycket ojämn med ca 62% av alla dödsfall i Afrika, söder om Sahara. Osäkra aborter har kallats ”The Preventable Pandemic”, den förebyggbara pandemin, eftersom sjuklighet och dödlighet till följd av dessa skulle gå att förebygga med tillgång till säkra aborter och aborteftervård inklusive legalisering och de-stigmatisering. Tillgång till moderna preventivmedel spelar också en avgörande roll. Utöver de reguljära preventivmetoder som finns har även akuta preventivmetoder utvecklats.

Med en akut preventivmetod menas en metod att förebygga oönskad graviditet efter det att ett oskyddat samlag ägt rum men innan ett eventuellt befruktat ägg har implanterat i livmodern och en graviditet uppstått. Det kan vara aktuellt med akutpreventivmedel t ex vid misslyckad preventivmedelsanvändning som när en kondom spruckit, om flera av ordinarie p-piller missats eller efter våldtäkt. Den mest effektiva akuta preventivmetoden med närmare 100% effektivitet är insättande av en kopparspiral upp till 5 dagar (120 tim) efter att ett oskyddat samlag ägt rum (eller egentligen 5 dagar efter ägglossningen om den är känd). En fördel med denna metod är att den kan sitta kvar och skydda mot graviditet under flera år framöver som en reguljär preventivmetod. Verkningsmekanismen är dels toxisk effekt på spermier och ägg men spiralen gör också att livmoderslemhinnan (endometriet) inte blir mottaglig för ett befruktat ägg om det trots allt skulle ha lyckats komma till livmodern. Denna metod är säker och effektiv men förutsätter tillgänglighet till en vårdinrättning för insättande och passar kanske inte heller alla av andra anledningar.

Det finns två dedikerade akut-p-piller på marknaden idag, dvs p-piller som förpackats och säljs i en styrka avsedd för akutprevention. Akut-p-piller har beräknats kunna förhindra ca 75% av oönskade graviditeter men beror på när i menscykeln det oskyddade samlaget skedde och hur långt senare man tar tabletten bland annat. Det preparat som funnits längst och är mest spritt runtom i världen är levonorgestrel (LNG), ett syntetiskt gulkroppshormon (gestagen) som kan tas upp till 72 tim (-96 tim) efter samlag och som fungerar genom att skjuta fram ägglossningen så att spermier inte kan befrukta ägget. Denna metod fungerar om den tas innan ägglossning men har ingen effekt precis innan eller under ägglossningen

och har heller inte någon effekt på endometriet, vilket medför att den inte skyddar mot önskad graviditet under hela menscykeln.

Det andra och senaste tillskottet till akut p-piller är en s.k progesteronreceptormodulator (PRM), ulipristalacetat (UPA) som har effekt upp till 120 tim efter oskyddat samlag. Den har också visat sig vara verksamt genom att skjuta fram ägglossningen i minst 5 dagar vilket gör att spermier därför inte hinner befrukta ägget under sin livstid. UPA är något mer effektiv än LNG vilket beror på att den har ett något längre tidsfönster där den har effekt innan ägglossningen, och kan skjuta fram den även efter att hormonet LH börjat stiga då LNG inte längre har effekt. LH frisätts och når en toppnivå ca 36 tim innan ägglossningen och vid eller efter denna topp är båda preparaten verkningslösa. UPA utövar sin effekt i vävnaderna genom att binda till progesteronreceptorn (PR) där den kan ha både hämmande och delvis stimulerande effekt, varav namnet PRM.

En annan typ av PRM är det först upptäckta anti-progesteronet mifepriston. Mifepriston har en något mer hämmande effekt på PR jämfört med UPA. Höga doser tillsammans med prostaglandin-analogen misoprostol (till exempel Cytotec) används för att medicinskt inducera abort. I låga doser fungerar mifepriston på samma vis som UPA, genom att skjuta fram ägglossningen och kan således fungera utmärkt som akut p-piller. Det är dock endast ett fåtal länder i världen där det är registrerat som akut p-piller pga politiska, religiösa och legala skäl eftersom den förknippas med abort, vilket också förmodas vara anledningen till att läkemedelsbolagen inte heller satsat på mifepriston för den indikationen. En del hävdar att anledningen till att UPA är mer effektivt än LNG är att det har effekt på embryots förmåga att fästa i endometriet samt att dess strukturella likhet med mifepriston gör att den fungerar som ett abortpiller, men effekten på endometriets mottaglighet för ett embryo eller dess förmåga att implantera i livmodern är inte tidigare studerat.

Det är viktigt att ta reda på verkningsmekanismen för UPA när det används som akutpreventivmedel för att kunna tillhandahålla kvinnor evidensbaserad information. Om verkningsmekanismen inte visar sig vara att förhindra det befruktade ägget från att fästa i endometriet skulle acceptansen för det akuta preventivmedlet öka runtom i världen och därmed tillgången för kvinnor. Acceptansen för preventivmedel hänger ofta ihop med verkningsmekanismen där graviditet på vissa håll räknas från befruktningsogonblicket och inte som oftast då embryot implanterats i livmoderväggen.

En daglig lågdosadministrering av PRM har potential att utvecklas till en reguljär preventivmetod och många kliniska studier har undersökt effekten av mifepriston i olika doseringar och behandlingsregimer för detta ändamål bland andra. I en 3-dimensionell provrörsmo- dell av mänskliga endometrie- celler har effekten av en mycket hög mifepristonkoncentration visat sig hämma embryoimplantationen. Det är dock inte tidigare studerat hur låga doser av mifepriston påverkar endometriets mottaglighet för embryot eller dess förmåga att implantera i livmodern.

För att kunna utveckla PRM som reguljärt preventivmedel eller som akut-p-piller med förstärkt effekt är det viktigt att undersöka effekten på endometriet i olika doser.

När en kvinna söker med behov av en akut preventivmetod är det ett gyllene tillfälle att erbjuda preventivmedelsrådgivning. Med tanke på att UPA skjuter upp ägglossningen kan ju behandlingen, även om den varit lyckosam vid det senaste oskyddade samlaget, leda till att ytterligare oskyddade samlag i samma menscykel ger oönskad graviditet då spermier ju kan befrukta det ägg som lossas vid den uppskjutna ägglossningen. Studier visar att det är ökad risk att bli gravid vid fortsatta oskyddade samlag i samma cykel efter intag av akut p-piller i jämförelse med att inte ha tagit tabletten. Det beror ju på att risken att bli gravid redan passerats för den som inte tog UPA eftersom de fick sin ägglossning som vanligt och fortsatta samlag efter äggets livslängd på ca 24 tim inte riskerar att leda till graviditet. Det är önskvärt att en effektiv reguljär preventivmetod påbörjas så snart som möjligt efter användande av akut-p-piller. Många kvinnor önskar påbörja p-pillerbehandling med vanliga kombinations-p-piller innehållande östrogen och gestagen, eller tar dessa i vanliga fall men har missat flera piller och vill nu fortsätta med det. Eftersom UPA binder till PR riskerar den att konkurrera med gestagenet i kombinerade p-piller och därmed påverka deras hämning på äggstocksfunktionen. Av denna anledning har råden varit att då reguljär p-pillerbehandling påbörjas i direkt anslutning till intag av UPA, s.k ”quickstart”, skall en barriärmetod användas som skydd mot oönskad graviditet fram till nästa menstruation innan p-pillren säkert har effekt. En möjlig interaktion mellan UPA och kombinations-p-piller har inte tidigare studerats.

För att kunna ge kvinnor evidensbaserad information om hur de på bästa sätt ska kunna skydda sig mot oönskad graviditet efter intag av akut p-piller är det viktigt att genomföra kliniska studier utformade för att undersöka detta.

Både UPA och mifepriston har en dosberoende effekt på endometriet, där högre dos ger tydligare påverkan. Båda preparaten kan också användas för behandling av myom, godartade muskelknutor i livmodern, där behandlingen ges i låg daglig dos. Behandlingen brukar krympa myomen och framförallt göra att de vanligt förekommande rikliga blödningarna upphör eller minskar betydligt. Preparaten har dock visat sig ge en förtjockning av endometriet med förbryllande utseende i mikroskop inklusive vidgade sekretfyllda körtlar bland annat. Detta typiska utseende som uppstår hos ca 60% av de som behandlas med en PRM kallas för PRM associated endometrial changes (PAEC). PRM-behandlingen gör de flesta kvinnorna blödningsfria och eftersom endometriet då inte stöts bort, vilket normalt sker vid menstruation, föreligger viss oro att PAEC i förlängningen skulle kunna leda till sjukdom eller cancer i livmodern, även om några sådana tecken inte setts hittills. PAEC har visat sig vara reversibelt, dvs försvinner när behandlingen upphör och efter att endometriet stötts bort i en blödning. Det har inte gjorts några molekylära studier på endometrium med PAEC som kan förklara vad det rör sig om för förändringar, eller om cellulära signaleringsvägar i vävnaden är associerade med risken att utveckla livmodercancer vid långtidsbehandling. Det är viktigt att förstå betydelsen av PAEC och om det finns molekylära tecken på att tillståndet skulle kunna leda till cancer i livmodern.

11.2 STUDIE I

I studie I ville vi undersöka hur äggstocken reagerar vid p-pilleranvändning som påbörjas direkt efter att kvinnan tagit UPA som akut p-piller, för att utröna om UPA försämrar den vanliga p-piller-effekten innan den gått ur kroppen. Studiens design skulle ge svar på om UPA påverkade äggstocken på ett annat sätt än sockerpiller (placebo). Den utfördes på 3 centra i Europa. Vi rekryterade 76 friska kvinnor mellan 18-35 som lottades till att få UPA eller placebo innan de påbörjade regelbunden p-piller användning under 21 dagar. Vi studerade sedan hur äggstocken reagerade genom att undersöka kvinnorna med ultraljud regelbundet samt hormonnivåer i urin och i blod. Kvinnorna fick också fylla i en dagbok där de skrev ner biverkningar och blödningsmönster. Totalt fick 39 kvinnor UPA och 37 fick placebo. Vi kunde visa att det inte fanns någon skillnad mellan hur äggstocken reagerade på p-pillren beroende på om kvinnan fått UPA eller placebo. Den viktigaste faktorn som avgjorde om kvinnorna fick ägglossning trots att de påbörjade p-pilleranvändning var hur stor äggblåsan var när de tog UPA eller placebopreparatet. Med anledning av vår studie (och sedan efterföljande studier) har riktlinjer för hur quickstart av reguljär preventivmetod efter akut p-piller ändrats.

11.3 STUDIE II OCH III

I studie II och III studerades effekten av PRM på endometriet och på förmågan hos det befruktade ägget att fästa till en tre-dimensionell provrörsmodell av endometriet. Dessa studier var experimentella till sin natur. Modellen bestod av celler som kom från en enkel provtagning där vi tog ut en liten del av endometriet från frivilliga friska kvinnor i fertil ålder strax innan den tidpunkt i menscykeln när kvinnan normalt sett kan bli med barn och isolerade och blandade sedan cellerna med kollagenlösning och byggde 3D modellen som efterliknar hur endometriet ser ut i verkliga livet. Befruktade ägg som bevarats i frys i 5 år och som enligt lag skulle kastas donerades från par som genomgått IVF-behandling (provrörsbefruktning). Vi studerade andelen av embryon som fäste till endometriemodellen efter att den behandlats med UPA (studie II) och två olika låga koncentrationer av mifepristone (studie III) och jämförde med en kontrollgrupp som inte fått någon PRM-behandling. Vi studerade även hur olika gener av betydelse för endometriets mottaglighet för det befruktade ägget uttrycktes i 3D-modellen. Man kunde inte se någon signifikant skillnad i hur många befruktade ägg som fäste mellan UPA och kontrollgruppen men med den högsta mifepristonkoncentrationen sågs en signifikant skillnad där inte några ägg lyckades fästa till endometriemodellen, vilket de kunde vid den lägre av koncentrationerna. UPA påverkade uttrycket av vissa av de studerade generna men flertalet var opåverkade medan båda mifepristonkoncentrationerna tydligt påverkade genuttrycken i en likartad riktning trots funktionellt så olika resultat när det gällde embryots förmåga att fästa vid endometriemodellen. Exakt vilken betydelse detta har i praktiken är osäkert i nuläget men tänkbart är att även låga doser dagligen under några dagar efter oskyddat samlag skulle kunna användas som en akutpreventivmetod, möjligen att utvecklas ”on-demand”, alltså att ta precis

inför el strax efter samlag. Studien är viktig för att demonstrera att den huvudsakliga verkningsmekanismen av UPA som akut p-piller inte är att påverka embryoimplantationen utan att förhindra att ägget befruktas genom att skjuta upp ägglossningen vilket tidigare visats.

11.4 STUDIE IV

I studie IV undersökte vi gener och proteiner i endometrium från kvinnor som lottats till 3 månaders behandling med mifepriston pga symtomgivande myom som skulle opereras bort. Endometrium från 13 kvinnor samlades ihop vid operationerna, varav 7 hade PAEC och 6 var utan dessa förändringar (non-PAEC). Vi jämförde uttrycken av gener och proteiner i de båda grupperna med olika avancerade tekniker samt analysprogram för att studera vilka signalvägar och processer som de förändrade molekylerna är involverade i. Studien visade att det var ökat uttryck av 142 gener och minskat uttryck av 39 gener i PAEC-gruppen jämfört med non-PAEC. Dessa analyserades sedan vidare med Ingenuity Pathway Analysis där det inte sågs någon påverkan på processer som är involverade i utveckling av livmodercancer. Det var ett ökat uttryck av 25 proteiner och ett minskat uttryck av 5 proteiner i PAEC-gruppen och vi noterade att de molekyler som påverkats av mifepristonbehandlingen är av vikt för vävnadens strukturella uppbyggnad, vilket kan förklara den typiska bild som ses vid mikroskopisk undersökning av PAEC. Denna studie underbygger resultat som visar att PAEC inte leder till cancer.

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