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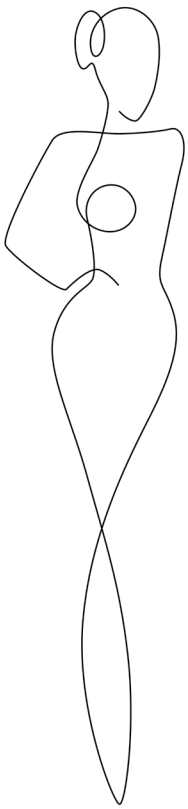
The effect of a progesterone receptor modulator on the endometrium and breast in premenopausal women



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THE EFFECT OF A PROGESTERONE RECEPTOR MODULATOR ON THE ENDOMETRIUM AND BREAST IN PREMENOPAUSAL WOMEN

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"Not everything that can be counted counts,
and not everything that counts can be counted"

Albert Einstein

To my mom and Andrianos

ABSTRACT

Background

The levonorgestrel intrauterine system, LNG-IUS 52 mg, is a highly effective and cost-effective contraceptive, entailing minimal patient effort. Irregular bleeding patterns are common during the first months of use and constitutes one of the main reasons for discontinuation. Up to date, no standard treatment approach has been proven to resolve this problem. Mifepristone is a synthetic steroid hormone, acting mainly as an inhibitor of the progesterone receptor (PR) thereby preventing the effect of progesterone. Administration of mifepristone has been proven beneficial in numerous fields in reproductive medicine and exerts various effects depending on the dosage and stage of the menstrual cycle at treatment. Administration of low daily doses of mifepristone results in anovulation and endometrial suppression with subsequent amenorrhea. Continuous treatment with PRMs cause endometrial alterations previously thought to be similar to unopposed estrogen exposure, given the PR antagonistic effects of PRMs. These alterations are today recognized as progesterone receptor modulator associated changes (PAEC). They are considered to be benign and dissolves with the cessation of treatment. The molecular alterations resulting in their development are still unknown. The mechanism of action of steroidal hormones on breast tissue remains largely unidentified. Epidemiological studies show a positive correlation between number of menstrual cycle exposure and hormone therapy to the risk of breast cancer. While this increased risk has been believed to be mainly caused by estrogen, a growing body of literature suggest progesterone and progestins to play a central role. PRMs can be used as a tool to study the effects of progesterone and holds potential to prevent breast epithelial cell proliferation.

Aim

The overall aim of this thesis is to explore the effects of the PRM mifepristone on the endometrium and on human breast tissue in premenopausal women. The specific objectives were to assess whether inducing amenorrhea with mifepristone, prior to placement of the LNG-IUS, could reduce the bleeding irregularities during the first months of use. Another objective was to evaluate the endometrial morphology after continuous treatment with mifepristone following insertion of the LNG-IUS, without prior endometrial shedding. Furthermore, we sought to explore how mifepristone alters the transcriptomic landscape in human beast *in vivo* and the epigenetic alterations observed in the breast tissue following PRM treatment.

Materials, methods and results

Study I was a prospective, randomized, placebo controlled, double-blind trial including healthy women with regular menstrual cycles opting for the LNG-IUS 52 mg for contraceptive purposes. Fifty-eight women were randomized whereof 29 to the mifepristone and 29 to the comparator group. Study participants received mifepristone, 50 mg every other

day or a comparator. The pretreatment period with mifepristone was 2 months, followed by the LNG-IUS insertion. Women kept bleeding diaries as per instruction for the pretreatment period and until 6 months after placement of the device. After removing drop outs and exclusions, 19 women in the mifepristone and 19 in the comparator arm contributed to the final analysis. Bleeding diary data were analyzed as rates of bleeding and spotting days (B/S%) per treatment cycle. The results showed a significant reduction of B/S% during the pretreatment period in the mifepristone arm compared to placebo. Following insertion of the device, no statistical difference could be seen between the two groups.

Women in ***Study II*** originated from Study I. Endometrial biopsies were retrieved at baseline, prior to the pretreatment period with mifepristone or the comparator. A second biopsy was retrieved at 3 months following LNG-IUS placement, with the IUS in situ. Nine paired biopsies from the mifepristone and 8 from the comparator group, contributed to the final analysis. The specimens were analyzed by an expert pathologist who was blinded to the treatment. All baseline biopsies where benign. The second biopsies were all benign and showed, as expected, changes due to progestin effect on the endometrium. There was no presence of PAEC.

Participants in ***Study III*** originated from Study I. Core needle breast biopsies were collected at baseline and after 2 months treatment with mifepristone or the comparator. Paired biopsies from 16 women in the mifepristone group contributed to the analysis. The changes on mRNA expression level at baseline compared to after mifepristone treatment were screened using RNA sequencing. Functional annotation and pathway enrichment analysis of the differentially expressed genes (DEGs) revealed genes mainly involved in extracellular matrix (ECM) remodeling.

In ***Study IV***, patient cohorts and databases were used to generate and validate a breast tissue specific epigenetic index. That index was subsequently used to assess breast tissue samples from three clinical trials, including Study I. Based on the results from this specific epigenetic index, PRM treatment could exhibit favorable results in the mammary gland from healthy women as well as women with increased risk for developing breast cancer.

Conclusion

The applied mifepristone treatment regimen could not demonstrate any significant improvement in bleeding disturbances following placement of the LNG-IUS compared to placebo. Continuous treatment with mifepristone and subsequent LNG-IUS insertion without prior endometrial shedding, could represent a safe alternative regarding PAEC endometrial safety. Transcriptomic alterations in the breast after treatment with mifepristone revealed pathways mainly involved in ECM remodeling. Furthermore, epigenetic and genetic alterations in the breast following PRM treatment seem promising and suggestive of further investigations regarding the potential beneficial effects of these compounds in the prevention of breast cancer.

LIST OF SCIENTIFIC PAPERS

I. Papaikonomou K, Kopp Kallner H, Söderdahl F, Gemzell-Danielsson K

Mifepristone treatment prior to insertion of a levonorgestrel releasing intrauterine system for improved bleeding control – a randomized controlled trial

Human Reproduction, 2018 Nov 1;33(11):2002-2009. Erratum in: Hum Reprod. 2019 Jul 8;34(7):1386-1387

II. Papaikonomou K, Frisendahl C, Williams ARW, Gemzell-Danielsson K

Effects of the levonorgestrel intrauterine system on the endometrium after long-term exposure to mifepristone: Secondary outcomes of a randomized controlled trial

European Journal of Obstetrics & Gynecology anf Reproductive Biology, 2020 Sep;252:330-335

III. Papaikonomou K, Boggavarapu NR, von Grothusen C, Lalitkumar PG, Gemzell-Danielsson K

Transcriptome profiling following treatment with the progesterone receptor modulator mifepristone in breast tissue of healthy premenopausal women – secondary outcomes of a randomized controlled trial

Manuscript

IV. Bartlett TE, Evans I, Jones A, Barrett JE, Haran S, Reisel, Papaikonomou K, Jones L, Simões BM, Clarke RB, Evans G, Ghezelayagh TS, Ponandai-Srinivasan S, Boggavarapu NR, Lalitkumar PG, Howell SJ, Risques RA, Flöter-Rådestad A, Dubeau L, Gemzell-Danielsson K and Widschwendter M

Targeting progesterone reduces epigenetic and genetic cancer surrogates in normal breast tissue

Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AR	Androgen Receptor
AUB	Abnormal Uterine Bleeding
cDNA	Complementary DNA
BMI	Body Mass Index
COC	Combined Oral Contraceptive
DNAme	DNA methylation
E	Estrogen
EC	Emergency Contraception
ER	Estrogen Receptor
FDR	False Discovery Rate
FNA	Fine Needle Aspiration
DEG	Differentially Expressed Gene
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EE	Ethinyl Estradiol
GR	Glucocorticoid Receptor
HPA axis	Hypothalamus-pituitary-adrenal axis
LARC	Long Acting Reversible Contraceptive
LH	Luteinizing Hormone
LNG	Levonorgestrel
LNG-IUS	Levonorgestrel-releasing Intrauterine System
MaSC	Mammary Stem Cell
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
NSAID	Non-steroidal Anti-inflammatory Drug
OC	Oral Contraceptive
P	Progesterone
PAEC	PRM Associated Endometrial Changes

PCR	Polymerase Chain Reaction
POP	Progestin Only Pill
PR	Progesterone Receptor
PR-A	Progesterone Receptor isoform A
PR-B	Progesterone Receptor isoform B
PRM	Progesterone Receptor Modulator
RANK	Receptor Activator of Nuclear Factor Kappa-B
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RNA-Seq	RNA Sequencing
RT-PCR	Real-time Polymerase Chain Reaction
SERM	Selective Estrogen Receptor Modulator
TNBC	Triple Negative Breast Cancer
TDLU	Terminal Ductal-Lobular Unit
UPA	Ulipristal Acetate
VTE	Venous thromboembolism
WHO	World Health Organization

1 INTRODUCTION

1.1 PROGESTERONE AND PROGESTERONE RECEPTORS

The progesterone receptor (PR) is a member of a class of proteins, the nuclear receptor superfamily of ligand-activated transcription factors, that regulates the expression of specific genes involved in female reproduction. There are two main forms of nuclear PR (PR-A and PR-B), arising from a single gene, exerting different biological effects in different tissue targets, also depending on their PR-A/PR-B ratio, the tissue content and on the hormonal status of the cell. The two isoforms are structurally similar except that PR-B has 164 N-terminal amino acids which PR-A lacks (1). The vast majority of our understanding regarding the role of the two isoforms derives from mice experiments although unbalanced expression ratio has been observed in endometrial and breast cancer in humans. In mice, PR-A seem mainly to control endometrial proliferation and PR-B breast epithelial proliferation and differentiation (1, 2).

Some additional PR have been described, namely PR-C, -M, -S and -T, but their translation *in vivo* could not be supported (3). Moreover, a membrane-bound PR has been implicated in rapid non-genomic action of progesterone although its physiologic actions remain unclear; its binding capacity to progesterone seems low and in some experiments, there were no evidence of membrane-bound PR activation upon binding to progesterone (2, 4).

The endogenous steroid hormone progesterone is the natural ligand of the PR and it is produced mainly in the ovary but also in the adrenal gland. Progesterone, derives from cholesterol, as the other steroid hormones, and it is a lipophilic molecule with a short half-life of only a few minutes. It is metabolized in the liver and its metabolites are excreted in the urine (5).

Progesterone plays a pivotal role in the female reproduction exerting its action on the uterus, ovary, mammary gland and the hypothalamic-pituitary axis. The main effects of progesterone include control of ovulation facilitating the luteinizing hormone (LH) surge, GnRH pulsatility modulation, endometrial differentiation, control of endometrial receptivity and implantation, maintenance of pregnancy and inhibition of tubal and uterine contractility, prevention of cervical ripening, as well as differentiation of the mammary epithelium (6-8). In addition to its action in the reproductive system, P exerts biological effects in other organ systems, such as the cardiovascular, the central nervous system and bone.

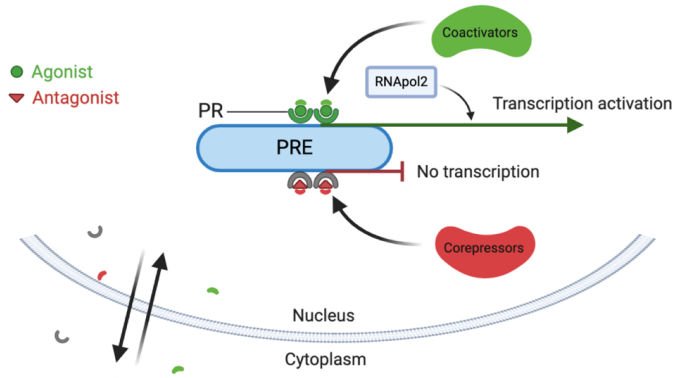

Figure 1. Upon ligand binding, the PR undergoes conformational changes and ultimately dimerization and DNA binding via specific progesterone response elements within target genes. Agonist-bound PR enhances transcriptional activation. Adapted from Chabbert-Buffet, Human Reproduction Update, 2005. Abbreviations: PR, progesterone receptor; PRE, progesterone receptor element; RNApol2, ribonucleic acid polymerase 2.

Since progesterone is proven to be crucial for the normal function of the female reproductive system, it is of no surprise that aberrant progesterone responses are implicated in various benign reproductive disorders such as fibroids, endometriosis, adenomyosis, abnormal uterine bleeding and miscarriage, making the PR an important therapeutic target (2). Numerous PR ligands have been developed displaying a broad spectrum of action ranging from full agonists (progestins), used in oral contraceptives (OCs) and hormone (replacement) therapies, and ligands with mixed activities through to full antagonists later also known as PR modulators (PRMs) (7, 8). Due to the similarities of their steroidal backbone, various PR ligands have the ability to exert a variety of cross-reactivity properties with other steroid receptors, depending on range of selectivity, affinity, their concentration in the tissue and the cellular context.

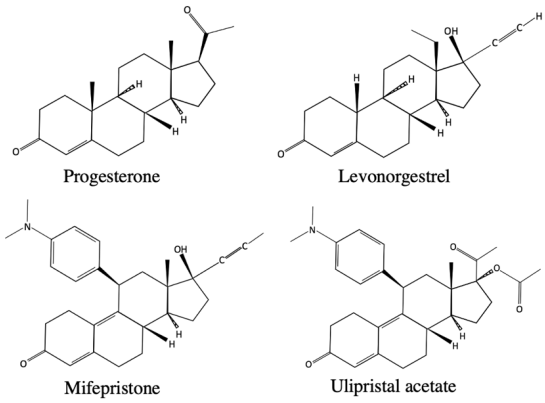

Figure 2. Chemical structures of progesterone (natural ligand of PR), levonorgestrel (progesterone receptor agonist – a progestin), mifepristone and ulipristal acetate (progesterone receptor modulators).

1.2 PROGESTERONE RECEPTOR MODULATORS

Progesterone receptor modulators (PRMs) are synthetic ligands, mainly steroidal, which bind to PR isoforms and display antagonist or mixed agonist/antagonist effects in a tissue specific manner (8). Mifepristone was the first PRM developed, in 1980s. Since then, numerous other PRMs have been synthesized and investigated in trials, mainly in reproductive medicine but also in endocrinology, psychiatry and various types of cancer (1, 9). Up to date, there are two compounds licensed for clinical use in reproductive medicine. Mifepristone for termination of pregnancy and emergency contraception, and ulipristal acetate (UPA) for emergency contraception as well as treatment of uterine fibroids. In addition, mifepristone is licensed in the United States for patients with Cushing's syndrome for control of hyperglycemia secondary to hypercortisolism (10).

There is some inconsistency in the literature regarding the nomenclature of PR ligands that exhibit a variety spectrum of mixed activities. Some authors differentiate and categorize the compounds with profound agonistic properties as antagonists, while utilizing the term selective progesterone modulators (SPRM) for the compounds with more mixed properties; others include all these compounds in the SPRM family. In this thesis, the term PRM will be used and restricted to PR ligands exerting some degree of mixed agonist-antagonist activity to the PR receptor.

Table 1. Clinical trials with PRMs in ClinicalTrials.gov. Trials with status completed, terminated or unknown are not included in this table. Search conducted on 18th of October 2020.

PRM	Name of trial	ClinicalTrials.gov Identifier	Status
Mifepristone	BRCA1/2 and Effect of Mifepristone on the Breast	NCT01898312	Recruiting
	Study of Pembrolizumab and Mifepristone in Patients with Advanced HER2-negative Breast Cancer	NCT03225547	Recruiting
	Abraxane® With or Without Mifepristone for Advanced, Glucocorticoid Receptor-Positive, Triple-Negative Breast Cancer	NCT02788981	Recruiting
	Study of Oral Mifepristone as Salvage Therapy in Patients with Advanced or Metastatic Non-Small Cell Lung Cancer	NCT02642939	Recruiting
	Enzalutamide and Mifepristone in Treating Patients with Metastatic Hormone Resistant Prostate Cancer	NCT02012296	Recruiting
	Efficacy of Mifepristone in Males with Type 2 Diabetes Mellitus	NCT03052400	Recruiting
	Improving access to abortion in the Republic of Georgia	NCT04458558	Recruiting
	Mifepristone for the Prevention of Relapses of Alcohol Drinking	NCT02243709	Recruiting
	Mifepristone and Misoprostol for 2nd Trimester Termination of Pregnancy in Burkina Faso	NCT03269279	Recruiting
	Mail Order Mifepristone Study	NCT03913104	Recruiting
	Assessing medical menstrual regulation in the United states	NCT03972358	Recruiting
	Telemedicine counselling for medical abortion	NCT03461653	Recruiting
	Mifepristone Induction for Fetal Demise (MIFD)	NCT02620904	Active, not recruiting
	Cervical Preparation with Mifepristone Prior to Osmotic Dilators	NCT03714880	Recruiting

Mifepristone	Evaluation of efficacy of two therapeutic strategies for cervical maturation before medical termination	NCT03194126	Recruiting
	Comparison of the Effectiveness of Treatment with Mifepristone and Misoprostol at the Same Time Compared to the Administration of Drugs at a 48-hour Interval for Medical Abortion.	NCT03440866	Active, not recruiting
	Study of Clinic-based Versus Self-use of Medical Abortion Pills (MOC)	NCT03727308	Recruiting
	Clinical Evaluation of Cervical Ripening in the Outpatient Setting	NCT04271722	Recruiting
	Pilot Study of an Ambulatory Medical Abortion Service at 13-18 Weeks of Gestation in Colombia	NCT04063904	Not yet recruiting
	Second Trimester Medical Abortion (RAPM)	NCT04160221	Recruiting
	Medical Termination of II Trimester Pregnancy (PRIMA)	NCT03600857	Recruiting
Ulipristal acetate	Liver Safety Assessment During Ulipristal Acetate Treatment for Uterine Fibroids (LISA)	NCT04004884	Recruiting
	Observation of Long-term Effects on Endometrium and Uterine Fibroids in Women with Ulipristal Acetate Therapy	NCT03972917	Recruiting
	Non-interventional Study to Evaluate Long Term Safety, Prescription Management Patterns of Esmya in a Long-Term Setting (Premium)	NCT02748460	Active, not recruiting
	Breast Cancer – Anti-Progestin Prevention Study 1 (BC-APPS1)	NCT02408770	Active, not recruiting
	Ovarian Function with ENG Implant and UPA Use	NCT04291001	Not yet recruiting
	Study Comparing Emergency Contraception Effectiveness in Women Who Weight ≥ 80 kg	NCT03537768	Recruiting
	Esmya Versus Surgery Before IVF/ICSI	NCT04028986	Recruiting
	Effectiveness of Orally Dosed Emergency Contraception in Obese Women – UPA (UPA-Obesity)	NCT02859337	Recruiting
Onapristone	A Study of Onapristone ER in Low Grade Serous Ovarian Cancer and Other Progesterone Receptor Positive Gynecologic Cancers	NCT03909152	Recruiting
	Onapristone as Preoperative Treatment for Postmenopausal Women with Hormone Receptor + and HER2- Breast Cancer (ONAWA)	NCT04142892	Not yet recruiting
Telapristone acetate	Transdermal or Oral Telapristone Acetate in Treating Patients Undergoing Mastectomy	NCT02314156	Active, not recruiting
	Oral CDB-4124 vs. Placebo in Stage I-II Primary Breast Cancer	NCT01800422	Active, not recruiting
Vilaprisan	Assess Safety and Efficacy of Vilaprisan in Subjects with Uterine Fibroids (ASTEROID 5)	NCT03240523	Active, not recruiting
	Assess Safety and Efficacy of Vilaprisan in Subjects with Uterine Fibroids (ASTEROID 6)	NCT03194646	Active, not recruiting
	Assess Safety and Efficacy of Vilaprisan in Subjects with Endometriosis (VILLENDO)	NCT03573336	Active, not recruiting
	Assess Safety and Efficacy of Vilaprisan in Subjects with Uterine Fibroids (ASTEROID 4)	NCT03400956	Active, not recruiting
	Assess Safety and Efficacy of Vilaprisan in Subjects with Uterine Fibroids (ASTEROID 3)	NCT03400943	Active, not recruiting
	A Study to Assess the Safety and Efficacy of Vilaprisan in Japanese Subjects with Uterine Fibroids and Heavy Menstrual Bleeding (ASTEROID 8)	NCT03476928	Active, not recruiting

Abbreviations: PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; ENG, etonogestrel; UPA, ulipristal acetate; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ER, extended release; CDB-4124, telapristone acetate.

1.2.1 Mifepristone

Mifepristone (RU 486) is the most studied PRM acting mainly as a PR antagonist (8). Its effect, as for other PRMs, is well known to be dependent on the dose and dosage given, the stage of the menstrual cycle and PR expression in the target tissue at time of administration (8, 11). Mifepristone in a single dose, administered immediately after ovulation have been shown to inhibit endometrial development and receptivity. When given in the mid luteal phase of the menstrual cycle it induces uterine bleeding in a dose-dependent manner (12). Low continuous doses cause anovulation and a high fraction of amenorrhea with decreased endometrial proliferation, also in a dose dependent manner (13, 14).

The binding affinity of mifepristone for the PR is about 2.5-5 times higher than that of the natural ligand progesterone (15). Given this ability and its antagonistic properties, it is of no surprise that mifepristone, as well as other PRMs, have been studied for numerous gynecologic indications. Mifepristone is currently approved as a single dose treatment for clinical use in abortion care in combination with a prostaglandin analogue, for cervical ripening prior to surgical abortion (16), induction of intrauterine fetal death (17) and emergency contraception (18). Long-term administration of mifepristone is not approved but nevertheless comprehensively studied in various clinical trials demonstrating its potential use for contraception (19), medical treatment of uterine fibroids (20, 21), endometriosis (22), improved bleeding patterns in women using progesterone only contraceptives (23, 24) and optimizing in vitro fertilization treatment (25). Furthermore, the potential anti-tumor properties of mifepristone have been assessed in women with metastatic breast and ovarian cancer (26) and in our previous study from healthy human breast tissue in vivo, continuous administration of mifepristone suggested a protective effect on the human mammary gland (27).

Mifepristone also binds to the glucocorticoid receptor (GR) in a dose dependent manner and with antiglucocorticoid effects occurring at continuous doses of 50 mg and single doses of >200 mg of the drug (28, 29). However, antiprogestin activity is apparent at much lower doses (29). The antiglucocorticoid effect of mifepristone has been explored in clinical trials for among others psychotic depression and to regulate hyperglycemia secondary to hypercortisolism in Cushing´s syndrome. For the latter indication, it is approved in the US since 2012 (Korlym®) with a recommended starting dosage of 300 mg/day (10). Mifepristone also binds to the androgen receptor (AR) to a lesser extent, but not to the mineralocorticoid or estrogen receptor (ER) (15).

Mifepristone exhibits a quick oral absorption with peak serum concentration after 1-2 hours. In humans, mifepristone binds to a_1-acid glycoprotein which regulates its serum kinetics and explains the low metabolic clearance rate. At intake up to 100 mg, distribution of mifepristone is linear, while at doses >100 mg a more rapid metabolization is seen. Mifepristone is further metabolized in the liver, by cytochrome P-450 enzyme CYP3A4, to mainly three metabolites, all maintaining considerable affinity to the PR. The elimination half-life of mifepristone is 24-48 hours measured with high performance liquid

chromatography, while other methods of measurement report a longer half-time of 54-90 hours probably due to the presence of its metabolites. (30).

1.3 LEVONORGESTREL INTRAUTERINE SYSTEM

Levonorgestrel is a potent progestin acting as a full agonist of the PR. The levonorgestrel intrauterine system (LNG-IUS), with a 52 mg LNG reservoir, is a safe and highly effective long-acting reversible contraceptive (LARC) (31). The initial LNG release rate is approximately 20 μg per day which gradually decreases to 10 μg per day after 5 years, with plasma hormonal levels much lower compared to other non-intrauterine progestin-only preparations (32). Hence, the primary contraceptive effect of the device is not the suppression of ovulation which needs higher serum levels to be achieved; LNG-IUS thickens the cervical mucus which impairs sperm migration and function (33, 34). Furthermore, LNG-IUS represses the endometrium, reduces menstrual blood flow and approximately 50% of women become amenorrhoic during its use (31, 32, 35). In addition, it represents one of the best accepted and most cost-effective contraceptive methods available and also provides numerous non-contraceptive benefits in women with heavy menstrual bleeding, adenomyosis, endometriosis, but also protection and treatment of endometrial hyperplasia and possibly early endometrial cancer (32). The characteristic endometrial morphology during use of the LNG-IUS consists of decidualized stroma, marked endometrial atrophy and inactivity of endometrial glands as well as alterations in vascular morphology such as suppression of spiral artery formation and large, thin walled, dilated vessels (36, 37).

Despite studies showing that the device can reduce overall menstrual blood loss by up to 97% after the first year of use (38), 25-62% of women experience initial unscheduled vaginal bleeding after the placement of the device, which constitutes one of the main reasons for the premature cessation of treatment (39, 40). Quite recently, two new lower dosed intrauterine systems with 19.5 and 13.5 mg levonorgestrel respectively in their reservoir, have been approved for contraceptive use.

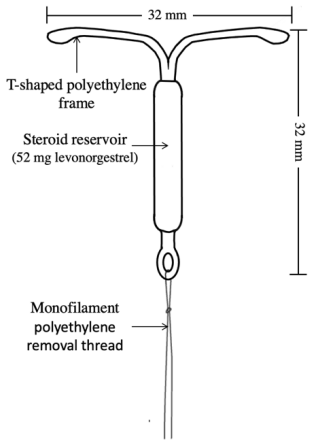

Figure 3. Levonorgestrel releasing intrauterine system with a 52 mg hormone reservoir.

1.4 BLEEDING IRREGULARITIES IN PROGESTIN ONLY CONTRACEPTIVE USERS

Progestin-only methods of contraception include oral preparations, intrauterine systems, injectables, subdermal implants and vaginal rings. A universal major drawback with the continuous progestin-only preparations is the bleeding irregularities induced in a significant number of users even though the extent of the problem is likely influenced by the dose and type of the progestin, the mode of delivery and resulting effects on follicular development and ovulation as well as their specific endometrial affinity/effects (41). Nevertheless, undesired bleeding patterns constitute one of the main reasons for discontinuation of these methods (42). The underlying mechanisms responsible for the bleeding irregularities remain largely unknown and seem to be multifactorial with evidence pointing towards superficial blood vessel fragility and alterations in their density, but also local changes in endometrial steroid response, structural integrity, tissue perfusion and local angiogenic factors (42, 43). A rise in cytokines and prostaglandins have been shown in the endometrium during initial use of the LNG-IUS (36). In addition, alterations in expression of vascular endothelial growth factors (VEGF) as well as their receptors were demonstrated in women with LNG-IUS and bleeding disturbances initiated after a problem-free period of use, suggesting fragile and dysfunctional blood vessel formation (44). It could though be hypothesized that initial bleeding irregularities with LNG-IUS and bleeding irregularities/disturbances that appear after an untroubled period of use, are due to different underlying mechanisms and necessitate therefore different treatment approaches (45).

Several pharmacological interventions have been tested with the intention to minimize the bleeding irregularities in progestin-only users and consequently increase patient satisfaction and reduce premature discontinuation of treatment. These interventions include non-steroidal anti-inflammatory drugs (NSAIDs), estrogens, progestins, vitamins, antifibrinolytic agents, matrix metalloproteinase inhibitors (doxycycline), the selective estrogen receptor modulator (SERM) tamoxifen and PRMs, either in single or combined regimens (41, 42). Despite these efforts there is up to date no effective treatment resolving the underlying problem in the long-term (42).

Recently a new progestin-only pill (POP) 24/4 regimen has been introduced for drospirenone mid dosed pills, with the aim to induce more regular withdrawal bleedings. Despite improved bleeding pattern compared with continuous POPs, irregular and unpredictable bleeding is still common (46). Apart from the addition of a combined hormonal method there is so far, no cure.

1.5 PROGESTERONE RECEPTOR MODULATORS AND CLINICAL APPLICATIONS IN REPRODUCTIVE MEDICINE

1.5.1 Medical abortion

Mifepristone is the only PRM approved for termination of pregnancy (15). It is licensed as a single dose treatment followed by misoprostol or gameprost, prostaglandin E1 analogues, for

medical abortion in over 60 countries worldwide (1, 15). The combination regimen of mifepristone and misoprostol is included in the List of Essential Medicines by the World Health Organization (WHO).

1.5.2 Emergency contraception

Emergency contraception (EC) is defined as any method utilized following intercourse to prevent pregnancy. It is an important strategy to prevent unintended pregnancy. Several pharmacological approaches have been described and utilized; initially administration of high doses of combined oral contraceptives (COC) - LNG/ethinyl estradiol (EE), the Yuzpe regimen, followed by LNG-only administration and later PRMs in a single dose regimen (47, 48). In contrast to LNG-EC with a narrower window of action up to the start of increase in LH, PRMs inhibit the LH surge even after its onset. PRMs block follicular rupture likely by direct inhibition of follicular PR-depended pathways (49). PRMs are associated with fewer pregnancies than LNG-EC, with comparable side effects (18) when used up to 120 hours after intercourse (48). The mechanism of the antiovulatory effect of PRMs seems to be multifactorial and complex involving effects on the hypothalamic pituitary axis, the preovulatory increase in progesterone but also direct inhibition of follicular development and rupture as suggested recently for UPA (49, 50). Mifepristone was the first PRM to be used for EC purposes (51) followed by UPA (48). Despite mifepristone's proven efficacy as an EC, it is today licensed on this indication only in China, Vietnam, Armenia, Moldova, Russia and Ukraine (10 mg, 12,5 mg, 25 mg and 50 mg respectively) while UPA is licensed in approximately 80 countries worldwide (30 mg) (52).

1.5.3 Contraception

Existing hormonal contraceptive methods include COC (estrogens plus a progestin), which are associated with increased risk of venous thromboembolism (VTE) but a very stable bleeding pattern (at least with EE), and progestin-only preparations which are not associated with an increased risk of VTE but are reported to have discontinuation rates up to 30% due to irregular and unpredictable bleeding patterns (53). Both methods are associated with suppression of ovulation in addition to reduced dysmenorrhea and blood flow. Since PRMs are proven to block follicular development and ovulation, and/or inhibit endometrial receptivity, they were very early predicted as potential novel long-term estrogen-free contraceptives with favorable bleeding patterns (54). A number of studies have explored these potential contraceptive effects of PRMs using different compounds, doses, routes of administration and regimens but up to date, no PRM is approved for regular contraception.

A proof of concept study showed the potential of mifepristone as a regular contraceptive method. A once a month administration of 200mg of mifepristone immediately postovulatory on LH+2, resulted in a non-receptive endometrium that prevented implantation. Since ovulation was not affected, serum estrogen and progesterone levels remained unaltered and menstrual bleeding remained cyclic and regular (55). In contrast, continuous low doses interfering with ovulation results in amenorrhea in a dose dependent manner (19, 22).

The threshold for daily oral dose of mifepristone capable of inhibition of ovulation is 2 mg (19, 56) and that of UPA is 5-10 mg (57). Despite their effect on ovulation and follicular suppression, PRMs are proven not to be associated with hypoestrogenism during treatment (56-58).

In a study exploring the contraceptive potential of lower continuous doses of mifepristone, no pregnancies occurred after 200 months of exposure to 2 mg or 5 mg daily in 50 women (19). Another study reported no pregnancies after 456 months of exposure to either 25 or 50 mg of mifepristone administered weekly, even though the weekly dose of 25 mg did not exhibit a consistent inhibition of ovulation but interfered with endometrial development (59). These data are in line with another study assessing the effect of continuous daily mifepristone dose of 1 mg, 5 mg or 10 mg. Even though the duration of the study was short (30 days), the group reported an inconsistent suppression of ovulation with 1 mg/day while treatment with 5 mg or 10 mg was associated with a coherent ovulation blockage. However, endometrial maturation was disturbed by all doses which implicates a differential effect threshold of the follicle and the endometrium by mifepristone (58).

Population Council conducted a dose finding study with a contraceptive vaginal ring delivering UPA and presented promising results (60). In a proof of principle study in rhesus macaques, a UPA impregnated intrauterine device induced endometrial atrophy and amenorrhea (61). The effects of PRMs delivered through an intrauterine device was also investigated in a small human study (62).

Based on available data from various studies, PRMs hold the potential to be utilized as estrogen-free long-term contraceptives without estrogen deprivation and with stable bleeding patterns.

1.5.4 Treatment of abnormal uterine bleeding

Abnormal uterine bleeding (AUB) is a common condition that can affect up to 25% of women during their reproductive life (63). Common causes of AUB include uterine fibroids and endometrial polyps, adenomyosis, malignancy and premalignant lesions as well as coagulopathies, ovulatory dysfunction and iatrogenic causes such as various types of progestin preparations for contraceptive purposes. Bleeding due to iatrogenic causes is commonly termed ‘breakthrough bleeding’ (64). While some of the above mentioned structural causes of AUB require surgical intervention, many are hormonally mediated and can therefore be subjected to medical treatments. PRMs hold great potential for medical treatment of these conditions.

Despite the differences in various PRMs, a common clinical feature in all is the large proportion of women who become amenorrhoic during continuous treatment, with the effect depending on dose and treatment duration. It seems that amenorrhea induced by PRMs is not only dependent on ovulation blockage (19). Some investigations suggest as contributing factors, downregulation of stromal growth factors (65), direct effect on endometrial blood vessels and in women with uterine leiomyomas, a moderate reduction in uterine artery blood

flow (1, 66). Furthermore, some studies have shown an amelioration of symptoms in women experiencing breakthrough bleeding following treatment with progestin only preparations as discussed in detail later in this thesis. In summary, the mechanism of amenorrhea following treatment with these compounds is poorly understood and more *in vivo* human studies are needed.

1.5.5 Uterine leiomyomas

High concentrations of ER and PR are expressed in leiomyoma cells relative to the adjacent myometrium and exposure to estrogen and progesterone promote leiomyoma growth (8). Therefore, treatment with PRMs have been evaluated for reduction of myoma size and as treatment of associated excess bleeding without estrogen deprivation. Mifepristone was the first PRM evaluated in clinical trials and later more studies with other PRMs also revealed a dramatic reduction in excess uterine bleeding and reduction in fibroid volume (20, 28, 67). Studies with UPA showed similar effects which resulted in the authorization of UPA as a treatment for moderate to severe symptoms of uterine fibroids (68, 69). The mechanisms contributing to leiomyoma growth reduction are not well elucidated. However, ample evidence from in vitro studies have shown that PRM induce apoptosis in leiomyomas through related pathways (70). In addition, PRMs can suppress collagen synthesis through modulation of extracellular matrix (ECM) enzymes like metalloproteinases (MMPs) (71). Long-term treatment with PRMs induce specific endometrial changes, now named 'PRM associated endometrial changes' (PAEC). Due to these changes and the lack of evidence regarding long term endometrial safety in their presence, the initial authorization was for preoperative treatment only (3 months). Following studies with UPA were designed to assess endometrial safety with regards to presence of PAEC which eventually led to authorization for intermittent long-term therapy but with endometrial shedding between treatment courses of three months (72, 73). It should be noted that the indication for the latter got suspended in March 2020 during ongoing review of liver injury risk and as per November 2020 restricted. Research on the use of other PRMs for the treatment of uterine fibroids are on hold. Currently, the Committee for Medicinal Products for Human Use of the European Medicines Agency has recommended the European Commission to re-approve UPA for intermittent use for moderate to severe symptoms of fibroids but with stricter indications. This issue is further discussed in section 5.

1.5.6 Endometriosis

Endometriosis is a common disease with a pathogenic complexity that still remains unclear. It is defined as the presence of endometrial glands and stroma in ectopic locations and it is mainly considered an estrogen-dependent condition. The medical treatment of the disease is currently dominated by agents that suppress the pituitary-ovarian axis and induce endometrial atrophy. Common medical treatments for endometriosis includes COCs, progestins or gonadotropin-releasing hormone agonists. The latter induce estrogen depletion with consequent adverse effects of vasomotor symptoms, urogenital tract symptoms and bone mass loss (65).

Considering PRMs in endometriosis therapy, concerns were raised regarding the difficulty to predict the effect of PRMs since the ectopic endometriotic tissue presents different steroid metabolism and enzyme physiology compared to the eutopic endometrium (8). Nevertheless, PRMs have shown a reduction of endometriotic lesions in animal models, and small studies in women utilizing 50 mg mifepristone daily for six months, indicated significant beneficial effects on symptoms and extent of the disease (22, 65). Several explanations have been offered to account for these observations including anovulation, suppression of menstruation, inhibition of endometrial proliferation, apoptotic effects and suppression of endometrial prostaglandin production with subsequent pain relief (8, 65). Even though PRMs appear to be promising for the treatment of endometriosis with the benefit of avoiding hypoestrogenism (56, 58), more studies are clearly needed for this indication. This will also include long-term follow up to define the specific role of PAEC that may develop in the ectopic lesions as observed in the eutopic endometrium described below.

1.6 PROGESTERONE RECEPTOR MODULATOR ASSOCIATED ENDOMETRIAL CHANGES (PAEC)

Since various PRMs have mixed or full antagonistic effect on the PR, concerns were raised on whether long-term administration of this group of compounds could induce endometrial alterations as in unopposed estrogen exposure. Lack of endometrial progesterone protection would result in actively proliferative appearances as seen in disordered proliferative endometrium or endometrial hyperplasia (74). Although early studies reported morphological endometrial changes described as glandular hyperplasia after long-term administration of PRMs, these changes were later, in 2008, widely recognized as PAEC – a distinct histological entity (75).

Trials have shown PAEC rates of close to 60% following repeated courses of UPA (72). The common and most prominent histologic features of PAEC include cystic dilatation of endometrial glands, irregular architecture lined by inactive gland cells and compact, non-decidualized stroma. Interestingly, none of these features are unique when occurring separately, but their combination does not occur in physiological states even though they have rarely been described in patients not treated with PRMs (74). While there is evidence that all family members of PRMs induce these histological changes, the characteristic features are varying, depending on the PRM used (37). Further features include vascular changes such as prominent but delicate anastomosing capillary networks but also thick walled vessels (75). PAEC have been proven to be reversible to a great extent following cessation of therapy and shedding of the endometrium. Therefore, treatment with UPA to decrease uterine fibroid volume was initially restricted to a 3-month treatment (68, 69). Since the development of PAEC was initially thought to be the consequence of unopposed estrogen action, a later study was designed to separate each 3-month course of UPA by a sequence of progestin (noretisterone acetate) to limit the development of PAEC. However, this regimen could not demonstrate prevention of occurrence, or induce faster resolution of PAEC (72), resulting in

the clinical treatment recommendations where intermittent courses with UPA were separated by two menstrual cycles (48, 73).

While PAEC are now well described, appear reversible and are considered to be benign due to the lack of cytological atypia (75), the mechanism by which they develop and their significance is still unknown (2, 74). More evidence is required to define if these changes persist over time, dissolve or progress after PRM exposure and why PAEC do not develop in all women (75).

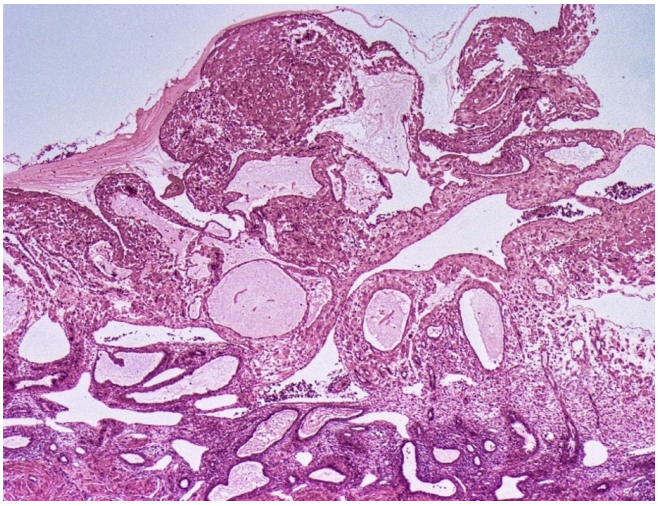

Figure 4. Endometrial biopsy (not from our study) to illustrate PRM-associated endometrial changes (PAEC). Endometrial glands show cystic dilatation, some with distortion of architecture. Glandular epithelial lining is inactive but not atrophic, and there is non-physiological secretion. The stroma is compact and non-decidualized. (Mifepristone 5 mg daily, 12 weeks). Image courtesy: Professor Alistair Williams.

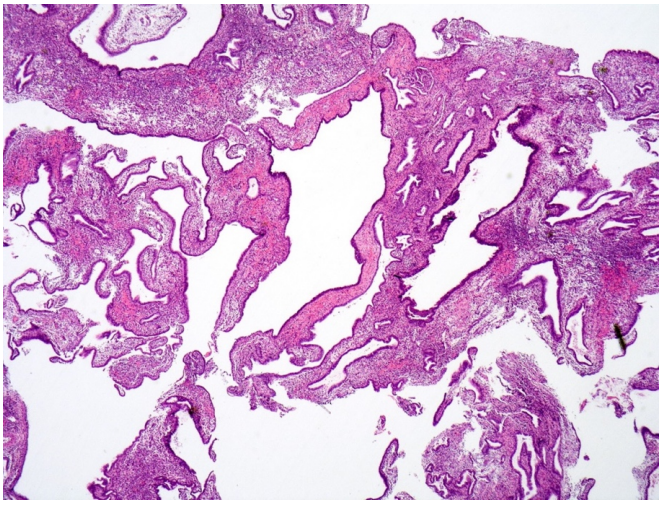

Figure 5. Endometrium showing progestin effect (not from our study). Some glands show cystic dilatation, and glandular epithelium is flattened and atrophic. The stroma shows confluent decidual change. Image courtesy: Professor Alistair Williams.

1.7 BREAST

1.7.1 Development, homeostasis and risk factors

Breast development starts during fetal life and undergoes dramatic changes during women's life, with the major changes occurring at puberty, pregnancy, lactation and in the postpartum period. The breast tissue is also susceptible to proliferation, differentiation, and involution (regression) with every menstrual cycle, orchestrated by fluctuations of the ovarian hormones estrogen and progesterone. Later in life, during the time of menopause, the breast undergoes involution, where epithelium is gradually replaced by adipose tissue. Full differentiation and maturation of the breast tissue is considered complete after the first full term pregnancy (76).

1. Chest wall
2. Pectoralis muscles
3. Lobules
4. Nipple
5. Areola
6. Milk ducts
7. Fatty tissue
8. Skin

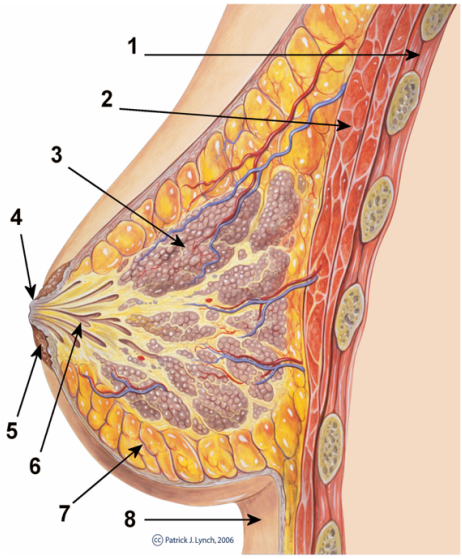

Figure 6. Schematic illustration of the female breast anatomy. Image courtesy: Wikimedia Commons, illustration by ©Patrick J. Lynch.

The mammary gland consists mainly of epithelium and stroma (inter- and intralobular), the latter comprised of adipose tissue, fibroblasts, extracellular matrix and immune as well as hematopoietic cells. Development and homeostasis of the breast are coordinated by complex reciprocal interactions between the different cell lineages and their microenvironment, accompanied by growth factors and cytokines (77).The granular tissue corresponds to a minor compartment in the breast and consists of 15-25 ducts, each giving rise to a lobe arranged in a branching system which ends in several terminal ductal-lobular units (TDLUs). The TDLUs are surrounded by the intralobular stroma and are the functional units of the mammary gland but also the primary source of most breast cancer (78).

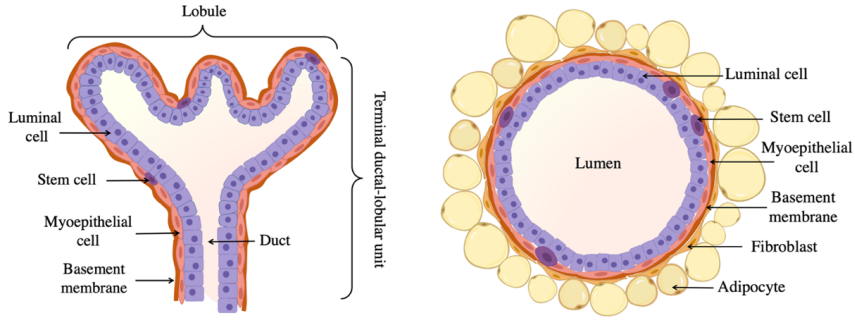

Figure 7. Schematic illustration of the terminal ductal-lobular unit (TDLU). Adapted from Dimri, Breast Cancer Research, 2005.

Initially, breast cancer was considered a cancer form of nuns, who may have led a healthy life but had an uncommon reproductive behavior. Epidemiological studies revealed a number of risk factors which solely or combined, seem to increase breast cancer susceptibility. The majority of those are positively correlated to the number of menstrual cycles a woman undergoes during her lifetime and thus the cyclic exposure time of the breast to ovarian hormones (79). However, the biological mechanisms of breast cancer susceptibility linked to the fluctuation of the ovarian hormones are not well understood. The mid-luteal phase of the menstrual cycle, where levels of estrogen reach a smaller peak and progesterone levels are high, is the proliferative phase in the mammary gland whilst during the late luteal phase the breast epithelium undergoes apoptosis and tissue remodeling (77). Even though the role of progesterone in the breast has been debated for many years, a growing body of literature suggests that the mitotic and subsequent apoptotic activity of the glandular tissue is associated with progesterone's rise and withdrawal during one menstrual cycle under the influence of estrogen. There is also a positive correlation between levels of serum progesterone and mitotic activity in the breast reflected by a higher level of mitotic activity assessed by cellular expression levels of the proliferation marker Ki67 (80, 81). Mitotic activity increases the risk of genomic instability, due to the risk of random genetic errors during DNA replication, and consequently the risk of tumorigenesis. In addition, estrogen induces proliferative effects in the breast by direct binding to the ER but is also responsible for upregulation of the PR during the luteal phase (77). Altogether, data supports that progesterone exerts proliferative effects in the mammary epithelium which is in sharp contrast to its effect on the endometrium, where it exhibits antiproliferative action.

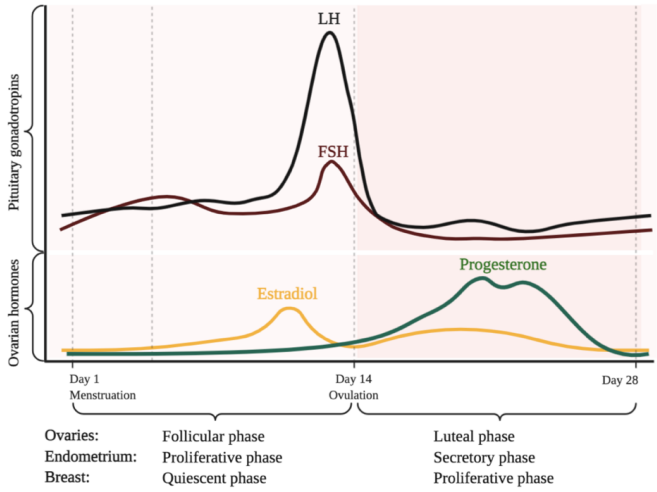

Figure 8. Ovarian and pituitary hormone fluctuations during one menstrual cycle together with tissue changes in the ovaries, uterus and breast. Notably, with regards to changes in tissue structure, the proliferative phase during the follicular phase corresponds to the uterus (endometrium) whilst the proliferative phase in the luteal phase of the cycle corresponds to the breast. Modified from Brisken, Nature Reviews, 2013.

Large observational studies conducted in postmenopausal women have linked use of menopausal hormone therapy to an increased risk of breast cancer when estrogen is combined with progestins (82, 83) and in particular when the progestin intake was on a daily basis rather than intermittently (84). Similarly, the effect of hormonal contraception in premenopausal women seems to slightly increase the risk of breast cancer in current users (85, 86).

Other factors associated with breast cancer risk include age, extensive breast density visible on mammogram, heredity and other genetic factors, personal history of breast pathologies, socioeconomic group and geographical location, life style factors and environmental exposures (86). Another parameter consistently associated with increased breast cancer risk is obesity. Interestingly, while population based studies demonstrate a positive association between obesity and postmenopausal breast cancer, the association in premenopausal women seems to be inverse, with fewer ovulatory cycles suggested as a plausible explanation (86, 87).

Despite decades of research and rapid advances in molecular diagnostics, breast cancer is still the most frequent female malignancy and a major health concern worldwide. It is estimated that approximately 276.480 new cases of invasive breast cancer and 48.530 new cases of adenocarcinoma in situ will be diagnosed in the US year 2020. The incidence rates show a slight increase in recent years (by approximately 0.3%/year) (88). Our efforts to treat but most importantly prevent breast cancer, are significantly hampered by our knowledge gaps in biology and developmental genetics of the healthy breast.

Table 2. Risk factors of breast cancer. Adapted from Veronesi, Lancet, 2005.

	Relative risk	High-risk group
Age	> 10	Elderly
Geographical location	5	Developed countries
Breast density	> 5	Extensive dense breast
Age at menarche	3	Before age 11
Age at menopause	2	After age 54
Age at first full pregnancy	3	First child after age 40
Family history	≥ 2	BC in first-degree relative
Previous benign breast disease	4-5	Atypical hyperplasia
Cancer in other breast	> 4	Previous breast cancer
Socioeconomic group	2	High and low socioeconomic status
BMI premenopause BMI postmenopause	0.7 2	High BMI High BMI
Alcohol consumption	1.07	7 % increase with every daily drink
Exposure to ionising radiation	3	Abnormal exposure to young girls after age 10
Breastfeeding and parity	RR falls by 4.3 % for every 12 months of breastfeeding in addition to a 7 % reduction for every birth	Women who do not breastfeed
Oral contraceptives MHT	1.2 1.66	Current users Current users

BC=breast cancer, BMI=body mass index, RR=relative risk, MHT=menopausal hormone therapy.

1.7.2 PR expression, distribution and paracrine mechanisms

The majority of our knowledge on how different factors of interest impact the breast tissue, have been assessed by markers of proliferation or apoptosis, evaluated by immunohistochemistry or immunocytochemistry. Although these methods have been useful, they cannot delineate the complex interplay within the mammary gland. The steroid hormone receptor expression and distribution in the normal breast have therefore been studied. Normal breast of premenopausal women seems to exhibit a sparse overall distribution of ER and PR, with PR detected in about 20% of luminal cells (89). There is though, a great heterogeneity in the distribution of receptor staining even within the same tissue sample, depending on the examined section, adding even more to the complexity of breast tissue assessment (90). Nevertheless, immunohistochemical staining in cryostat or paraffin sections from women

biopsied due to mainly fibroadenomas, showed staining predominantly in the TDLU and while the proportion of PR positive cells was unaffected with OC pill use, the intensity of PR detection increased (90). Furthermore, not only the ER but also the proportion of PR expression was significantly decreased in the breast tissue of healthy women treated with the SERM tamoxifen (91). With more advanced technologies, some studies could show a reduced expression level of PR mRNA transcripts in parous women compared to nulliparous, a relation that have been postulated to contribute to the protective effect of full-term pregnancy regarding breast cancer risk (92).

As previously discussed, there are mainly two isoforms of PR, namely PR-A and PR-B. While animal and in vitro data have demonstrated different actions of the two isoforms in various developmental stages of the mammary gland, the *in vivo* actions in humans still remain unclear. In addition, concerns have been raised regarding our ability to reliably distinguish the two isoforms from each other. While the differentiation between the two isoforms seems to be consistent by western blot due to their difference in size, their similarity in structure have made it difficult to distinguish them by immunohistochemical methods. The antibodies used have been proven not be as selective as anticipated (93).

Recently, studies in mice have shown that PR activation of luminal breast epithelial cells drives proliferation in adjacent PR negative cells through paracrine mechanisms, with receptor activator of nuclear factor kappa-B ligand (RANKL) being an important paracrine mediator (94). This has gained immediate interest, not the least due to the existence of an antibody already in clinical use (denosumab) licensed for treatment of patients with different bone disorders. Denosumab binds to RANKL and inhibits its downstream receptor activation. A small study reported detection of RANKL expression, assessed by immunohistochemistry, in reduction mammoplasty specimens in women who displayed high serum progesterone levels at the time of the surgery. In the same study, expression of the RANKL protein could not be detected in women with low serum progesterone levels (95). Larger studies are needed to prove this pathway to be consistently involved in humans and breast carcinogenesis. This could pave the way for new treatment modalities in breast cancer patients as well as new risk reduction strategies not least in women with high breast cancer susceptibility.

1.7.3 Mifepristone effects in the breast

In the clinical setting, apart from the histological classification and grade of breast cancers, large genomic analyses have helped to introduce the molecular classification of these malignancies (96). This molecular classification is today mainly based on expression levels of ER, PR and HER2 and divided into 4 main subtypes that guide therapy and predict prognosis (96, 97). Even if this classification provides a very important framework, it is far from perfect. New information is constantly being added with the help of high-throughput technologies. In addition, expression of ER and PR is measured by immunohistochemistry. This per se, introduce errors in the assessment. It is a semi-quantitative method using a low cut off for positive scoring (1-10%) (98) and not all antibodies detect steroid receptor isoforms equally (97). It has further been suggested that loss of protein expression could

merely reflect its rapid degradation and that loss of its expression is difficult to interpret when mRNA is present or not measured in each and every patient (97).

The epidemiological studies linking ovarian steroid hormones to increased breast cancer risk provided the rationale for a series of approaches investigating the potential beneficial effects of ER and PR antagonism. The first study assessing the effect of mifepristone was conducted in breast cancer cell lines and the group reported a dose dependent growth inhibition of the PR+ cell lines (99). Thereafter, a number of *in vitro* studies demonstrated similar effects, with outcomes depending on the dose, the cancer cell lines used and the expression levels of the PR (26). Studies in rodents have also shown inhibition of growth rates of mammary tumors, and other PRMs have exerted similar effects, in particular those with marked PR antagonistic activity (26, 100). In small studies including patients with metastatic breast cancer and failed third- or fourth-line endocrine therapy, mifepristone has shown partial responses and it has been suggested that mifepristone may be useful in combined therapies in this patient group (100).

In terms of breast cancer prevention, there is only one previous study examining the effects of mifepristone on the human healthy breast tissue *in vivo,* showing an inhibition of breast epithelial proliferation and hence a possible protective effect (27). This potential protective effect of PRMs in the breast is of great interest in addition to their other therapeutic properties in particular when used long term in current or future indications.

1.8 BRCA

The vast majority of breast and ovarian cancer cases are sporadic but approximately 7-10% of these malignancies are hereditary and estimated to be due to breast cancer susceptibility genes. BRCA1 and BRCA2 are the two major susceptibility genes and mutations in these tumor suppressor genes account for a life time risk of developing breast cancer by 54-85% and 45% respectively (101). There is no single management strategy in reducing the risk of breast and ovarian cancer for BRCA mutation carriers and hence various risk reducing strategies such as surveillance, risk reducing surgery and chemoprevention are utilized, making the clinical management of these individuals very complex and challenging (102, 103).

Until to date, we have no answers regarding the organ-specific caner penetrance of these mutations (97). Quite recently, a study conducted in BRCA1/2 carriers, reported 121% and 33% higher progesterone and estradiol serum levels respectively, in the luteal phase compared to women with no mutations (104). These higher exposures may be a possible link to the increased breast cancer susceptibility in the mutation carriers. In an animal model of BRCA1 mutation utilizing BRCA1/p53-deficient mice, treatment with mifepristone prevented mammary tumor development (105). As described, we have previously shown an antiproliferative effect of low dose mifepristone in human breast tissue *in vivo* (27). Thus, a protective effect of PRMs in breast tissue could be hypothesized providing an additional plausible non-invasive risk-reduction strategy in women with increased risk of breast cancer.

2 AIM AND OBJECTIVES

The overall aim of this thesis is to explore the effects of the progesterone receptor modulator mifepristone on the endometrium and on human breast tissue in premenopausal women.

The specific objectives of each study were as follows:

Study I: To assess the effect of pretreatment with mifepristone on the bleeding pattern after placement of the LNG-IUS for contraceptive purposes.

Study II: To evaluate the endometrial morphology including presence of PAEC in mifepristone primed endometrium followed by placement of the LNG-IUS with no prior endometrial shedding.

Study III: To explore the molecular responses associated with progesterone receptor antagonism in breast tissue *in vivo* of healthy premenopausal women.

Study IV: To investigate the effect of PRMs on genetic and epigenetic surrogate markers for the development of breast cancer.

3 MATERIALS AND METHODS

A comprehensive overview of materials and methods are explained in this section. More details are provided in Study I-IV articles and manuscripts.

3.1 TABULATED OVERVIEW OF STUDIES

Table 3. Overview of study designs, participants, methods and statistics.

Study	Design and participants	Outcome	Participants contributing to final analysis	Method	Data analysis
I Effect on bleeding pattern following treatment with continuous low dose mifepristone prior to LNG-IUS insertion	Randomized double-blind placebo controlled trial Healthy women opting for a LNG-IUS for contraception, age 18-43	Rate of bleeding and spotting days reported during 3 months after the LNG-IUS placement	Mifepristone n=19 Comparator n=19	Daily diary of vaginal bleeding and adverse effects Transvaginal ultrasonography + blood samples for screening and safety	Descriptive statistics. Two group comparison with Mann-Whitney U test (non-parametric). Per protocol analysis.
II Endometrial evaluation after continuous treatment with mifepristone and immediate placement of a LNG-IUS	Secondary outcome of RCT Endometrial biopsies at baseline, followed by a 2-month mifepristone treatment, LNG-IUS placement and second biopsy after 3 months with the device in situ Healthy women from trial I	Endometrial morphology and presence of PAEC 3 months after LNG-IUS placement with prior mifepristone treatment and without endometrial shedding Secondary outcome Study I	Mifepristone n=9 Comparator n=8	Hematoxylin-Eosin staining Light microscopy for histological evaluation	Descriptive statistics. Two group comparison with Mann-Whitney U test (non-parametric).
III Molecular alterations following mifepristone treatment in healthy premenopausal breast tissue *in vivo*	Secondary outcome of RCT Breast biopsies from healthy premenopausal women before and after a 2-month treatment with mifepristone Healthy women from trial I	Alterations of the transcriptomic profile of healthy breast following mifepristone treatment Secondary outcome Study I	Mifepristone n=16	RNA extraction cDNA library construction for new generation sequencing RNA-seq data processing and analysis Gene Ontology and pathway analysis	Descriptive statistics FDR Inbuilt algorithm
IV Epigenetic alterations in breast following PRM treatment and assessed by surrogate markers	Experimental, hypothesis generating study. Patient cohorts from Study I, BRCA RCT and ulipristal acetate trial	Effects of mifepristone and ulipristal acetate on surrogate markers for development of breast cancer	Study I: M: n=9, C: n=11 BRCA trial: M: n=11, C: n=4 UPA trial: n=9	Establishment and validation of a breast specific epigenetic index, then used to assess the patient cohorts	Non-paired sample t-test. Paired t-test. Z-tests. Multivariate cox proportional hazards model. Pearson's correlation test.

3.2 STUDY DESIGN

3.2.1 Study I, II and III

This thesis is mainly based on data from a prospective, randomized, placebo controlled, double-blind trial that we conducted at the Karolinska University Hospital, Stockholm, Sweden. The study was designed to assess the effect of pretreatment with the progesterone receptor modulator mifepristone on bleeding patterns, following placement of a LNG-IUS 52 mg for contraceptive purposes ***(study I)***. A total of 68 healthy women opting for a LNG-IUS were assessed for eligibility and 58 were randomized. The first participant was screened in November 2009 and the last screened in in November 2013, with the final study contact in January 2015.

A secondary objective of the trial, presented in ***study II***, was to delineate the endometrial morphology in women using the LNG-IUS in the mifepristone primed endometrium. Further, to assess the presence of progesterone receptor modulator associated endometrial changes (PAEC) with this combined treatment regimen, with no endometrial shedding prior to the insertion of the device.

Another secondary objective of the trial, presented in ***study III***, was to explore the molecular alterations in breast tissue *in vivo*, following treatment with mifepristone.

3.2.2 Timeline of intervention and data collection events

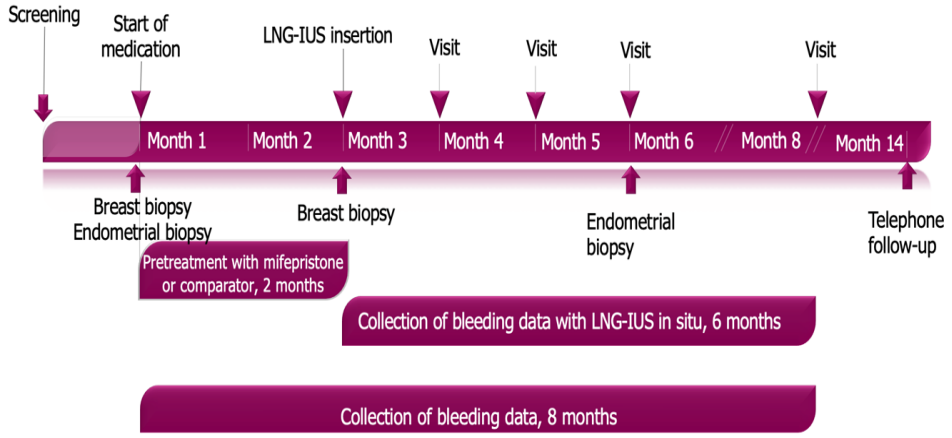

Figure 9. Schematic timeline presentation of the trial resulting in study I-III. The mifepristone pretreatment period corresponds to months 1 and 2 in the timeline, followed by the LNG-IUS insertion. The first 3 months contributing to the bleeding data analysis for Study I corresponds to months 3-5 in the timeline. The endometrial biopsy at baseline and 3 months after placement of the IUD was assessed in Study II. Breast biopsies, at baseline and at the end of the pretreatment period were assessed in Study III and were also part of Study IV.

3.2.3 Study IV

This was a hypothesis generating experimental study including several patient cohorts of healthy women (unknown BRCA status), women with known BRCA mutation and women with high risk for developing breast cancer. One cohort consisted of BRCA mutation carriers and confirmed non-carriers for assessment of sex steroid hormones measured in saliva. A DNA methylation signature surrogate for replicative age, named WID-Brest29 index, was established and validated using data sets from breast tissue of BRCA 1 and 2 mutation carriers, healthy controls, and triple negative breast cancer (TNBC) patients. Thereafter, the impact of mifepristone was assessed using the WID-Breast29 index using 2 cohorts. The first cohort consisted of women from study I. The second cohort included women with BRCA 1 or 2 mutations, participating in another RCT conducted at the Karolinska university hospital, a prospective, randomized, placebo controlled, double-blind trial assessing the effect of mifepristone, 3 months treatment, versus a comparator on the breast. An additional third cohort was included to assess the impact of UPA on breast tissue in women with increased risk for breast cancer. These women are also from an ongoing RCT conducted in Manchester, UK. Finally, the impact of mifepristone on TP53 mutations was evaluated in women deemed to respond to mifepristone based on the reduction in WID-Breast29 index.

3.3 STUDY SUBJECTS

3.3.1 Study I, II and III

Eligible women for the randomized controlled trial were healthy, ≥18 years of age with regular menstrual cycles, opting for a LNG-IUS 52 mg for contraception. Table 4 explains in detail the inclusion respectively exclusion criteria for the trial.

Table 4. Inclusion and exclusion criteria study I

Inclusion criteria	Healthy Age ≥ 18 Request of LNG-IUS for contraception Regular and normal menstrual cycles 25-35 days Willing and able to participate after giving informed consent Can and will use barrier methods for contraception during pretreatment period and + 7 days after the LNG-IUS insertion
Exclusion criteria	Hormonal treatment or IUD use within 2 months prior to study start Any contraindication for mifepristone or LNG-IUS Pregnancy or breast feeding within 2 moths prior to study start Current signs of pelvic inflammatory disease Unexplained irregular vaginal bleeding History of malignant disorder of the breast History of other malignancies Abnormal laboratory values at baseline Abnormal gynecological ultrasound at baseline Abnormal Pap smear findings at baseline
Exclusion criteria for per protocol analysis	Violation of the study protocol Lack of essential data

LNG-IUS=levonorgestrel intrauterine system, IUD=intrauterine system

3.3.2 Study IV

Saliva sample collection

BRCA1/2 mutation carriers (n=12) and confirmed non-carriers (n=8) included for the saliva sample collection were recruited as part of the UK, BRCA Unite Research study (http://www.brcaunite.org). The inclusion criteria for women participating were: age 18-45 years, no current pregnancy or breast feeding, hormonal medication 3 months prior to recruitment, previous cancer diagnosis or previous risk reducing bilateral salpingo-oophorectomy.

Breast samples/DNA methylation data sets

The following cohorts/databases were used for the DNA methylation (DNAme) microarray analyses:

1. To establish and validate the WID-Breast29 index:
    a. Breast tissue samples from healthy women (n=14) opting for cosmetic breast surgery. The participants had no family or personal history of breast cancer and the average age of the group at surgery was 31 years.
    b. Breast tissue from BRCA1/2 mutation carriers (n=14). The average age at breast surgery was 36 years.
    c. Breast tissue from TNBC patients (n=14). Tissue was collected from the tumor and from non-cancerous sites surrounding the tumor, in the same individual. The average age at breast surgery was 43 years.

2. To assess the WID-Breast29 index in ER+/PR+ cancers:
    Breast tissue from ER+/PR+ stage T1-T2 breast cancers and from non-cancerous tissue surrounding the tumor, in the same individual (n=31). The average age at surgery was 51 years. These datasets were available from GEO (Gene Expression Omnibus – a public functional genomics data repository)

3. To assess the performance of the WID-Breast29 index for evaluation of mifepristone effects
    a. Part of the women from our trial (Study I) were included in this analysis. Nine samples from the mifepristone and 11 samples from the comparator group respectively, provided sufficient DNA in both biopsies (baseline and end of treatment) for the downstream analysis (extended data Figure 3, Study IV).
    b. Part of the women from our ongoing BRCA1/2 trial were included in this analysis. The ongoing study is a prospective, randomized, placebo controlled, double-blind trial in BRCA1 or 2 mutation carriers treated with mifepristone 50 mg or the comparator Triobe® (as in Study I) every other day for 3 months. In brief, eligible women are 18 years or older, with regular menstrual cycles and no hormonal medication 2 months prior to reqruitment, not pregnanant, breastfeeding or with a history of malignancy. Core needle biopsies are collected at baseline and

at the end of treatment. Eleven women from the mifepristone and 4 women from the comparator group provided sufficient DNA in their paired biopsies for downstream analysis (extended data Figure 3, Study IV).

4. To assess the performance of the WID-Breast29 index for evaluation of UPA effects.
   Women (n=9) with an increased risk of breast cancer who are part of an ongoing "Breast Cancer Anti-Progestin Prevention Study 1" conducted in the UK were included. This is a single arm pilot study designed to test the efficacy and safety of UPA in breast cancer prevention. Eligible are women at increased risk of breast cancer (≥1:6 lifetime risk assessed by Tyrer-Cuzick model version 8) and with regular menstrual cycles. A breast biopsy was retreived in the luteal phase of the menstrual cycle, and the second biopsy after treatment with UPA 5 mg daily, for 3 months from the contralateral breast (extended data Figure 6, Study IV).

Impact of mifepristone on TP53 mutations

To assess whether Tumor Protein 53 (TP53) mutation frequency can be altered by mifepristone treatment, breast tissue from women deemed to respond (n=5) or not (n=3) to mifepristone based on the reduction in WID-Breast29 index, was evaluated. TP53 is a tumor suppressor and upon mutation, implicated in over 50% of human cancer and highly prevalent in TNBCs (106). TP53 has further been described in mutated form in a variety of normal tissues including breast (107). Eight samples were analyzed.

3.4 METHODS STUDY I

3.4.1 Randomization, study medication and interventions

Women who were willing to participate and fulfilled all the inclusion and none of the exclusion criteria were randomized at a ratio of 1:1 in blocks of 10, by dispensing opaque identical, sequentially numbered pill packs prepared according to a computer-generated randomization list containing the allocation.

The screening visit included detailed assessment of women's medical and reproductive history, routine physical examination including blood pressure, height and weight measurements, gynecological examination including transvaginal ultrasound, breast palpation and pregnancy test. As a safety parameter, blood samples were collected at baseline and after the pre-treatment period (complete blood count, follicle-stimulating hormone (FSH), LH, prolactin, estradiol and progesterone).

According to the allocation, women received mifepristone (Mifegyne®, Exelgyn, Paris, France) 200 mg or visually indistinguishable vitamin B (TrioBe®, Recip, Stockholm, Sweden). One TrioBe® tablet contains cyanocobalamin 0.5 mg (B12), folic acid 0.8 mg (B9) and pyridoxine hydrochloride 3 mg (B6). A study nurse, not participating in any other parts of the study, divided the tablets into 4 parts and instructed the women to take one quarter of the comparator or mifepristone (which equals to approximately 50 mg mifepristone) orally,

every other day starting on the first day of the menstrual cycle. The duration of the pre-treatment period was 2 months, corresponding to two menstrual cycles (2x28 days) prior to the LNG-IUS (Mirena®, Bayer) insertion and until 3 days (±2 days) after the insertion. Following the pre-treatment period, pregnancy test and chlamydia samples as per clinical routine were taken and the LNG-IUS was placed.

Endometrial biopsies were collected at baseline, prior to the pre-treatment period and three months after the LNG-IUS insertion, with the device in situ. The rationale and assessment of these biopsies are presented and further discussed in *Study II*.

Breast biopsies were collected at baseline and at the end of the 2-month pre-treatment period. The rationale and evaluation of these biopsies are presented and further discussed in *Study III* and constitutes also part of *Study IV*.

3.4.2 Bleeding diaries

The study participants were instructed to keep daily diaries of vaginal bleeding during the 2-month pre-treatment period and until 6 months after the LNG-IUS insertion. One treatment cycle corresponded to 28 days in the diary. Bleeding was graded on a five-point scale as none (0), spotting (1), mild (2, less than normal menstruation), moderate (3, similar to normal menstruation) or severe (4, heavier than normal menstruation). Women were also asked to keep records of adverse effects and potential concomitant medication during the same study period. The diaries were monitored at every visit by a study coordination midwife.

The bleeding diary data were summarized as percentage rate of bleeding and spotting (B/S%) per treatment cycle (28 days) for comparison between the treatment groups. In the five-point scale provided, spotting, mild, moderate and severe bleeding was classified as a bleeding event (1+2+3+4 in the bleeding scale). A separate analysis was conducted to compare the proportion of women with normal or heavy intensity bleeding (3+4 in the bleeding scale).

3.5 METHODS STUDY II

3.5.1 Study participants

The participants for study II originate from the randomized control trial (study I). Nine paired samples from the mifepristone and 8 paired samples from the comparator arm respectively, contributed to the final data analysis for the endometrial assessment. Paired samples implicate endometrial sample at baseline, prior to the pre-treatment and 3 months after the LNG-IUS placement, from the same woman. The two groups were comparable in terms of age, pregnancies, parity, menstrual characteristics and body mass index (BMI). Flow chart and a detailed description of the demographic characteristics of the current cohort are available in the published article.

3.5.2 Endometrial biopsies

The first endometrial biopsy was retrieved at baseline, prior to the pre-treatment with mifepristone or the comparator and the second one at 3 months after placement of the LNG-IUS with the device in situ. The LNG-IUS placement was commenced directly after the pretreatment period, with no prior endometrial shedding. An endometrial disposable plastic suction curette was used for retrieval. One part of the biopsy was sent for routine pathological evaluation as a safety measure, while one part was fixed, sectioned, processed and stained with hematoxylin and eosin by standard methods and stored at +4°C.

3.5.3 Histological assessment

The stained samples were histologically assessed in Edinburgh, by an expert pathologist in the field of PAEC, who was blinded to the treatment. The evaluation was made according to 1) primary diagnosis (benign, hyperplastic or malignant), 2) whether morphological appearances represented a physiological state or not and if physiological, record the stage of the menstrual cycle and 3) the presence or absence of PAEC. Free text comments were given if other histological features were present.

3.6 METHODS STUDY III

3.6.1 Study participants

The participants contributing to the paired breast biopsies evaluated in study III derive from the enrolled subjects of the randomized controlled trial (study I). Only women from the mifepristone arm were included in the current cohort. Flow chart and a detailed description of the demographic characteristics of the cohort are available in the published article.

3.6.2 Breast biopsies

Core needle breast aspiration biopsies were collected by one radiologist at baseline, and at the end of the pretreatment period of 2 months (paired samples). Local anesthesia was applied in the skin to minimize patient discomfort. The specimens were collected under ultrasound guidance from the upper outer quadrant of one breast using a 14-gauge needle with an outer diameter of 2.2 mm. The collected breast tissue was divided into 2 parts, snap-frozen and stored at -180°C until further processing.

3.6.3 General workflow of RNA-seq and data analysis

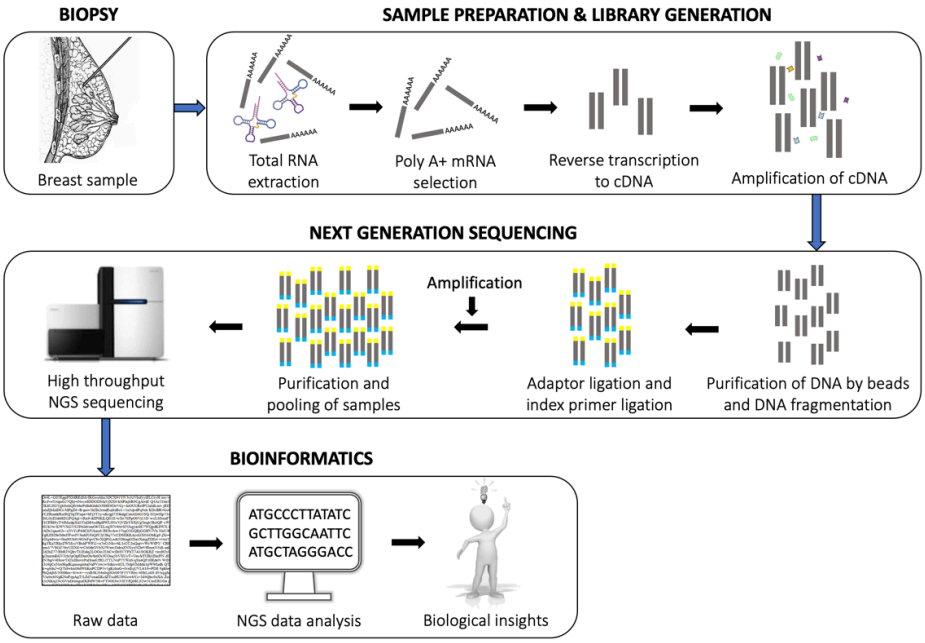

Figure 10. General workflow of RNA-seq and data analysis used in this study. Following sample preparation and library generation, NGS reads were sequenced with the Illumina NextSeq 550® and subsequently aligned to the hg38 genome. Differential expression of read counts was analyzed using DEseq2. Gene Ontology (GO) and pathway analysis were performed to interpret the results.

3.6.4 RNA extraction

For the transcriptomic analysis, RNA extraction was performed from 16 randomly chosen paired breast samples (i.e. 32 samples) using whole tissue. Briefly, for the extraction, the Purelink™ RNA Micro kit in conjunction with TRIzol® was used, in order to isolate the total RNA from the tissue samples. For purification of the total RNA retrieved, each sample was deoxyribonuclease (DNAse) treated to eliminate the contaminating genomic DNA. The final concentrations of the RNA samples (quantification) was done using the Qubit™ RNA High Sensitivity Assay Kit.

3.6.5 cDNA library construction and sequencing

cDNA libraries for next generation sequencing (NGS) were constructed using 1 ng RNA each for the 16 paired samples, using the well-established Smart-seq2® protocol (108). NGS library construction was done based on the Qubit™ quantification. Tagmentation of the cDNA was performed using Nextera® XT Kit (Illumina®) followed by addition of adapters and index primers. The resulting DNA libraries after Nextera® reactions were purified using AMPure XP beads (Beckman Coulter, USA) and quantified (Qubit flex™, Life

technologies). 10 ng of DNA from each post Nextera library was pooled and sequenced on Illumina NextSeq 550® instrument using 1x75 cycles High output kit at the Bioinformatics and Expression Analysis Core facility, Karolinska Institutet, Stockholm.

3.6.6 RNA-seq data processing and analysis

Quality check of raw sequencing reads was performed with FastQC and MultiQC (109). RNA sequencing data analysis was performed with the Partek® Flow® Genomic Analysis Software (Partek Inc., St. Louis, Missouri, USA). Briefly, the FASTQ files were processed to filter the contaminants such as ribosomal DNA and mitochondrial DNA using Bowtie 2 aligner followed by trimming the standard Nextera Transposase adapter (CTGTCTCTTATACACATCT) from the raw reads. The filtered reads were then aligned to the hg38 genome using STAR aligner with default settings. Total alignment rate was in the range of 95-99% of which the unique alignment rate was 80-92% with an average Phred quality score of 34 per base post alignment. The filtered alignments were quantified to hg38 Ensembl Transcripts release 100. The obtained gene features were filtered off where the value was less than 1 count in at least 80% samples. A total of 26.581 (78%) genes passed the criteria. Differential expression analysis was performed using DEseq2 on the Partek platform.

3.6.7 Gene Ontology and pathway analysis

For interpretation of the differentially expressed genes (DEGs) in our data set, we conducted functional annotation and pathway gene set enrichment analysis using the g:Profiler online database (version e101_eg48_p14_baf17f0) (110).

Gene Ontology (GO) analysis was performed for the functional annotation of the DEGs. GO is an online computational tool providing a systematic and regulated vocabulary to explain the current best representation of the normal functions of genes. It consists of different ontologies, with each composed of an assembly of terms with well-defined associations between the terms. The most common ontologies used to explore the associations of DEGs to GO terms in our study were: a) Biological Process (BP) that describe the pathways and processes where the DEGs contribute (e.g. transcription and apoptosis), b) Cellular Component (CC) describing the location in where the DEGs are active (e.g. lysosome, nucleus) and c) Molecular function (MF) describing the molecular activities of each DEG (e.g. ligand, transporter).

For the pathway analysis of the DEGs in our data set, we used Reactome to assess the biological pathways put into a broader context. Reactome is a peer-reviewed database, cross-referenced to different online resources.

3.7 METHODS STUDY IV

Saliva sample collection and hormone analysis

BRCA mutation carriers and wild-type controls collected a daily morning saliva sample (~1ml) during one full menstrual cycle. Samples were immediately frozen at ~ -20^{o}C and

later transferred into -80^{o}C. Salivary hormone levels were measured using enzyme immunoassay kits for progesterone and estradiol, following the manufacturer's instructions.

DNAme analysis

In brief, DNA samples were normalized and bisulfite-modified. The bisulfite-converted DNA was then subjected to methylation analysis on Illumina InfiniumMethylation EPIC BeadChip (Illumina, CA, USA) as per manufacturer's standard protocol. The DNAme data were background-corrected and normalized (111). Probes were removed if they had < 95% coverage across samples and any remaining probes with detection *p*-value > 0.05 were replaced by *k*-NN imputation, with *k*=5. The same quality control and normalization procedure was followed for 257 breast cancer invasive carcinoma samples with associated clinical data and 38 healthy control breast tissue samples with matched gene-expression data from a publicly available data bank (The Cancer Genome Atlas) repository (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga).

Bisulfite-sequenced DNAme data for purified breast epithelial cell subtypes were downloaded from the European Genome-Phenome Archive (EGA) and reads were aligned and counted using Bismark (112) with default settings. Only reads mapping to CpGs represented on the Illumina EPIC array were retained and which had a total number of mapped reads (methylated+unmethylated) of at least 20.

Gene expression analysis

Gene-expression data from 38 healthy control breast tissue samples with matched DNAme data available were downloaded from the TCGA repository (The Cancer Genome Atlas, https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) and were then quantile-normalized using the preprocessCore package in R.

RNA extraction, cDNA synthesis, and RNA sequencing and analysis

RNA extraction from paired mifepristone treated samples was conducted and cDNA and DNA library preparations were performed as in Study III. Post-quality control reads were mapped to the reference genome hg38 using STAR aligner with default settings, further filtered and quantified to coding transcripts/genes using hg38 assembly and Ensemble transcripts release 91. Gene counts were obtained after filtering for regions fully or partially spanned within exon regions.

Transcriptome data for purified breast epithelial cell subtypes

Transcriptome data for purified breast epithelial cell subtypes as for the DNAme data were downloaded from the European Genome-Phenome Archive (EGA). Transcriptome data for purified fat cell samples were downloaded from the ENCODE (Encyclopedia of DNA Elements) repository (https://www.encodeproject.org). Read-counts were obtained from the archived bam (binary alignment map) files for the RNA-seq libraries (as downloaded from

the online repositories) using Samtools featureCounts. TPM (transcripts per million)-normalized counts were then used for all downstream analyses.

Real-time PCR

cDNA was used in triplicates in real-time polymerase chain reaction (RT-PCR) with TaqMan® fast advanced master mix and TaqMan® gene expression probe/primer for RANKL (Cat no. Hs00243522_m1) and analyzed using StepONE RT-PCR (Applied Biosystems, USA). Ribosomal RNA 18s was used as a housekeeping gene to normalize the gene expression of RANKL and the relative expression fold change were calculated for the above paired treatment groups using the standard formula 2-ΔΔCT.

TP53 mutational analysis by Duplex Sequencing

TP53 mutations were analyzed using ultra-accurate Duplex Sequencing technique (113) in 3 non-responders and 5 responders (according to the WID-Breast29 index) using DNA extracted from normal breast tissue collected before and after mifepristone treatment. Duplex Sequencing kits were used according to the manufacturer's instructions (TwinStrand Biosciences, Seattle, WA, USA). TP53 coding regions were captured by hybridization with 120 bp biotinylated probes. Two successive rounds of captures were performed to ensure sufficient target enrichment (114). Indexed libraries were pooled and sequenced in an Illumina MiSeq using v2 300 cycle kits. Data analysis was performed using the standard Duplex Sequencing pipeline (113) with updated modifications (https://github.com/Kennedy-Lab-UW/Duplex-Seq-Pipeline).

For each sample, we sequenced an average of 5.2M duplex nucleotides in coding TP53 exons and calculated TP53 mutation frequency as the number of identified mutant positions/total number of nucleotides sequenced in the coding region; TP53 mutation burden as overall number of mutant alleles (sum of mutant alleles in all positions) divided by the total number of nucleotides sequenced in the coding region. Intronic mutations, except for splice sites, and single nucleotide polymorphisms (SNPs) were excluded from mutation analysis. TP53 hotspot mutations were defined as the top 1% most frequent mutations in breast cancer according to the COSMIC database.

3.8 STATISTICAL ANALYSIS

3.8.1 Study I

The current treatment regimen of mifepristone was also used in our previous study where it induced amenorrhea in 86% of women after a treatment period of two months (20). Further, 62% of women reported unscheduled bleeding the initial months after the LNG-IUS 52 mg placement (40). To reach 80% power applying a two-tailed test, we had to include 19 women in each treatment arm, assuming a reduction of the bleeding/spotting rate from 62% to 21% (i.e. reduced to one third). A total of 29 women in each group ought to be recruited, to allow for possible drop outs. Descriptive statistics of the study participants are presented with

median and range and Mann-Whitney *U* test was used for comparison between the groups. Mann-Whitney *U* test is a non-parametric test that can be used when variables are not normally distributed. A p-value of <0.05 was considered statistically significant. Statistical analysis was conducted using the statistical software R version 3.4.3.

3.8.2 Study II

No power calculation was conducted for this secondary outcome of the RCT (Study I) and no similar investigations have been conducted previously. Descriptive statistics are presented with median and range. The Mann-Whitney *U* test was used for comparison between the groups. A p-value of <0.05 was considered statistically significant and data were analyzed using SPSS version 25 software (IBM).

3.8.3 Study III

No power calculation was conducted for this secondary outcome of the RCT (Study I). The filtered gene list was tested for differential gene expression by DeSeq2 between the control and treated samples. The DEGs where the fold change was up or downregulated by 2 and false discovery rate (FDR) <0.05, were considered statistically significant and were used for the downstream analysis using g:Profiler. The Benjamini-Hochberg FDR multiple testing correction was used in g:Profiler, with a significance threshold of 0.05 for the functional annotation and enrichment pathway analysis of the DEGs (110). FDR (adjusted p-value) is a statistical approach commonly used in multiple hypothesis testing, such as high-throughput experiments, to correct for multiple comparisons. FDR adjusts the statistical confidence based on the number of tests performed.

3.8.4 Study IV

The composition of each tissue sample in terms of proportions of epithelial, fibroblast, immune and fat cells, based on the DNAme profile of each sample was analyzed using a well-validated algorithm (111). The breast epithelial cell subtype inference method for DNAme data is available as an R package from:
https://github.com/tombartlett/BreastEpithelialSubtypes. The composition of cells in each tissue sample was analyzed using transcriptome data applying a thoroughly tested algorithm (115).

Non-paired sample t-test was used to analyze DNAme microarray data (with n>10) to compare healthy tissue with the tumor, to compare the salivary hormonal levels E2 and P and to compare the WID-Breast29 test between the BRCA mutation carriers and control groups. Paired t-test was used to compare breast cancer biopsies with the surrounding normal tissue obtained from the same women. The significance of the WID-Breast29 index was assessed by z-tests on the Wald statistics after fitting a multivariate Cox proportional hazards model. Correlation of RANKL expression in luminal progenitor cell fraction was calculated with Pearson's correlation test. All analyses were carried out using the R software, version 4.0.

4 RESULTS AND DISCUSSION

4.1 STUDY I

4.1.1 Results

During the study period, 68 women were assessed for eligibility. Of those, 10 were excluded prior to randomization due to: ovarian cyst (n=1), positive pregnancy test (n=1), declined participation (n=1), cervical dysplasia (n=4), long menstrual cycle (n=3). Consequently, 58 women were randomized, 29 to mifepristone and 29 to placebo. In the mifepristone arm, 26 women completed the pretreatment period since three withdrew their consent. In the placebo arm, 23 women completed the pretreatment period since 3 withdrew their consent and 3 got pregnant. Twenty-three women in each group returned for the LNG-IUS insertion: in the mifepristone arm 3 did not receive the device due to failed insertion in 2 and 1 declined insertion. All women in the placebo arm received the device. Finally, 4 participants were excluded from the bleeding data analysis in the mifepristone group due to; missing bleeding data (n=2), chlamydia infection (n=1) and a partial LNG-IUS expulsion (n=1). In the placebo group, 4 women were excluded due to missing bleeding data. This resulted in a per protocol population of 19 women per treatment arm contributing to the final analysis. The flow chart in the published article also provide the details of the women excluded from the trial.

During the first 2 months of the pretreatment period, a highly significant difference in mean B/S% was reported between the comparator and mifepristone group ($p<0.001$) with mifepristone treated women reporting fewer B/S days. Following the first month after insertion of the LNG-IUS 52, women in the mifepristone group still reported lower B/S% but with a difference that did not reach statistical significance ($p=0.077$). A higher proportion of women in the mifepristone group reported less moderate or heavy intensity bleeding the first month after insertion of the intrauterine device in relation to the comparator group with a difference that did not reach statistical significance ($p=0.36$). A reverse difference was seen at 3 months post the LNG-IUS insertion, with more days with normal or heavy bleeding in the mifepristone arm compared with the comparator group ($p=0.044$). During the remaining observational period (up to 6 months' post insertion) the B/S rates where low with no statistical differences between the groups. Furthermore, there was no difference between the groups in in the proportion of women with LNG-IUS removal. No serious adverse events were reported during the study period except from the pregnancies that occurred in the comparator arm presumably due to lack of adherence to the contraceptive method used. The side effects where mild with no differences between the study arms.

4.1.2 Discussion

Our study is the first to explore whether pretreatment with a PRM resulting in amenorrhea, could also prevent unscheduled bleeding in new users of a progestin only method (LNG-IUS 52 mg) in regular cycling women. Women treated with mifepristone reported a significant reduction in menstrual bleeding during the two pretreatment months compared to the comparator group. Following the pretreatment period, the mifepristone group experienced a

reduced but statistically not significant difference in B/S the first month after the LNG-IUS placement. The following months, bleeding patterns were similar in the two study arms. Furthermore, even though the mifepristone group reported lower rates of normal and heavy bleeding the first month after the LNG-IUS placement the difference was non-significant with an inverse effect, this time significant, three months after insertion of the device.

The mifepristone treated women reported 79% of amenorrhea at the end of the pretreatment period. This is in line with other studies reporting a high fraction of amenorrhea during continuous treatment with PRMs. Studies conducted in women with uterine fibroids and heavy menstrual bleeding have shown induction of amenorrhea in 80-90% of continuously PRM treated women (69, 116). In a study conducted in our group using the same mifepristone treatment regimen as in this study, 86% of women with fibroids reported amenorrhea after two months of treatment and close to 100% after three months respectively (20). The factors contributing to cessation of bleeding during continuous PRM treatment are currently still not well elucidated. Various effects are suggested to contribute, such as inhibition of ovulation, compound specific vascular effects and thickening of the arterial wall, altered expression of metalloproteinases and endometrial leucocytes as well as altered regulation of the ER and PR signaling (116). Treatment duration is also a contributing factor. It could also be postulated that the measured treatment effect could be particularly evident in women with uterine fibroids and heavy menstrual bleeding compared to normal cycling women.

A literature review revealed a total of ten studies using a PRM to improve bleeding patterns in women using a progestin for contraceptive purposes (Table 5) and one study assessing the effect of a PRM on bleeding disturbances in women with an LNG-IUS placed due to menorrhagia. These studies could further be categorized into PRMs given as prophylaxis or as therapy. In the studies exploring the potential prophylactic effect of PRMs, all new progestin-only users with regular menstrual cycles were included to prevent unscheduled bleeding. The first study by Gemzell-Danielsson *et al*., included women starting with a progestin-only pill, (Cerazette® 75 µg desogestrel daily) in combination with an investigational PRM, Org 31710, 150 mg (Organon, Oss, The Netherlands), once every 28 days for four to seven cycles. A more regular (cyclic) bleeding pattern was observed with addition of the PRM compared to placebo (24). Another study, by Jain *et al*., showed a significant decrease in the percentage of days with breakthrough bleeding in new starters of the injectable progestin depo-medroxyprogesterone acetate when combined with mifepristone 50 mg every 2 weeks for 6 months compared to placebo (117). The third study by Massai *et al*., included new users of a subdermal progestin, levonorgestrel releasing implant (Norplant®) and mifepristone 100 mg/day administered for 2 consecutive days every 30 days, or placebo. They reported the same frequency of bleeding episodes but a total number of 35% lower bleeding days compared to the placebo group (118). Another placebo controlled study by Warner *et al*., compared the effect of 150 mg UPA, administered over three consecutive days starting on days 21, 49 and 77 after insertion of the LNG-IUS. This regimen showed an initial reduction in bleeding/spotting % yet subsequently actually increased compared with

that for placebo treated controls (119). During the design of our study we hypothesized that pretreatment with endometrial suppression and amenorrhea and subsequent LNG-IUS insertion could counteract the increased B/S observed with PRM withdrawal. This effect could not be proven, since the mifepristone treated group in our study reported a non-significant lower occurrence of normal and heavy menstrual bleeding the first month after the LNG-IUS insertion compared to the comparator group, while a reverse difference was reported at 3 months. Nevertheless, whether adjusted treatment protocols and treatment continuing some months after the LNG-IUS insertion could display more favorable results, remains to be shown. It could be speculated that maintenance of the suppressed endometrium by a PRM until the LNG effect on the endometrium was in place may result in a continuously suppressed endometrium and less bleeding, even if ovulation resumes.

Table 5. Trials using PRMs as prophylaxis or therapy for unscheduled bleeding in progestin-only users.

		PRM and regimen	**Progestin**	**Type of study**
Prophylaxis	Gemzell-Danielsson *et al* 2002	Org 31710, 150 mg every 28 days, starting on day 1 for 4-7 cycles	Desogestrel 75µg (Cerazette®)	RCT
	Jain et al 2003	mifepristone 50 mg every 2 weeks for 6 months starting 2 weeks **after** DPMA injection	DMPA 150 mg	RCT
	Massai *et al* 2004	mifepristone 100 mg/day for 2 consecutive days, on days 30, 60, 90, 120, 150, 180 **after** implant insertion	Levonorgestrel subdermal implant (Norplant®)	RCT
	Warner *et al* 2009	UPA 150 mg in divided doses (50 mg/day) given over three consecutive days on days 21, 49 and 77 **after** LNG-IUS insertion	LNG-IUS, additional information not provided	RCT
	Papaikonomou *et al* 2018	mifepristone 50 mg every other day for two months **before** LNG-IUS insertion	LNG-IUS 52 mg (Mirena®)	RCT
Therapy	Cheng *et al* 2000	mifepristone 25 mg, two tablets once every 4 weeks for 6 months	Levonorgestrel subdermal implant (Sino-Implant)	RCT
	Glasier *et al* 2002	mifepristone 200 mg as a single dose on day 3 of a bleeding episode	Levonorgestrel subdermal implant (Norplant®)	RCT
	Weisberg *et al* 2006 pilot study and larger follow-up trial 2009	mifepristone 50 mg for one day, followed by EE 20 µg/day for four days, or placebo for five days	Levonorgestrel subdermal implant (Norplant®)	RCT
	Fava et al 2020	5 mg UPA for five consecutive days	LNG-IUS 52mg (Mirena®)	RCT

PRM=progesterone modulator, DMPA=depo-medroxyprogesterone acetate, UPA=ulipristal acetate, LNG-IUS= levonorgestrel intrauterine system, EE=Ethinyl estradiol.

In contrast to the above described studies using PRMs as prophylaxis as in our study, three trials used PRMs to improve progestin-only contraceptive bleeding patterns in women already experiencing bleeding disturbances thus using PRMs as therapy (23, 120-123). While all studies reported a favorable bleeding pattern with the PRM treatment in comparison to placebo, none of these studies found an improvement that reached statistical significance.

Finally, in an open-labelled, non-randomized comparative study, women with heavy menstrual bleeding and an LNG-IUS received 100 mg mifepristone every 30 days for 3 months after placement of the device (not included in Table 5 since the indication for the LNG-IUS was heavy menstrual bleeding). Their results showed a significant reduction in frequency and duration of intermenstrual spotting compared to the control group which was composed after a review of medical records of women who had an LNG-IUS placed (124).

A recent systematic review was conducted to assess the medical treatment options for amelioration of bleeding irregularities in women with LNG-IUS 52 mg (45). Besides the studies of Warner *et al.* (119), Fava *et al*. (123) and our trial (Study I) as presented in Table 5, three more RCTs were identified in women receiving LNG-IUS for contraceptive purposes. In one of the trials, new users of LNG-IUS were treated with the SERM tamoxifen or placebo, with no statistically significant differences between the groups. The rationale was that this SERM could improve bleeding patterns by antagonism of ERβ and consequent downregulation of endometrial angiogenesis (125). The second study also included new users of the LNG-IUS and women were treated with either an NSAID, an estradiol patch or placebo. The group reported a reduction in bleeding and spotting days in the NSAID treatment arm while an inverse effect was shown in the estradiol treatment group, compared to placebo (126). The third study failed to show a statistical significant difference in bleeding irregularities in LNG-IUS users treated with two different antifibrinolytic agents (tranexamic acid or mefenamic acid) or placebo (127). The comparison of various studies conducted to assess the effects of different agents on bleeding patterns of progestin only users is very challenging. Even if most trials use bleeding and/or spotting days as a primary outcome, almost all use different definitions for those bleeding irregularities as well as different time periods for evaluation, different progestins, interventional agents and treatment regimens.

Since progestins are PR agonists, there have been concerns regarding the plausible pharmacokinetic interactions with any PRMs given because of their antagonistic actions on the PR. As previously discussed, continuous administration of sufficient doses of PRMs hold the potential to be utilized for contraceptive purposes due to their ability to suppress ovarian follicular development. The same contraceptive mechanism of action is adopted by various progestin-only preparations including pills, injectables and implants. As a result, even if PRMs and progestins exhibits antagonistic or agonistic effects on the PR respectively, in sufficient doses they exert the same effect, namely follicular suppression. In the above-mentioned study by Gemzell-Danielsson *et al*., a cyclic bleeding pattern was observed in women treated with desogestrel 75 μg daily and the PRM Org 31710 once a month compared to the women who received desogestrel and placebo (24). This effect on the bleeding pattern

could be due to a direct effect on the endometrium and/or a result of the increased rate of ovulation observed in the PRM treated group. Nevertheless, no pregnancies occurred in that study, nor in the study by Cheng *et al*., where 300 cycles in women with a levonorgestrel subdermal implant and mifepristone were included (23). Furthermore, even with a single dose PRM administered directly after ovulation, secretory endometrial changes are avoided which could be sufficient to prevent implantation (128). Another study explored whether start with desogestrel 75µg immediately following the day after UPA intake for EC could jeopardize the effect of the latter. The authors reported a reduced ability of UPA to delay ovulation and concluded that this treatment regimen could potentially decrease UPAs efficacy as an EC. However, they also concluded that the action of desogestrel was not affected by UPA intake (129). In addition, UPA could not affect ovarian quiescence induced by a COC (EE+LNG) compared to placebo (130). In summary, the theoretical risk that PRMs may reduce efficacy of hormonal contraception have not been demonstrated to date. Properly powered trials assessing actual pregnancy incidence with a progestin/PRM combination regimen are needed. Even then, given the differences in various progestins and PRMs in progesterone receptor affinity and effect depending on which phase of the cycle they are administered, any extrapolation to other PRMs or progestins than those assessed should only be concerned tentative. The main contraceptive mechanism of the LNG-IUS used in our study is thickening of the cervical mucus and not primarily blockage of follicular development.

In an attempt to reduce the time to LNG-IUS placement, we decided the pretreatment period to last 2 months and the women were carefully instructed to use barrier methods for contraception. Despite these efforts, 3 women, all in the placebo group, had unintended pregnancies. This underlines the major drawback of any pretreatment regimens that do not hold contraceptive properties. Even though mifepristone and other PRMs are not licensed for contraceptive use, their potential as contraceptive agents have previously been discussed in this thesis and even a much lower dose than the one we used, namely 25-50 mg once a week, demonstrated high contraceptive effectiveness (59).

Whether a different treatment regimen, prolonged pretreatment duration or treatment stretching over a longer period after LNG-IUS insertion would exhibit a significant amelioration of the bleeding disturbances and ultimately lead to higher continuation rates, remains to be investigated. Obviously, only a subset of women would actually suffer from unscheduled bleeding after LNG-IUS placement and thus could benefit from the use of prophylactic treatment with PRMs, such as in our study design. Finding a way to overcome this major problem - from a compliance point of view, would be beneficial for a large proportion of LNG-IUS as well as other progestin-only contraception users.

4.2 STUDY II

4.2.1 Results

Following randomization in study I, 29 women were assigned to the mifepristone and 29 to the comparator arm, respectively. Endometrial samples at baseline were successfully obtained

from all study participants in the mifepristone group (29) while retrieval failed in 4 women in the comparator group due to narrow internal cervical os, resulting in 25 retrieved biopsies. The second biopsy, retrieved 3 months after the LNG-IUS 52 mg treatment with the device in situ, was possible in 20 women and 17 women from mifepristone and comparator arm respectively. For the histological analysis, one woman from the mifepristone group was excluded because of a chlamydia infection and one biopsy was lost in transition to the lab after retrieval from a woman in the comparator group. Due to an unfortunate loss of samples during the move of our research laboratory, 9 paired biopsies in the mifepristone and 8 in the comparator arm respectively, contributed to the final histological analysis. The flow chart in the published article also provides the details of women excluded from this study. Demographic and reproductive characteristics between study participants in the two study arms were similar.

All endometrial biopsies at baseline were diagnosed as benign, with no signs of hyperplasia or atypia. Further, all but one were assessed as physiological, with the majority classified to be in secretory phase. The biopsy in the comparator group that was classified as non-physiological, was overall secretory with occasional dilated glands with subtle architectural alterations and compact stroma. No biopsies in either study arm revealed any presence of PAEC at baseline.

The second biopsies with the LNG-IUS in situ were all diagnosed as benign with no presence of PAEC. The classification of all biopsies was assessed as non-physiological due to the progestin effects of the LNG-IUS. In addition, gland, epithelium and stroma were described and vessel evaluation was conducted. The findings are summarized in Table 6.

Table 6. Summary of the histological classification of endometrial follow-up biopsies collected after the 2-month pretreatment period with mifepristone or the comparator followed by 3 months of LNG-IUS in situ.

	Mifepristone	**Comparator**
Primary diagnosis: benign	9/9	8/8
Non-physiological due to progestin effect	9/9	8/8
Absence of PAEC	9/9	8/8
Gland dilation	Absent 8/9 Occasional 1/9	Absent 3/8 Non-assessable 2/8 Occasional 2/8 Frequent 1/8
Gland architecture	Normal 9/9	Normal 6/8 Non-assessable 2/8
Epithelium	Inactive 9/9	Inactive 6/8 Non-assessable 2/8
Stroma, decidualized	9/9	8/8
Vessels	Ectatic 9/9	Ectatic 5/8 Non-assessable 2/8 Thick-walled 1/8

4.2.2 Discussion

The objective of this study was to assess the endometrial morphology and presence of PAEC in women treated with a continuous low dose mifepristone preceded by insertion of the LNG-IUS 52 mg with no endometrial shedding prior to placement of the device. The endometrial biopsies at baseline, prior to commencement of mifepristone, were all benign. The follow-up biopsies retrieved 3 months after placement of the LNG-IUS revealed progestin effects upon histological assessment and no presence of PAEC in either treatment group. The glandular architecture in the specimens that could be evaluated upon this feature, was normal in both groups. There were one and two biopsies with occasional gland dilation in mifepristone and comparator group respectively. In addition, one biopsy in the comparator group displayed frequent occurrence of gland dilation. These features were judged as progestin effects and not considered a sign of PAEC since the other common and most prominent histologic features of PAEC, such as compact non-decidualized stroma, irregular gland architecture and abnormal secretion were absent.

In order not to interfere with the primary outcome of our study (bleeding patterns after the LNG-IUS placement), no biopsy was retrieved following the pre-treatment period with mifepristone. Consequently, the rate of PAEC developed after mifepristone treatment in our cohort is unknown. Studies conducted before the establishment of PAEC as a histological entity in 2008, describe morphological features associated with PAEC but often designated as glandular hyperplasia. Today, it is widely accepted among pathologists that upon lack of information regarding continuous treatment with PRMs, the alterations may be confused with endometrial hyperplasia associated with unopposed estrogen effect (74). Hence, comparison with these early studies prior to PAEC definition is unfortunately problematic and uncertain. In investigations conducted after establishment of the PAEC criteria, the most utilized PRM treatment period is three months' cycles. A trial conducted in our group, assessed the impact of the same mifepristone regimen as in our current study on uterine leiomyoma and found PAEC present in 54% of the treated women (20). In another trial from 2003, assessing the contraceptive potential of mifepristone, the endometrium of participants displayed endometrial alterations with cystic dilations but inactivity, in a dose depended manner, already after 30 days of treatment and with much lower doses than ours (2 mg versus 5 mg) (14). Further, 3 months' treatment with UPA could display PAEC in 65-74% of the women (68, 69, 74). According to these data, we can postulate that PAEC can develop after 2 months with the currently used dosage.

Treatment with PRMs for uterine fibroid volume reduction and associated excess bleeding has been explored and gained even more attention after the introduction of UPA (28, 69). Cessation of menstrual bleeding has shown to be rapid and confirmed in 90% of patients treated with 5 mg UPA daily, and 88-90% in women treated with 2 mg and 5 mg mifepristone respectively (19, 69). In our previous study that used the same mifepristone regimen as in the current trial, 86% and nearly 100% of women reported amenorrhea after 2 and 3 months respectively (20). The mode of action resulting in this rapid induction of amenorrhea is still uncertain with some suggestions involving effects on vasculature and

especially PR expressing perivascular cells (74). Given the well-established antiproliferative and protective endometrial effects of progesterone and progestins, the PR antagonistic properties of PRMs have raised concerns about if antagonizing the PR could result in unopposed estrogen influence with consequent risk for endometrial hyperplasia and cancer development. For this reason, the presence of PAEC remained a key issue regarding endometrial safety and with the potential of clinical approval of a PRM as continuous treatment, this concern had to be further addressed. There are only two studies evaluating the role of progestins on the endometrium after a continuous PRM treatment regimen on PAEC development. The first study was conducted in women with fibroids and assessed whether presence of PAEC could be reduced by the progestin noretisterone acetate when used for 10 consequent days after a 3-month treatment course of UPA (72). The second study explored the potential of a vaginal ring containing UPA as novel estrogen-free method of contraception and utilized a single dose of levonorgestrel to reduce endometrial thickening (PAEC) and subsequent heavy bleeding (60). Neither study could, with the progestin regimen, type and dose used, display any significant reduction in presence of PAEC despite the fact that shedding of the endometrium was reported to be more effective after treatment with a progestin. Some limitations should be considered. The endometrial samples were retrieved approximately 3 months after cessation of PRM treatment and therefore, related changes might have been missed. In contrast to these studies, no endometrial shedding was part of the protocol in our trial, after continuous treatment with mifepristone and before placement of the LNG-IUS.

Taken together, all PRMs studied until today, in continuous regimens for various indications, seem to be associated with the development of PAEC. It has been suggested that the variety of these endometrial features might differ by agent and dose over time (75). However, in a small study (n=9) exploring the endometrial effect of UPA in women with fibroids, the degree of PAECs was not correlated to the duration of treatment (131). Another study could not demonstrate differences regarding development of PAECs with two different doses of UPA (5 mg vs 10 mg) indicating, in this particular study, an absence of a dose-dependent effect (74). However, the vast majority of the literature reports a dose- and duration-dependent effect.

Our lack of knowledge regarding the specific affinities for various PRMs and their interaction with the different PR isoforms in human and consequently the molecular alterations underpinning PAEC development currently makes it difficult to compare the plausible differences on the molecular level between these compounds. Further, the rate of regression of PAEC on cessation of treatment also seem to be variable (72) and not all women develop PAEC, which accordingly implicates individual variations. Even though PAEC following treatment with PRMs are now considered benign due to lack of atypia and mitotic features, the molecular alterations underpinning their development are not yet delineated. Adopting therefore a more prudent approach, we need a greater understanding of the molecular and cellular mechanisms responsible for these histological features and also distinguishing the different compounds and assess them separately. Thus, more studies are required to better

define PAEC development, long-term effects possibly even without cessation of treatment as well as possible treatments to counteract their appearance in order to expand the use of PRMs and hence allow major improvements in women's health. It can be concluded that PAEC can be ceased by shedding of the endometrium. Our study indicates that placement of a LNG-IUS directly after continuous treatment with mifepristone, could be a safe alternative regarding the endometrium. Whether PAEC could resolve without need of endometrial shedding or even be reversed by the influence of the LNG-IUS, has to be investigated in larger trials.

4.3 STUDY III

4.3.1 Results

Following randomization, 29 women were allocated to mifepristone or the comparator respectively. Only women from the mifepristone arm were included in this sub-study. At baseline, 28 core needle biopsies were retrieved due to one woman withdrawing her consent. Following the 2 months' mifepristone treatment, 25 women provided biopsies since 2 withdrew their consent and one dropped out. Thereafter, one participant was excluded since she did not take the study medication according to the protocol. Finally, 16 randomly chosen paired breast samples contributed to the final analysis.

To study the molecular alterations in the breast following mifepristone treatment, we assessed the changes on mRNA expression levels at baseline compared to after treatment. After RNA sequencing of whole breast tissue, we found 27 differentially regulated genes (DEGs) of which 19 were upregulated and 8 downregulated respectively. The results of the top enriched Gene Ontology terms in each of the three categories (BP, biological process; CC, cellular component; MF, molecular function) for upregulated DEGs were 'extracellular matrix organization', 'collagen-containing extracellular matrix' and 'platelet-derived growth factor binding'. The functional annotation for the downregulated DEGs revealed one significantly enriched term, in category MF, namely 'active borate transmembrane transporter activity' with the involvement of solely one gene.

The Reactome pathway analysis showed that the upregulated DEGs were significantly enriched in 54 biological processes, of which the top 10 were mainly associated with extracellular matrix organization. The downregulated DEGs revealed only two genes involved separately in 6 pathways.

4.3.2 Discussion

The objective of this study was to explore the *in vivo* molecular response of the mammary gland to progesterone antagonism in healthy women. We used next-generation whole transcriptome sequencing (RNA-seq) for the paired samples at baseline and after 2 months' treatment with mifepristone. Subsequent bioinformatics analyses were conducted. The upregulated DEGs were mainly enriched in 'extracellular matrix organization', 'collagen-containing extracellular matrix' and 'platelet-derived growth factor binding' and the

significantly altered pathways in biological processes were also mainly related to the extracellular matrix (ECM) organization.

It is widely accepted that the ECM plays an important role in tissue homeostasis. A constant remodulation is achieved through a complex but far from well elucidated crosstalk between ECM components and adjacent cells during development and the monthly cyclical proliferation and apoptosis of the breast tissue (132). It is therefore of no surprise that aberrant responses in ECM dynamics are proven to be associated with various pathologic conditions, tumorigenesis and cancer invasion (133). In our study, most of the DEGs involved in the enriched pathways code for pro-collagens. Collagens constitute the major structural proteins of the ECM. Evidence points at both collagen content and alignment within the ECM playing a role in tumor cell invasion (134). Furthermore, breast density as assessed by mammography constitutes an independent risk factor for breast cancer development although the underlying mechanisms responsible for this observation are not well understood (86, 134). Studies have shown an increase in mammographic density in women using hormone replacement therapy (97). It has been further suggested that increased collagen production by stromal fibroblasts lead to a firmer ECM resulting in alterations in focal adhesions within the tissue, leading to altered signaling pathways and ultimately distorted epithelial cell behavior (134). In our material, the enriched pathways reflect an increased remodeling of ECM compared to baseline, with both 'collagen degradation' as well as 'collagen formation' emerging as enriched pathways. These findings suggest that ECM may play an important role in PR antagonism in breast tissue which is in line with previous evidence advocating the ECM as an important regulator of homeostasis, tumorigenesis and cancer progression in the mammary gland (132, 133).

In addition to DEGs encoding for pro-collagenases, other DEGs involved in the enriched pathways presented are metalloproteinases which process pro-collagens that will subsequently be deposited as collagen fibrils into the ECM. Metalloproteinases are suggested to exhibit an important role in the physiological process in the breast but also in tumorigenesis, cancer progression and metastasis (132, 135). We found ADAMTS2, a matrix metalloproteinase to be involved in the enriched pathways 'collagen biosynthesis' and 'collagen formation'. Even though members of ADAMTS family have been demonstrated to be involved in angiogenesis and cancer, ADAMTS2 seems to inhibit angiogenesis and consequently tumor growth (136). Furthermore, no significantly altered expression levels could be seen in ADAMTS2 in malignant compared to healthy breast tissue (137). MMP2 is another metalloproteinase involved in the enriched pathways 'degradation of the extracellular matrix' and 'collagen degradation' in our material. Investigated in breast cancer, increased levels of MMP2 have shown an inverse correlation to prognosis while other studies suggested a dual role depending on the stage of the disease (132, 138). In a study conducted in healthy women donors, 15 breast samples were investigated using RNA-seq as in our study (139). The stage of the menstrual cycle was retrospectively assessed by serum progesterone levels collected by the time of the breast biopsy. One biopsy was collected from each woman and therefore the intra- and interindividual variation could not be taken into account. The group

reported a higher expression of matrix metalloproteinases MMP3 and ADAMTS9 in the luteal phase of the women's menstrual cycle. Since the luteal phase represents the proliferative phase in the breast, these findings may suggest that MMPs and consequently the ECM play an important role in breast proliferation. Hence, their counteraction could be hypothesized as a breast protective approach. If this could be achieved by PRM treatment, remains to be demonstrated in larger *in vivo* trials. MMP3 is implicated to be involved in mammary gland branching morphogenesis in mice (139).

Due to availability limitations of mammary gland from healthy women, the vast majority of human studies are conducted from tissue deriving from breast cancer patients. Even though the results may have shed light into different potential models regarding cancer development and progression, the complexity and heterogeneity of the tumors are major obstacles in interpretation of the results. With advanced technologies, a growing number of potential important genes with altered expression are emerging to exhibit dual roles in different stages of the disease making interpretation even more complex. In addition, many comparisons are made between breast cancer tumors and healthy breast, with the latter however excised from the periphery of the breast cancer. This tissue may display benign histopathological features but could already express unfavorable molecular alterations due to the tumor proximity. There is only one previous study exploring the effect of a PRM on breast tissue in healthy women *in vivo* (27). That trial, conducted in our group, used the same mifepristone regimen as we did in the current study but for 3 instead of 2 months and the breast biopsies were collected with a fine needle. The expression of the proliferation marker Ki-67 in the epithelial cells, assessed by immunocytochemistry was downregulated, indicating a potential protective effect of mifepristone in the breast. The current study is the first to explore the molecular alterations, and in particular the transcriptomic landscape in heathy breast tissue *in vivo*, following progesterone antagonism. We used paired biopsies in an attempt to reduce the interindividual variability of the response. Even though the sample size was quite limited, and the samples were not pooled into different cell lineages, given the lack of similar data this study still provides us with unique findings with the ECM seemingly playing an important role in the potential protective effects of PRMs.

A study exploring the impact of UPA in normal breast tissue could not exhibit any impact on the mitotic index (140). Even though the tissue used derived from healthy women, the study was conducted in vitro and in xenografted athymic mice with a treatment duration of 28 days. The results should therefore be interpreted with caution. Furthermore, the differences between mifepristone and UPA should also be considered in terms of affinity towards the other steroid receptors beside the PR. As previously described, mifepristone binds to some extent to the AR, as does UPA albeit to an even lesser extent (15). The androgen testosterone, has shown suppression of proliferation in human breast cancer cell lines (141). In addition, reduced serum androgen levels were found in women after 2 months treatment with COC (EE/levonorgestrel) while their breast biopsies showed increased breast proliferation in comparison to the baseline and the comparator group with no COC use (142). Furthermore, in our previous study where mifepristone showed a significant reduction in the proliferation

marker Ki-67 in the breast, there was also a significant elevation of testosterone (27). These data may suggest that increased androgen levels may have an inverse effect on breast proliferation and that consequently AR antagonism may also play a role.

Epithelial cells in the breast are thought to derive from a pool of precursor cells, the mammary stem cells (MaSCs) which are necessary for the hormonally driven response of the mammary gland (97). Following identification of the mammary stem cell, a lot of studies have been conducted but despite numerous experimental observations the location of MaSCs within the glandular epithelium and their differentiation potential still remains elusive (143). As previously discussed, only a minor fraction of epithelial mammary cells in the normal breast express PR and MaSCs are reported to lack PR (139). Consequently, paracrine signal mediation of progesterone and other PR ligands into adjacent PR- epithelial cells with the help of RANKL and the involvement of ECM could be hypothesized to constitute a central concept.

4.4 STUDY IV

4.4.1 Results

Estradiol and progesterone were measured from daily saliva samples over the course of one menstrual cycle in BRCA1/2 mutation carriers and confirmed non-carriers. Both hormones showed significantly higher levels in the BRCA1/2 group compared to the control group.

High prevalence of DNAme in polycomb-group target genes has been implicated in cancer (144) and with increasing age (145). Potential DNAme sites (CpG dinucleotides) within polycomb-group target genes have been identified and are suggested to define a tick rate that correlates with the estimated number of lifetime accumulated stem cell divisions (mitotic age/replicative age) in normal tissues (146). This mitotic clock, named pcgtAge, has further been refined in this study to generate a more breast specific epigenetic index. This new index, named WID-Breast29 (Women´s risk Index for Breast 29), includes a subset of 37 CpGs in 29 genes selected from the CpGs in the original pcgtAge panel. The CpGs included, were selected based on increased DNAme scores in normal breast tissue from BRCA1/2 women compared to confirmed non-carriers. For validation, WID-Breast29 was applied and scored higher in breast tissue from the vicinity of TNBC than breast tissue from healthy volunteers. Similarly, the index score was higher in tissue from the tumor of TNBC patients compared to its adjacent tissue. In addition, the index was higher in ER+ and PR+ breast cancers compared to that of the adjacent histologically normal breast tissue.

Bulk breast biopsy tissue was used for the above-mentioned comparisons. To minimize the risk of the differences in replicative age being merely a reflection of differences in cellular composition, all tissue samples were assessed again, this time using a well validated algorithm (147). This algorithm was applied sequentially, by first estimation of the proportions of four main cell-types (epithelial, adipose, stromal and hematopoietic) and then further by decomposition of the epithelial compartment into luminal progenitor, mature luminal, and basal (myoepithelial) subtypes. Then, a new custom-defined DNAme was

assembled with reference profiles for these epithelial subtypes based on previously published whole-genome bisulfite-sequencing data (148) by adapting the original algorithm (147) used to obtain these profiles for each individual cell-type. With this new classifier, a marked increase in the number of luminal progenitor cells was demonstrated in TNBC compared to adjacent breast tissue, while the number of mature luminal cells remained unchanged and that of basal cells decreased.

Further, we assessed whether PR antagonism with mifepristone could modulate the DNAme index for replicative age and luminal progenitor cell proportion. WID-Breast29 in conjunction with the epithelial cell-subtype classifier was applied in breast samples of 15 BRCA mutation carriers and 20 controls at baseline and after 2-3 months treatment with mifepristone (11 BRCA carriers and 9 controls) or the comparator (4 BRCA carriers and 11 controls). These volunteers originate from Study I and our ongoing BRCA study. The WID-Breast29 score did not differ in women treated with the comparator, while it was significantly lower in women treated with mifepristone. In the mifepristone treated BRCA mutation carriers, 7/11 (64%) showed a reduction in WID-Breast29, while in the control group the reduction could be detected in all women (9/9). In addition, luminal progenitor cell fraction was not affected in the comparator treated women, while mifepristone exhibited a decrease in 9/9 (100%) of control volunteers and 8/11 (73%) in BRCA mutation carriers. Two of three of the BRCA mutation carriers who did not show a decrease in the fraction of luminal progenitors showed no decrease in their WID-Brest29 index either.

The effect of UPA on breast in women judged to have a higher breast cancer risk, was also evaluated. The comparison between baseline and after treatment biopsies revealed a significant reduction in WID-Breast29 in some women.

Further, we wanted to assess the impact of mifepristone on TP53 mutation frequency in women deemed to respond to mifepristone based on the reduction in WID-Breast29 index. T53 mutations could be detected in 7 of 8 samples analyzed. The TP53 mutation frequency was found to be unaltered in 3 volunteers who showed an increase in WID-Breast29 index. The TP53 mutation frequency showed a significant decrease in 5 volunteers who also had a decrease in WID-Breast29 index.

4.4.2 Discussion

Elevated estradiol and progesterone levels measured in saliva were demonstrated in BRCA1/2 mutation carriers and are in line with previous observations when measured in serum (104). These elevations could be implicated in enhanced progesterone signaling and consequently mitotic action in the breast of BRCA mutation carriers compared to non-carriers. Furthermore, higher WID-Breast29 score in BRCA mutation carriers suggests an accelerated replicative (mitotic) age of their mammary epithelium as in tissue adjacent to cancer. This observation may explain their increased breast cancer susceptibility.

In our two clinical trials (Study I and the BRCA trial), none of the women in the comparator arm showed any alterations in WID-Breast29. The healthy women from Study I, treated with

mifepristone, showed all a reduction in WID-Breast29. In BRCA1/2 mutation carriers, 7 out of 11 (64%) showed a reduction. This could imply a PR independency adopted in these particular women. Further, luminal progenitor cell fraction was not altered after the comparator intake, while in the mifepristone group, 100% and 73% of the control and BRCA group respectively showed a decrease. PR activation of luminal epithelial cells have been shown to drive proliferation of adjacent PR- cells in mice through paracrine mechanisms via RANKL (94). In humans, RANKL expression has been demonstrated in breast tissue of women with high serum progesterone levels (95). Hence, a protective effect following mifepristone could be hypothesized. Upon assessment in women judged to have a high risk for breast cancer, UPA, another PRM, could demonstrate similar effects assessed by WID-Breast29. These results are in contrast to the studies of UPA in breast tissue from healthy women assessed in vitro and xenografted in athymic mice (140). This discrepancy may reflect the differences between the *in vivo* and *in vitro* milieu. Further, the duration of treatment was one month in the above-mentioned *ex-vivo* study and the assessment was based on immunohistochemistry. However, the number of participants were few in both studies and the results have to be further confirmed.

The WID-Breast29 index presented in this study is proposed as a DNAme based index reflective of the mitotic age in the breast. It seems mainly correlated with the change in luminal cell progenitor fraction. Moreover, WID-Brest29 score is highest in breast cancer cells, followed by breast tissue from tumor adjacent sites and is lowest in mammary epithelium from healthy women. The number of luminal progenitor cells and the WID-Breast29 index is reduced following PRM treatment, in BRCA1/2 mutation carriers, non-carriers and women with a higher risk for breast cancer. These data strengthen the prospects of progesterone receptor antagonism as an attractive approach to be further evaluated, in particular in BRCA mutation carriers in which the most effective means of cancer prevention in still the surgical removal of organs at risk. Monitoring patient responsiveness of risk reduction trials could be facilitated using the WID-Breast29 index.

4.5 METHODOLOGICAL CONSIDERATIONS

4.5.1 Study I

Study I, is a randomized double-blind placebo controlled trial and as such provides one of the most robust levels of scientific evidence. When well conducted, the randomization inclusively blinding process reduces selection and allocation bias and subjects as well as investigators expectations, and thus known and unknown confounding variables. Hence, it improves internal validity by reducing random as well as systematic errors (bias) and consequently improves external validity (generalizability) (149, 150). The per protocol analysis and possible bias from women that were lost to follow-up (attrition bias) or discontinued for various reasons is a limitation of our study.

The mifepristone dosage used was 50 mg every other day. The rationale for this treatment regimen was that we aimed for the lowest possible dose with proven effect on amenorrhea.

However, mifepristone tablets below 200 mg are not available in Europe. A high dose-dependent induction of amenorrhea ranging from 63-100% has been reported during mifepristone treatment with low continuous doses, varying between 2-50 mg daily (19, 151). In addition, in the previous study conducted in our group, the same treatment regimen as in Study I, resulted in 86% of amenorrhea within two months (20). Given the long half-life of mifepristone, we assumed that 50 mg every other day would correspond to a daily dose of approximately 25 mg. We could at the same time overcome our lack of access to low dose tablets by dividing the available 200 mg tablets into 4 parts. According to the available data presented above, even a lower continuous dose could induce amenorrhea but at a lower rate.

The effect of pretreatment with a PRM on bleeding disturbances following progestin treatment had never previously been explored and appeared as an attractive approach to assess. At the same time, it is well known that commencement of contraception as prompt as possible after decision making is beneficial for women by reducing their time at risk for undesired pregnancy. With this fact in view, we decided to shorten the pretreatment period to 2 months. Since amenorrhea following PRM treatment is also dependent on duration of treatment, we postulated that an even shorter pretreatment period would be too brief to induce amenorrhea in a sufficiently high proportion of women in our cohort. Despite these efforts and comprehensive counseling regarding the need of contraceptive use (condoms) throughout the pretreatment period and in addition 7 days after the LNG-IU placement, 3 study participants got pregnant. All the undesired pregnancies were in the comparator arm. These unfortunate events stress the need of any eventual pretreatment regimen to possess contraceptive properties.

We used a quarter of a table TrioBe® every other day as a comparator in our study. Investigations regarding possible effects of B vitamins on the menstrual cycle are scarce. One study reported a statistical significant difference on bleeding and spotting in women with a copper IUD treated with daily vitamin B1 100 mg compared to placebo (152). No measurements of micronutrient status were conducted at baseline and therefore, it is not known if some women had a B1 deficiency at study start which have been corrected by B1 vitamin intake and may thereby influenced the study results. B1 is not included in TrioBe®. Another study assessed the dietary intake of B2, B6 and B12 on ovarian function of healthy premenopausal women. No statistical significant associations could be observed between intake of B vitamins and ovarian cycle function (153). In our study, we assumed that the effect of TrioBe® on menstrual bleeding patterns, if any, would be negligible with the current dose used.

No liver function evaluation was conducted during our trial. Cases with transient elevations of serum liver transaminases have been reported and varied depending on the PRM used, the dose and duration of treatment. Our study was conducted prior to the alarm regarding liver failure during UPA treatment for uterine fibroids. In hindsight, measurements of transaminases during study treatment would obviously be reassuring. Nevertheless, no elevation of transaminases was measured during the 3-month treatment with the same PRM

and treatment regimen in our previous study (20). Moreover, no participant reported symptoms suggestive of liver injury during our trial with the last telephone follow up 12 months after the LNG-IUS insertion.

Study participants estimated their vaginal bleeding according to the bleeding scale provided. There are several methods to measure menstrual bleeding more precisely such as the alkaline hematin and different pictorial assessments. Even if self-assessment is of course objective, it is ultimately the one that accounts for satisfaction, acceptability and possible premature discontinuation of the LNG-IUS in the general population.

4.5.2 Study II

There are several strengths in this trial. The participants were derived from the RCT of Study I and the endometrial assessment was conducted by one pathologist who was blinded to the treatment. He is also one the most recognized pathologists regarding PAEC.

Since this was a secondary outcome and in a sense a pilot study and no previous similar studies are reported in the literature, no power estimation has been conducted. Further, in an attempt not to interfere with the primary outcome of Study I, no endometrial biopsy was retrieved after the 2 months treatment with mifepristone and prior to placement of the LNG-IUS. As previously discussed, due to this reason, we are not aware of the proportion of women that actually had developed PAEC by the end of the pretreatment period. Given the lack of similar studies though, our results implicate that a larger, properly powered trial, would be of great interest to further test the hypothesis that LNG-IUS for contraceptive purposes could be adopted without prior endometrial shedding after continuous treatment with PRMs.

4.5.3 Study III

A significant strength of Study III is the use of human *in vivo* biopsies from a randomized controlled trial. As discussed throughout this thesis, breast biopsies from healthy women are valuable since they are extremely scarce.

The breast biopsies were retrieved under ultrasound guidance. Experienced radiologists are able to, by means of sonography, distinguish the various anatomical structures within the breast and retrieve targeted biopsies from the most glandular areas within the mammary gland (154). One experienced radiologist collected all breast biopsies on our trial, minimizing thus the interobserver variability introduced by several investigators. The follow-up biopsies were collected from the same area also under ultrasound guidance. The same approach has been adopted in other studies previously, in an attempt to minimize variations in localization, due to the well-known heterogeneity and regional differences within the breast (27, 80). Furthermore, biopsies were collected with a core needle providing us with enough material for our downstream analysis. Fine needle aspiration (FNA) biopsies provide exclusively cells as material, often with low cellular content (27) instead of tissue, as in core biopsies.

Molecular alterations precede morphological and biological modifications of various responses. With advanced technologies, attention has been able to shift from functional and morphological changes of various diseases, drug responses and developmental stages in physiological conditions, to the molecular mechanisms underpinning these changes. NGS technology has the last decade revolutionized the field of differential gene expression studies since they offer scalability, high-throughput and consequently speed. High-throughput sequencing (massive parallel sequencing) allow for measurement of gene expression quantification of tens of thousands of genes in one experiment (155). RNA-seq is of particular interest since it reflects a snapshot of the entire transcriptional profile instead of a predetermined subset of genes as with reverse transcription PCR as well as microarrays. In addition, with RNA-seq, it is possible to detect novel transcripts and sequence variations of the transcribed regions (156). Consequently, for the above-mentioned reasons and due to the scarcity of healthy breast tissue, we considered RNA-seq as the best available option to assess our material.

Despite the advantages of high-throughput technologies, analysis and interpretation of the gene lists generated are challenging, and would be practically impossible to assess by manual literature search. Therefore, there is today hundreds of databases providing pathway enrichment analysis which makes interpretation more feasible and offer useful insights into biological mechanisms. We used the free-online database g:Profiler for the functional annotation and pathway enrichment analysis. This software is currently one of the most up-to-date databases (157). GO was used for the functional annotation (attachment of biological functions and their relationships to gene sets) of the DEGs in our data set. Further, Reactome was used for the pathway enrichment analysis. Reactome is a human pathway database manually curated (experimentally validated) and peer-reviewed. Pathway enrichment analysis identifies biological pathways that are enriched (over-represented) in the gene list, as compared to all genes in the human genome, more than it would be expected by chance (157). Given the plethora of databases, there is an ongoing debate regarding which ones are more appropriate to use. The answer, if there is one, is complicated. There is a number of factors that should be considered upon selection for downstream analysis depending on the hypothesis and the experiment. In experiments dealing with human tissue, a database of human pathways should be utilized. Furthermore, an updated and curated peer-reviewed database is considered better to use, rather than a non-curated one which implies minimal standardization and a wide degree of freedom for data documentation. There are also some points to be considered in general. First, the pathway boundaries of various databases tend to be arbitrary, giving occasionally diverse results. Some scientists argue that different databases should be tested upon to decide which interprets the experimental data better. Second, the gene lists incorporated are biased towards well-known pathways. In fact, genes with no systematic pathway annotation are ignored in pathway enrichment analyses. Results are therefore also changing overtime as more data are integrated into the databases. Third, genes that are multi-functional may lead to enrichment of many biological pathways. Some of them may not be necessarily relevant to the conducted experiment and therefore, some suggest that

those genes should be excluded from the analysis. Fourth, enrichment analysis is more effective for pathways where various differentially expressed genes (genes with strong biological signals) cluster. As a consequence, pathways controlled by only a small number of genes will not be detected as enriched. That doesn't necessarily mean that these genes are not important regulators. Fifth, the statistical analysis incorporated, most commonly FDR (a multiple testing correction method) is often more or less conservative than optimal. In any case, statistical significance or not, may not always equal to absence or presence of biological significance. Nevertheless, it should still be utilized for exploratory analysis and hypothesis generation (157). In summary, these quite new methods provide a huge amount of new information with tremendous possibilities. The interpretation of the data though, is still challenging with potential of improvement. Until further, validation with more conventional methods of any given results should be considered.

The current mifepristone dose and dosage used, has previously shown a reduction of breast epithelial cell proliferation in healthy breast after 3 months of treatment (27). In our study, the treatment period was 2 months, in attempt to reduce the time to the LNG-IUS insertion as described for Study I. Trials on COCs have demonstrated breast epithelial cell proliferation after two months of treatment (142) and breast biopsies in healthy women with no hormonal treatment could show an increase in proliferation in mid-luteal phase compared to early follicular phase within one menstrual cycle (80). Based on these observations, we assumed that a 2 month treatment with mifepristone would be enough to demonstrate changes in the mammary gland.

Even though breast biopsies in our study were retrieved under ultrasound guidance in an attempt to include as much glandular tissue as possible, the specimen retrieved for downstream analysis should be considered bulk (mixed) tissue. Bulk tissue in RNA-seq experiments is a widely-adopted method to study transcriptomic alterations in different conditions. However, given bulk tissue heterogeneity, the specific cell types cannot be discerned and spatial information is therefore not preserved (158). Hence, our results may be biased due overrepresentation of a certain cell type in the biopsy and we cannot rule out that additional significant results are diluted and therefore not represented in the list of DEGs in our study. Nevertheless, since transcriptomic signature following PR antagonism has never previously been explored, investigation of the whole tissue may provide a more comprehensive approach and reveal specific points of penetration for future research.

4.5.4 Study IV

Changes in the epigenetic landscape is commonly assessed by evaluation of the total amount of methylation present in the DNA which subsequently affect gene expression. In this study, a breast specific epigenetic index was developed and used as a surrogate marker in 3 different patient cohorts to further assess the impact of PRMs in breast tissue. This was a collaboration with well-known experts in the field and established algorithms were used. Another strength is the *in vivo* biopsies included. Having BRCA mutation carriers participating in this study is also a strength since these patients have a higher risk for breast cancer in young ages. They

may represent “an *in vivo* model” with accelerated processes leading to cancer development. It is therefore interesting to evaluate any impact of treatment to gain deeper insight in steroid-related alterations in the mammary gland. There are several limitations such as the small cohorts included. Further, the BRCA mutation status is not known in the women included from Study I and the duration of mifepristone treatment differs. As previously discussed, the treatment duration in Study I and the BRCA trial was 2 and 3 months respectively. This difference could have an impact treatment response, even if it was not reflected in the results of this particular study. The algorithms used as surrogate markers for risk of breast cancer development need further evaluation in bigger cohorts to prove consistency.

5 SAFETY OF LONG-TERM TREATMENT WITH PROGESTERONE RECEPTOR MODULATORS

Side effects following continuous low dose PRMs are in general mild and transient with the most common reported to be headache and hot flushes (73). In patients with Cushing's syndrome that receive ≥300 mg mifepristone daily, the most common adverse effects reported are nausea, fatigue, headache and hypokalemia (159).

As previously described, mifepristone, as well as UPA, also bind to the GR in a dose dependent manner. Single doses of mifepristone exceeding 200 mg are capable of activating the hypothalamus-pituitary-adrenal (HPA) axis, reflected by rises in serum concentrations of cortisol, adrenocorticotropic hormone (ACTH) and dehydroepiandrosterone sulfate (DHEAS). When mifepristone is given in lower continuous doses, 50 mg daily is reported to exhibit a raise in serum concentration of cortisol and DHEAS at 12 weeks of treatment (28). Mifepristone at a dose of 25 mg every other day (27) and daily doses of 10 mg (58) did not affect cortisol levels. It should though be noted that another trial could not detect any significant changes in cortisol levels with 50 mg daily compared to placebo (22) and no clinical signs of adrenal failure have been reported with low treatment doses despite the alterations of serum cortisol levels (160). However, the studies are rather few with limited number of participants.

PRMs have been proven not to induce hypoestrogenism despite their ability to block follicular growth and induce amenorrhea (57, 161). Bone mineral density remained unaltered in a small study (n=9) in women treated with mifepristone 50 mg daily for 6 months (22).

Subjects with elevation of liver enzymes have been reported in trials using continuous doses of different PRMs for various indications. The assessment of this side effect and whether it is a class effect and to which extend, is tremendously impeded by a number of factors. The affinity, selectivity and pharmacokinetic as well as pharmacodynamic differences between different compounds. Further, the variations in dose, dosage and duration of treatment adopted in various trials. While most studies report mild and transient elevation of liver enzymes even without cessation of PRM treatment, phase 3 trials have been suspended due to liver toxicity in women treated with the PRM telapristone acetate 50 mg daily (1, 116). Clinical trials have thereafter been employed using lower doses of telapristone acetate and alternative routes of administration (Table 1, section 1.2) (1). The development of another PRM, onapristone, was also stopped due to hepatotoxicity. Recently, an extended-release oral formulation of onapristone has been developed and is currently tested in a phase 2 trial in PR+ gynecological cancers (Table 1, section 1.2) (162). UPA was authorized in the European Union in 2012 for treatment of moderate to severe symptoms of uterine fibroids. In 2018, five cases of lever injury were reported in women treated with UPA, of which four required liver transplantation. The European Medicines Agency decided upon repeated measurements of liver transaminases to minimize the risks, with the recommendation of cessation of treatment when transaminases exceeded three times the upper normal limit. Despite these measures, one more liver transplantation was eventually required, which led to the current

recommendations of UPA being prescribed for treatment of uterine fibroids only when surgery is not possible. In summary, five cases of liver transplantations due to serious liver injury occurred in over 900.000 women treated with UPA (163). Single-dose treatment with UPA for EC is not affected by the restrictions described above.

Continuous doses of PRM are also associated with PAEC, as reviewed and discussed previously in this thesis.

6 ETHICAL PERMITS AND CONSIDERATIONS

The study protocols were designed according to the recommendations in the CONSORT statement. They were approved by the ethical committee at Karolinska Institutet, Stockholm Sweden. ***Study I, II, III:*** Dnr: 2009/144-31/4. ClinicalTrials.gov identifier: NCT01931657. Further, approval was obtained from the Swedish Medical Products Agency (EudraCT number 2009-009014-40).

In ***Study IV***, participants from Study I were included as well as BRCA mutation carriers from clinical trial with Dnr: 2012/729-31/1, 2014/703-32 and ClinicalTrials.gov identifier: NCT01898312. Approval was also obtained from the Swedish Medical Products Agency (EudraCT number 2012-003703-35). For the other participants, ethical permits have been obtained from respective regulatory authority.

All participants gave written informed consent prior to randomization and received oral and written information inclusively the lack of contraceptive properties in both the active as well as the comparator substance. Participants past medical and reproductive history was reviewed and they were carefully examined. They received contact details (telephone number and e-mail address) to the WHO center for any additional contact if needed, apart from the planned return visits. Women with abnormal Pap-smear findings were excluded from the study and referred to our gynecological department for further investigations as per clinical routine. The LNG-IUS is a well-documented, highly effective LARC. The insertion cause patient discomfort which usually resolves rapidly. All women were offered paracetamol prior to the insertion. A vaginal ultrasound was conducted before and after placement of the device to ensure optimal localization. No uterine perforations occurred. Mifepristone side effects with the dose and treatment regimen used are mild and usually transient. To reduce the time to LNG-IUS placement, the pretreatment period in the trial was reduced to 2 months. Despite these efforts, three pregnancies occurred in the comparator group which emphasizes the need for a reliable contraceptive potential in any pretreatment regimen used in clinical practice. The participants that got pregnant decided to terminate the pregnancy and were referred to our gynecological clinic for further care.

All biopsies were coded after retrieval to ensure anonymity before transportation to our research laboratory. The collection of biopsies are invasive procedures which cause patient discomfort. Study participants received a detailed explanation of the study procedures, local anesthetics were used for the retrieval of the breast biopsies and all patients were informed that they could deny further participation in the study even without stating reason. This would by no means affect possible future treatments. Study participants that did not wish for a LNG-IUS placement or wanted their IUD removed, were offered comprehensive counselling regarding other contraceptive alternatives.

7 CONCLUSIONS

- Mifepristone given as pretreatment prior to LNG-IUS insertion resulted in amenorrhea. After pretreatment the difference in bleeding and spotting days did no longer reach statistical significance and the vaginal bleeding patterns were similar within the two study groups.

- Following placement of a LNG-IUS 52 mg directly after continuous treatment with mifepristone, with no prior endometrial shedding, no PAEC was observed. Larger appropriately powered trials are needed to confirm this reassuring finding.

- Transcriptome analysis of human breast tissue exposed *in vivo* suggest that the PRM mifepristone alters the structural organization and ECM composition of the mammary gland.

- Epigenetic alterations in breast tissue following PRM treatment, may suggest a potential favorable effect in healthy, but also BRCA mutation carriers and other women with increased risk for breast cancer. Validation of the surrogate markers used in this study is warranted prior to further clinical investigations.

8 FUTURE PERSPECTIVES

Undesired bleeding irregularities in progestin-only contraceptive users remain an unsolved issue despite the attempts that have been adopted to find a treatment. Surely, new approaches, or agents already investigated in various trials, may exhibit more favorable results if utilized in different treatment regimens. Several candidate components or processes have emerged as contributing factors to these bleeding disturbances. With advancing technological development, new possibilities for investigations arise. It is important to distinguish between bleeding irregularities in new progestin-only users and those that develop after some period of use. The underlying mechanisms are probably different and should therefore be addressed separately.

As previously discussed, our knowledge regarding normal breast development and differentiation is scarce, in particular during fetal and pubertal life due to, for obvious reasons, access limitations to healthy tissue. The mouse has become a widely-used model to study molecular and cellular interactions within the tissue, in an attempt to understand the biological mechanisms responsible for various events in breast development and ultimately cancer initiation. However, even though studies in mice have addressed various issues, analogies should be made with caution, in particular considering the complexity of endocrine and paracrine signaling. High-throughput technologies can contribute enormously to the amount of information. A newer technological advantage, single-cell sequencing, can provide the opportunity of studying single cells, which could be more informative than bulk tissue where information can be diluted. Interpretation of the data these technologies generate have great opportunities for improvement and further standardization. The recent possibility to conduct profiling studies on formalin fixed archival tissue will definitely open up for analysis of the healthy human breast tissue shedding light into our large knowledge gaps of breast tissue homeostasis, regulation and consequently cancer formation.

Although more investigations are necessary to explore the potential beneficial effects of PRMs in the human breast, PR antagonism sheds light into progesterone action. The mammary gland of BRCA mutation carriers may serve as a model where regulation or dysregulation will help us gain information on healthy breast but also hopefully guide as towards risk-reduction strategies for women with BRCA or other mutations in women with high risk for breast cancer. In addition, the ability of PRMs to block ovulation and ultimately follicular development could hypothetically reduce ovarian cancer risk either by these mechanisms per se or by direct impression.

Continuous treatment with PRMs holds the potential to be used in clinical applications covering a broad field within the female reproductive system and PRMs with more specific steroid receptor specificity are under development. If an antiproliferative effect of mifepristone and other PRMs in the breast could be proven, it would be tremendously beneficial in view of expanding indications of PRMs such as for contraceptive purposes and more. Prospective clinical trials are necessary to prove this potential.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund till studierna

En hormonspiral frisätter levonorgestrel (LNG), ett syntetiskt gulkroppshormon (gestagen). Det finns numera hormonspiraler med tre olika LNG koncentrationer. I vår studie använde vi en spiral (IntraUterine Device – IUD) som innehåller 52 mg LNG (LNG-IUS 52 mg). LNG-IUS 52 mg ger ett effektivt skydd mot befruktning genom att livmoderhalsens sekret förtjockas och spermietransport samt funktion försämras. Dessutom har den en uttalad effekt på livmoderslemhinnan som hämmas och minskar menstruationsblödningarna trots att ägglossningarna är opåverkade. Hormonspiral är ett mycket säkert preventivmedel som används av en hög andel kvinnor i hela världen. Förutom fertilitetskontroll är rikliga blödningar en viktig indikation för användning av LNG-IUS 52 mg. Studier har visat att blödningsmängden kan minska med upp till 97% under första behandlingsåret och upp till 50% blir helt blödningsfria under behandlingstiden. En känd orsak till att kvinnor inte finner behandling med hormonspiral tillfredsställande är att de första månaderna under behandlingen karakteriseras av ett oregelbundet blödningsmönster och småblödningar (spotting) vilket drabbar ca 25–62% av användarna. Olika preparat har testats för att minska de besvären men än idag finns ingen etablerad behandling. I tidigare studier har man observerat att kvinnor som efter behandling med sin första hormonspiral får sin andra insatt, har mindre besvär med oregelbundna blödningar och att andelen kvinnor som blir helt blödningsfria ökar. Vår teori är att detta beror på att den första hormonspiralen orsakat en tunn och hämmad livmoderslemhinna. Om man därför, innan insättning av en hormonspiral, kunde ge en förbehandling som orsakar en hämmad slemhinna och blödningsfrihet, skulle det initiala blödningsmönstret efter spiralinsättningen förbättras.

Som förbehandling innan spiralinsättningen har vi använt läkemedlet mifepriston. Mifepriston är en så kallad progesteronreceptor-modulator (PRM). Det är ett syntetiskt framställt hormon som verkar främst som ett antiprogesteron, d v s hämmar gestagenets effekter på receptornivå. Mifepriston utövar ett flertal effekter på det reproduktiva systemet beroende på när under menscykeln och i vilken dos det administreras. Studier visar att kontinuerlig behandling med mifepriston bland annat kan minska blödningar samt inaktivera livmoderslemhinnan och därmed leda till blödningsfrihet efter några dagars behandling. Studier har också visat att mifepriston kan krympa muskelknutor i livmodern och minska endometriosförändringar och besvär som följd av det. Mifepriston kan även potentiellt fungera som ett östrogenfritt preventivmedel.

Långtidsbehandling med olika PRM har visats orsaka en förtjockad livmoderslemhinna vid underökning med ultraljud. Vid undersökning i mikroskop har man sett att de förändringarna verkar ha ett typiskt utseende och de uppkommer efter PRM behandling. Förändringarna kallas ”progesterone receptor modulator associated endometrial changes” (PAEC). Det innefattar bland annat vätskefyllda körtlar och inaktivitet i cellerna trots att

livmoderslemhinnan vid undersökning med ultraljud ser förtjockat ut. PAEC anses vara ett godartat tillstånd men p g a att mekanismerna bakom dess uppkomst idag är okända vill man undersöka dem vidare samt hitta sätt att motverka förändringarna tills vi vet med säkerhet att de inte har en negativ inverkan.

Vidare har gulkroppshormonets och gestagenernas effekt på bröstvävnad genom åren varit omdiskuterad med studier som har visat på en möjlig negativ effekt på vävnaden. I en mindre klinisk studie i vår grupp kunde vi se en tillväxthämmande inverkan av mifepriston på bröstvävnad vilket kan tyda på en skyddande effekt. Vi planerade därför även att undersöka mifepristonets effekt på bröstvävnaden hos friska kvinnor och kvinnor med känd ökad risk för bröstcancer.

Studie 1

I studie 1 undersökte vi hur mifepriston som förbehandling påverkar blödningsmönstret efter insättning av LNG-IUS 52 mg. Vi rekryterade 58 friska kvinnor som önskade hormonspiral som preventivmedel. Av dessa, lottades 29 till två månaders förbehandling med mifepriston och 29 till en kontrollbehandlings- (placebo-) grupp innan spiralinsättningen. Kvinnorna fick skatta sina blödningar enligt en förbestämd skala (0: ingen blödning, 1: spotting, 2: sparsam blödning, 3: normal blödning och 4: riklig blödning) i en blödningskalender, under förbehandlingen med mifepriston/placebo till och med sex månader efter spiralinsättningen. Kvinnorna följdes upp regelbundet med ultraljud och blodprover. De fick också fortlöpande dokumentera och rapportera in biverkningar. Efter studien kunde vi se att de flesta kvinnorna under förbehandling med mifepriston blev blödningsfria. De tycktes också ha lite färre blödningsdagar första månaden efter spiralinsättningen än gruppen som erhöll placebo, men skillnaden blev inte statistiskt säkerställd. Resterande studietiden var blödningsmönstren mellan de två studiegrupperna jämförbar. Inga allvarliga biverkningar rapporterades och majoriteten av kvinnorna var nöjda med valet av preventivmedel. Vi kunde således med vår behandlingsregim inte påvisa några statistiskt säkerställda skillnader mellan de två studiegrupperna.

Studie 2

Studie 2 innefattar samma studiedeltagare som Studie 1. Målet med den studien var att undersöka livmoderslemhinnan ur en säkerhetsaspekt efter två månaders behandling med mifepriston och därefter direkt insättning av hormonspiralen utan att tillåta en menstruationsblödning emellan. Ett vävnadsprov från livmoderslemhinnan togs vid studiestart, innan kvinnorna började med mifepriston eller kontrollbehandling. Ett nytt prov togs 3 månader efter insättningen av hormonspiralen med spiralen på plats. Vävnadsproverna bedömdes av en patolog som är expert på PAEC. I den slutliga analysen ingick prover från 9 kvinnor i mifepriston- respektive 8 från kontrollgruppen. Alla prover var godartade och PAEC kunde inte påvisas i någon utav grupperna. Denna behandlingsregim kan således vara ett säkert alternativ för livmoderslemhinnan. Med tanke dock på att antalet deltagare som ingick i vår analys var få, krävs större studier för att bekräfta denna slutsats.

Studie 3

Studie tre innefattar samma studiedeltagare som Studie 1. Tidigare har en studie visat att 3 månaders behandling med mifepriston minskar andelen bröstceller som aktivt delar på sig. Ökad celldelning i vävnaden kan ge upphov till genetiska fel och betraktas därför som en riskmarkör för uppkomst av cancer. Målet med studien var att utföra analyser med nyare metoder och undersöka mifepristonets effekt i bröstvävnaden. Vävnadsproven från bröstet togs med en provtagningsnål i lokalbedövning med hjälp av ultraljud. Ett prov togs före behandlingsstart och det andra efter 2 månaders behandling med mifepriston eller placebo. I slutanalysen ingick 16 kvinnor från mifepriston gruppen. Vi analyserade mRNA (molekyler som fungerar som mellanhänder för kodningen av gener till proteiner). När vi jämförde proverna innan och efter behandlingen kunde vi se att det mRNA som var överrepresenterat i vårt material efter mifepristonbehandling tillhörde extracellulär matrix (ECM). ECM är den substans som finns mellan celler. Reglering av ECM, där flera komplexa och ofta okända mekanismer och processer är iblandade, spelar en central roll i vävnadens homeostas (balans och stabilitet). Obalans i ECM-reglering har visats vara av stor betydelse för cancerinitiering, tillväxt och spridning. Framtida studier designade för att undersöka vidare den eventuellt skyddande effekten av PRM på bröstvävnad borde ta hänsyn även till ECM och dess påverkan.

Studie 4

Studie 4 är en hypotesgenererande studie med internationella samarbeten. Huvudfokus var att undersöka PRMs epigenetiska effekter i bröstvävnaden hos friska kvinnor men även kvinnor med känd ökad risk för att utveckla bröstcancer. Epigenetik undersöker förändringar i genuttryck (om en viss gen är aktiv eller inte), oberoende utav förändringar i själva generna. Specifika epigenetiska förändringar har visats vara associerade med utveckling av olika typer av bröstcancer. Flera experimentella metoder användes för att undersöka effekten av mifepriston. Effekterna av mifepriston jämfördes också med effekten av ulipristal acetat, en annan PRM. Resultaten talar för att PRM kan ha en skyddande effekt i bröstvävnaden genom både epigenetiska och genetiska effekter. De använda metoderna bör dock valideras (utvärderas) och större studiegrupper krävs för att kunna bevisa ett säkert samband.

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