MIFEPRISTONE FOR PREOPERATIVE TREATMENT OF UTERINE LEIOMYOMA

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ABSTRACT

AIM: To explore the clinical impact and its molecular regulation on uterine leiomyomas in preoperative treatment with mifepristone, a progesterone receptor modulator (PRM).

BACKGROUND: Uterine leiomyomas are highly prevalent in fertile women, increasing with age up to 35-50 % in a population approaching the age of 50. These most often benign tumors, frequently cause menorrhagia, and may interfere with fertility and the outcome of pregnancy. Progesterone and estrogen have a role in leiomyoma growth regulation, as well as in endometrial and breast cell proliferation and the development of endometrial and breast cancer. The objective of the current project was to study the effect of mifepristone on leiomyoma growth, as well as on cell proliferation in human endometrial and breast tissue in premenopausal women.

METHODS: Thirty premenopausal women scheduled for surgical treatment due to uterine leiomyoma were randomized to either 50 mg mifepristone or non active treatment every other day, for 12 weeks before surgical intervention. Uterine and leiomyoma blood flow and leiomyoma volume were measured once a month until surgery. Endometrial biopsies were obtained and analyzed before and at end of treatment. Breast biopsies were assessed at baseline and at the end of the study for the expression of Ki-67 by immunocytochemical analysis in order to evaluate mammary epithelial cell proliferation. On surgery biopsies were collected from the periphery of the dominant leiomyoma. In order to investigate the gene expression leading to volume change in myoma, microarray analysis followed by Real time PCR analysis was performed. The degree of apoptosis was studied by TUNEL. Functional studies using primary cell cultures from fresh and untreated leiomyoma biopsies were performed to investigate the antiglucocorticoid response of mifepristone in the Integrin pathway.

RESULTS: There was a significant difference in percentual volume regression of the dominant leiomyoma between the treatment groups (p=0.014). The controls (N=15) had a percentual n.s. increase in volume of mean (± 95% Confidence interval), +8% (-10%, +26%) over time. The mifepristone group (N=12) had a significant volume regression of -27% (-47%, -8%), p=0.028 within the mifepristone group and between the treatment groups at the end of study (p=0.014). Mifepristone treatment significantly reduced the number of bleeding days (p<0.001) and increased blood haemoglobin values (p=0.046). The breast Ki-67-index was significantly reduced by mifepristone treatment (p=0.012). Breast symptoms, like soreness (p=0.035), swelling (p=0.028) and the score for sense of increased volume (p=0.043), were reduced within the mifepristone treated group. The incidence of hot flushes was more frequent in the mifepristone treated group (p=0.012). Endometrial morphology showed no hyperplasia or atypia. Microarray displayed 17 pathways significantly changed by mifepristone exposure, among which Integrin, EGF, NRF-2 mediated Oxidative Stress response and Ephrine pathways were the top 4 most significant. In a subgroup analysis of good (N=4) versus poor (N=4) responders, with regard to the effect of mifepristone in induction of volume regression, the Glutathione pathway was the second most significant. Among good responders GSTM1 was expressed, while it was not detectable by Real time PCR in non responders. In primary cell cultures, the genes PIK3R1 and PAK3 proved to respond to the antiglucocorticoid effect of mifepristone.

CONCLUSION: Mifepristone may offer an effective, well tolerated preoperative treatment option for women with uterine leiomyoma and the associated uterovaginal bleeding. Our results also show an antiproliferative effect of mifepristone in normal premenopausal breast epithelium, implicating a possible protective effect. It is suggested that GSTM1 may be of importance for the response in leiomyoma volume regression as induced by mifepristone, and could have a role as a biomarker for tailoring of the medical treatment of uterine leiomyomas.

LIST OF ORIGINAL PUBLICATIONS

I. **Engman M, Skoog L, Soderqvist G, Gemzell-Danielsson K** 2008 The effect of mifepristone on breast cell proliferation in premenopausal women evaluated through fine needle aspiration cytology. Hum Reprod 23:2072-2079

II. **Engman M, Granberg S, Williams AR, Meng CX, Lalitkumar PGL, Gemzell-Danielsson K** 2009 Mifepristone for treatment of uterine leiomyoma. A prospective randomized placebo controlled trial. Hum Reprod 24:1870-1879

III. **Engman M, Byström B Varghese S, Lalitkumar P.G.L, Gemzell-Danielsson K** (2011) Glutathione pathway gene expression determines good or poor response to mifepristone in myoma volume regression (Submitted).

IV. **Engman M, Brett G, Varhese S, Gemzell-Danielsson K, Lalitkumar P.G.L** (2011) Ephrin and Integrin pathways in uterine leiomyoma during mifepristone treatment (Manuscript).

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CONTENTS

ABBREVIATIONS

AF- 1,2,3	Activating functions 1, 2 and 3. AF-3 is specific for PRB
BSI	Breast Symptom Index
COC	Combined Oral Contraception
CREB5	cAMP responsive element binding protein 5
DBD	DNA Binding Domain
E2	Estradiol
EA	Endometrial ablation
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FNA	Fine Needle Aspiration
GRIN2A	Glutamate receptor, ionotropic, N-methyl-D-aspartate, subunit 2A
GSTM1	Glutathione- S- transferase mu 1
HMB	Heavy menstrual bleeding
HPF	High power field in the microscope
HSP	Heat shock proteins
HT	Hormone Treatment
ID	Inhibitory Domain
IGF-1	Insulin like Growth Factor-1
LBD	Ligand Binding Domain
DBD	DNA Binding Domain
MEC	Mammary Epithelial Cell
MF	Mitotic figures
MI	Mitotic index
P4	Progesterone
PAK3	p21-activated kinase 3
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1
PRA	Progesterone receptor A
PRB	Progesterone receptor B
PRE	Progesterone response element
PRM	Progesterone Receptor Modulator
ROR1	Receptor tyrosine kinase-like orphan receptor
RTK	Receptor Tyrosine Kinase
SPRM	Selective Progesterone Receptor Modulator
SRC-1, SRC-2	Steroid receptor coactivator-1 and -2
TMX	Tamoxifen
TVS	TransVaginal Sonography

1 INTRODUCTION

Uterine leiomyomas are composed of fibro muscular monoclonal fusiform smooth muscle cells, clones of a single mutated myometrial cell. This was concluded by non random inactivation of X chromosome, demonstrated by electrophoresis of glucose 6 phosphate dehydrogenase isoform expression (G-6-PD) (1). Myocytes and fibroblasts are intermingled and oriented in different interconnected planes creating a randomly arranged multidirectional network of tissue bundles in a rounded tumor. Usually the leiomyoma is of limited size, less than 4 cm in diameter, without any need for medical attention (2). The exposure to estradiol (E2) as well as progesterone (P4) is of fundamental importance in the proliferation and growth of leiomyomas. The tumor almost invariably declines at menopause. Estrogen (ER) and progesterone (PR) receptors are over expressed in leiomyomas compared to adjacent myometrial tissue (3). Cyclic myometrial cellular changes of proliferative activity are the basis for increased incidence of leiomyoma induction by means of leiomyoma cell mutation (4). There appears to be genetic variation in leiomyoma prevalence as African American women in United States have a 3-fold higher risk of developing leiomyoma (5). Leiomyoma related clinically recognized symptoms in terms of bleeding, mechanical discomfort or dysfertility occur in up to 50% of women with leiomyoma (6).

1.1 PREVALENCE

Uterine leiomyomas are highly prevalent and increase with increasing age. A Swedish study reported 18 patients with myomas, with a diameter of 9-80 mm, from a random sample of 335 women in the age between 25-40 years. The overall prevalence was 5.4% (0.95 CI: 3.0-7.8 %), and in a subcohort of women aged 33-40 years, the prevalence was 7.8% (3.6%-12%) (7). Higher prevalence of leiomyomas > 2 cm, 254/641 (40%), was seen on ultrasound screening of a population sample of women in the USA. Subsets for ethnicity showed 144/314 (46%) for African American women and 110/327 (34%) for Caucasian women, between 35-49 years of age (2). Stratified for age, the prevalence of clinically relevant tumors, defined as enlarged uterus > 9 week pregnancy was 30% for African American women and 10% for Caucasian women up to 39 years of age. For women up to the age of 50, the prevalence was 50% and 35% respectively.

1.2 INCIDENCE

In the Nurse´s health study II, a study carried out on a cohort of 116,678 female nurses between the age of 25 and 42 years who were premenopausal and with intact uterus, the subjects were followed with questionnaires every second year between 1989 and 1993. In 327 065 women years 4181 myomas were diagnosed. The incidence per 1000 women years was 12.5 (12.1-12.9) for Caucasians and three fold higher (37.9) for the African American cohort. The incidence increased with premenstrual age. An incidence of 9.0 cases per 1000 women years was seen between 30-34 years of age, compared to a more than doubled rate, 22.5 between the ages of 40-44 (5). The yearly hysterectomy incidence per 1000 women years, due to leiomyoma, was 2.9 for women between 25-44 years (8). In Sweden, during 2008, the overall abdominal surgical incidence due to heavy menstrual bleeding (HMB) or leiomyomas, among women 25-44 years of age was 1520 out of 1198681 women, thus 1.3/1000 women years. Treatment included hysterectomy, supracervical amputation and leiomyoma enucleation but not uterine artery embolisation (UAE) or hysteroscopic resection (registry extract Socialstyrelsen).

During 1997 in the USA, the proportion of leiomyoma diagnosis and bleeding disorder in relation to the choice of surgical technique (9):

Surgical approach (% of all in the category)	Indication: leiomyoma	Indication: bleeding disorder
Abdominal subtotal	49.7%	7.3%
Abdominal total	40.2%	9.5%
Laparoscopic	28.7%	15.7%
Vaginal	17.1%	13.6%

1.3 LOCALIZATION

The localizations of leiomyomas, (prevalence in brackets) are: submucosal (5%), bulging into the uterine cavity with more than half of its size; intramural (75%), mainly engaging the uterine wall; or subserous (20%), with more than half of the volume located exterior to the uterine wall and occasionally stalked. Stalked intracavitary myomas may protrude through the cervix, and cause inversion of the uterine cavity as it is delivered through a dilated uterine cervix. The localization is of importance for eliciting symptoms such as bleeding disorder or dysfertility, as well as for potential mechanical impact upon adjacent organs. Bleeding disorder is mainly associated with submucous or intramural tumors (6).

1.4 LEIOMYOMA SUBTYPES

In order to guide in the evaluation of whether a malignancy should be considered or not leiomyoma subtypes are classified according to the World Health Organization (WHO) classification for mesenchymal tumors of the uterus as; mitotically active, cellular, hemorrhagic cellular, atypical ,epiteloid and myxoid. Patterns of necrosis, relatively acellular zones of hyaline degeneration, are frequently seen (60%), following infarctions in benign tumors. Hyaline degeneration is seen as clear amorphous areas in leiomyoma sections for microscopy (10). The vascular support to myomas is arranged through a vessel network embracing the tumor, branching into centripetal radial vessels, towards the central part of the tumor which is often visible upon Doppler ultrasound investigation.

1.4.1 Signs of malignancy

The issue of malignancy must be kept in mind, and is defined as >10 mitotic figures per 10 high power fields (HPF) in the microscope. Modifications up or down, as in cellular (up to 20 MF) or in myxoid (<2 MF) subtypes, are applied in accordance with the WHO classification. Leiomyosarcoma comprise 1.3% of uterine malignancies, and 30% of the sarcomas. One in 800 leiomyomas proves to be a sarcoma. Gross pathology of sarcomas includes: size >10cm; solitary lesions (50-70%); soft, fleshy and yellowish cut surface, frequently with the presence of necrosis or hemorrhagic areas.

1.4.2 Benign tumor characteristics

Benign tumors are characterized, on the other hand, as frequently multiple, smaller (3-5 cm), with a firm white whorled cut surface, infrequently appearing necrotic or with hemorrhagic areas. Diffuse enlargement of the uterus is also seen in rare conditions, like leiomyomatosis, with multiple nodules up to 3 cm increasing the uterine volume. The uterine weight may exceed 1000 grams; still without any association to malignancy (10).

Fig 1.
Uterus and leiomyoma opened longitudinally, with a 6 cm transmural leiomyoma with a rather large intracavitary portion.
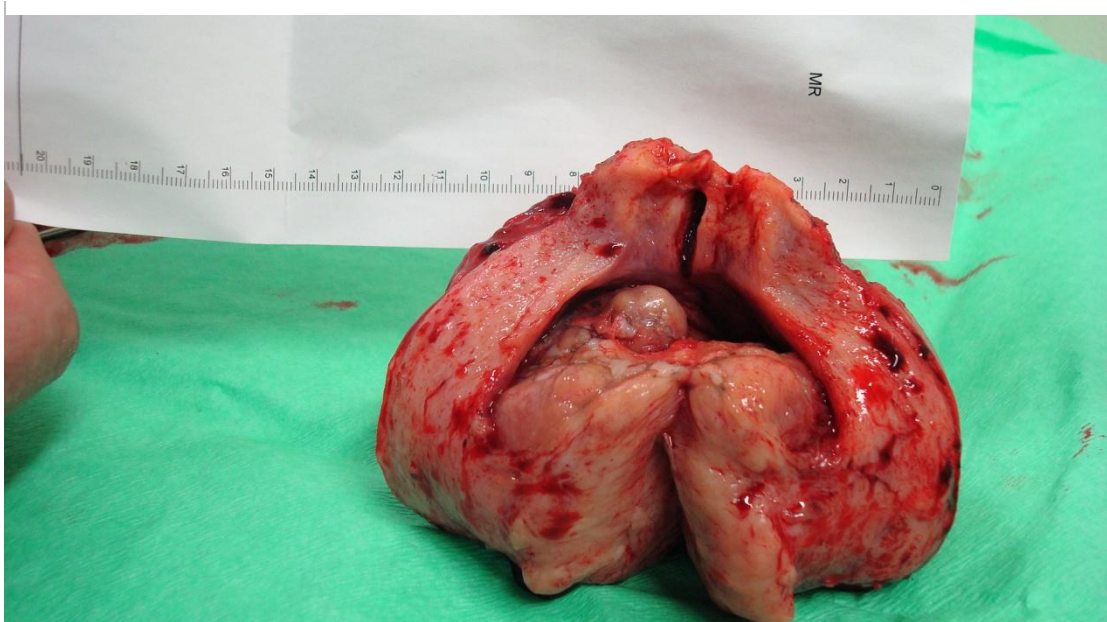
1.5   SYMPTOMATOLOGY

The clinical symptoms caused by uterine leiomyomas are dominated by an increased amount and duration of menstrual bleeding. Less frequently reported is discomfort and dysfunctional impact caused by pressure upon adjacent organs such as the bowel, urinary bladder or ureter. Dysfertility with decreased chance of pregnancy and an elevated risk of spontaneous abortion is concluded in mainly retrospective studies (11).

1.5.1 Menstrual pattern
Women with leiomyoma have on average one extra day of bleeding; 5 days, compared to women without leiomyoma; 4 days. The relative risk, RR (0.95 confidence interval) for bleeding discomfort increases with tumor size. RR 1.9 (1.5 to 2.5) for women with leiomyomas >5 cm, among whom a nearly doubled occurrence of gushing flow was also seen, compared to women without leiomyomas. Intramural or submucous location did not affect the relative amount of bleeding in this study (12). The patophysiological mechanism behind excessive bleeding is not yet known. The mechanism could be multifactorial involving inflammatory, atrophic or vascular alterations in endometrium covering a submucous leiomyoma. The endometrial area from which bleeding is released is increased by an intracavitary or transmural leiomyoma. Intramural leiomyomas are considered to block the venous return from endometrium by pressurizing the venous vasculature at the venule level.

1.5.2 Pelvic pain
Pain is not a frequent complaint associated with leiomyoma and any differential diagnosis as the cause of abdominal pain should be ruled out. Pain is generated whenever perfusion of a leiomyoma is compromised. Occasionally a pedunculated leiomyoma may rotate around its own axis and torsion of such a leiomyoma may be accompanied with pain. Accelerated growth of the tumor may cause outgrowing of its vascular supply, clinically occasionally associated with malignancy. Cervical dilatation, due to delivery of a submucosal leiomyoma, is likely to be painful. In an Italian population it was reported that among women with leiomyoma, 7% reported severe to moderate pain. The OR for dyspareunia was 2.8 (95% CI: 0.9-8.3), and for non cyclic

pelvic pain 2.6 (95%CI: 0.9-7.6), compared to women without leiomyoma. No difference was found for dysmenorrhea (13).

1.5.3 Mechanical impact on adjacent organs
Myomas exceeding 5 cm represent space occupancy and weight that may cause pressure and impingement of adjacent organs. A sense of pressure or weight in the lower abdomen is a common feature in women with leiomyomas. A myomatous tumor with proximity to bowel, bladder or ureter, may elicit significant obstruction and further dysfunction such as constipation, urinary urgency, frequency or hydronephrosis (14).

1.5.4 Dysfertility
Fertility is more likely to be affected by submucous intracavitary lesions (15). However transmural location could interfere with the tubal passage, endometrial receptivity and implantation. Leiomyoma is occasionally involved in recurrent spontaneous abortions. A currently debated issue is the benefit of myomectomy prior to assisted reproduction technique (ART), with or without dislocation of the cavity. Surgical removal improves the pregnancy rate following IVF, when there is a cavity involvement or deformation. Furthermore intramural leiomyoma without cavity impact has been shown to reduce the live birth rate, as well as clinical pregnancy rates, with strong significance (16). The IVF pregnancy and abortion rate after removal of myoma prior to IVF was similar to that of women without leiomyoma (17). In a population with at least one intramural myoma >5cm diameter, without submucous component, IVF was significantly more successful in a surgically treated subgroup compared to women who had not undergone surgery before the scheduled IVF procedure (18). There is a lack of prospective randomized studies in a fertility context. Available studies summarize the outcomes from retrospective observations.

Pregnancy complications such as peripartum hysterectomy OR 13.4 (95%CI: 9.3 - 19.4), malpresentation OR 2.9 (95%CI: 2.6-3.2), dystocia OR 2.4 (95%CI 2.1-2.7), premature delivery OR1.5 (95%CI: 1.3-1.7), placenta abruption OR 3.2, (95%CI: 2.6-4.0), and caesarean section OR3.7 (95%CI: 3.5-3.9) are significantly associated with intramural leiomyomas (17).

1.6 DIAGNOSIS

1.6.1 Manual pelvic examination
The classical finding is a solid, non tender, rounded tumor with smooth surface, palpable at bimanual abdomino-vaginal examination, or as an asymmetrical diffuse enlargement of the uterine corpus. It is not always possible, in these cases, to exclude a tumor originating from the adnexae, bowel or retroperitoneal space.

1.6.2 Ultrasonography
Vaginal and abdominal ultrasound examinations are useful tools in order to confirm the occurrence of a leiomyoma which appears as an echo dense, rounded tumor in association with the uterine corpus. Multiple leiomyomas >4 and sized >375 ml are difficult to evaluate by ultrasonography. In contrast MagneticResonance Imaging (MRI) is useful in these cases (19).

1.6.3 Computed tomography (CT)
Radiation exposure limits the use of CT for scientific purposes. Where malignancy is suspected or there is an inconclusive diagnosis, CT may be used for further tumor characterization, in order to describe the involvement of adjacent organs for preoperative evaluation.

1.6.4 Laparoscopy

Laparoscopy, optionally in combination with hysteroscopy, could be considered as a second line standard procedure, following ultrasonography, or MRI, for the diagnosis of uterine leiomyoma. The procedure permits complete visualization of the major proportion of significant pelvic tumors, and provides evaluation of the peritoneal surfaces, with occurrence of peritoneal adhesions and/or coexisting endometriosis. Abdominal laparoscopic visualization is compulsory, when non invasive investigations are inconclusive. Hysteroscopic examination is of value for diagnostic verification of intracavitary leiomyomas as well as lesions, such as polyps and anomalies of the uterine cavity. It is possible to carry out various treatments such as extirpation of polyps, resection of leiomyoma or a septum within the same surgical procedure.

1.7   COST FOR THE SOCIETY AND INDIVIDUAL

In the USA, a database from nine insurance companies, covering 1, 2 million people was utilized. Between January 1999 and December 2003, 5122 women between 15-64 years were diagnosed with leiomyoma. Matched controls without leiomyoma diagnosis were randomly selected from the database. The cases were followed during 1 year after receiving the diagnosis. Costs for hospitalization, medication and disability claims due to absenteeism from work, were registered. During the first year after diagnosis the relative risk for hysterectomy was 50 fold greater (21% of women with myoma) among diagnosed cases, compared to controls. Direct costs plus indirect costs such as disability claims were 6515+ 1677 (total 8192) dollars, compared to 2268+ 844 (total 3112) dollars for controls. The excess cost was 5080 dollars (2, 6 fold greater), during the first year after diagnosis. In- and outpatient care represented 93% of the total cost for diagnosed cases and 80% for controls (20).

1.8   STEROID HORMONES IN LEIOMYOMA GROWTH REGULATION

Steroid hormones such as cortisol (C21), progesterone (C21), androgens (C19) are all derived from cholesterol while estrogens (C18) are synthesized from androgens by aromatase enzyme. Ovarian steroid hormones, estradiol (E2) and progesterone (P4) are essential for the growth of leiomyomas. After menopause leiomyomas decline in size and invariably go into a process of involution. The steroid hormones are hydrophobic compounds that are made soluble in blood by binding to carrier proteins, such as transcortin or SHBG, from which they dissociate before entering target cells, by ready diffusion across cell membranes. In the target cell interior the nuclear receptor ligates to the hormone ligand. The receptor ligand complex dimerizes, release its inhibitory HSP, and functions as a transcription factor on promoters for a set of genes involved in the biological response to the specific hormone.

1.8.1 Progesterone

Progesterone is produced in the theca cells of the ovary and adrenal cortex. The compound is of major importance for the regulation of the menstrual cycle and is a key hormone for endometrial function and receptivity, embryo implantation and maintenance of pregnancy (21).

1.8.2 Progesterone receptor modulators (PRM)

A 19-nor, 11β -steroid compound was synthesized and reported in 1981 by Philibert and Moguilewsky. It was described as a strong glucocorticoid receptor ligand. The antiprogestin property of the compound was found useful for termination of early pregnancy, as reported soon thereafter (22) (23). Progesterone receptor modulators (PRM) ligate to the PR and inhibit the agonist mediated structural and functional reconfiguration of the receptor. Depending on the local tissue prerequisites, such as

lack or relative abundance of co activators and co repressors, a selective response to PR ligation is seen. Progesterone is considered to be a pure agonist; in contrast, mifepristone is regarded mainly as an antagonist and onaprisone (ZK98 299 Schering AG, Berlin) as a pure antagonist. Several compounds with different degrees of antagonist properties such as mifepristone, asoprisnil and ulipristal have been pharmacologically and clinically evaluated, and found to be potentially useful for a wide spectrum of clinical indications, including leiomyoma uteri, endometriosis and contraception (24) (25, 26) (27-29).

Fig 2.

Mifepristone
$C_{29}H_{35}NO_2$
(8S, 11R, 13S, 14S, 17S)-11-(4-dimethylaminophenyl)-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta [a]phenanthren-3-one
CID: 55245
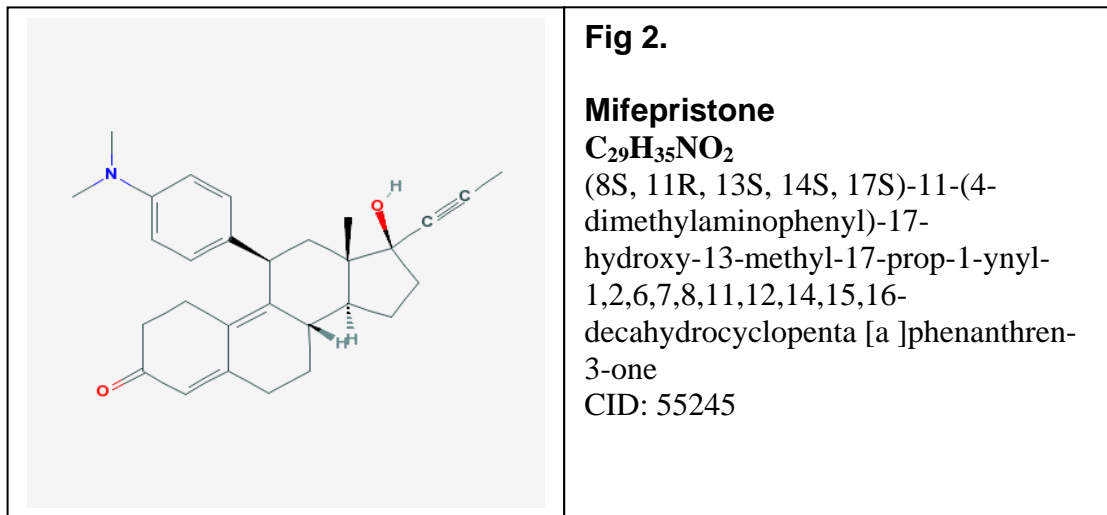
1.8.3 Pharmacokinetics of mifepristone
Mifepristone remains bound to serum proteins to a degree of 94- 99%. AAG (α1-acid glycoprotein) is the main human serum carrier for mifepristone, up to a dose of 100mg. A higher dose of more than 100 mg is characterized by a quick demetylation and hydroxylation in the liver by the Cytochrome P450 enzyme CYP3A4. The serum half time for mifepristone in a dose of 100 mg daily is 26-48 hours. Mifepristone is, in a small distribution volume, metabolized linearly. Metabolites are excreted with bile and very low urinary concentrations are detectable. The distribution is non linear at a dose of more than100 mg (30). This infers that an oral intake of 100, 400, 600 or 800 mg mifepristone, results in the same serum concentration, around 2.5 µmol/L. At a dose of> 400mg of mifepristone, mono and di demetylated and hydroxylated metabolites exceed the levels of the mother compound in serum. The metabolites bind more weakly than mifepristone, but with twice the strength of dexametasone to the glucocorticoid receptor (GR) and may be biologically active. GR affinity could be responsible for the antiglucocorticoid effect seen at higher doses, with a subsequent activation of the HPA (hypothalamus-pituitary-adrenal) axis (31).

1.9   CELL COMMUNICATION

The molecules in a signaling pathway behave as switches. The "turning off" is just as important as the "turning on" event, in offering options for recovery of the molecules so that they are ready for the next activation. In humans there are 520 different kinases and 150 phosphatases. Two main types of intracellular proteins act as signaling substances: one category is serine /threonine kinase that phosphorylates proteins on serine and threonine residues; the other is tyrosine kinases that phosphorylate proteins on tyrosine residues. The other possibility for phosphate load exchange is to convey GTP binding or hydrolysis to proteins. Large trimeric G-proteins take part in submembranous signal transduction from G protein coupled receptors (GPCRs) (32).

1.9.1 Growth factors-cytokines

Growth factors or cytokines are signal proteins that apply impact on the cellular functions, structures, movements, growth or apoptosis by paracrine cell to cell communication, mediated by signal molecules in the extracellular matrix (ECM). Each cytokine has its own receptor in the cell membrane, mediating a signal specific response to the cellular interior through activation of secondary pathways.

It is known that a combination of estrogen and progesterone mediated effects are necessary to elicit a response in cellular proliferation. This is mediated by the cytokine in question. Epidermal growth factor (EGF) is promoted by P_4 and its membrane receptor (EGF-R) is promoted by E_2 (33), both of which are essential for induction of proliferation in leiomyoma cells. The sex steroid effect on myocytes is mediated through cytokines similar to EGF, and their specific receptors at the cell membrane. Cytokines ligate to receptor tyrosine kinases (RTK) that activate G-proteins and further induce mitogen activated plasma kinases (MAPKs) with effects on the cellular function and cell cycle (34). The autocrine and paracrine abundance of different growth factors like EGF and IGF as well as apoptosis protective agents like bcl-2 are likely to determine the proliferative net response to steroid hormone stimulation (33). Gene expression of EGF in leiomyoma is no different than that the in myometrium during the proliferative phase, but in the secretory phase there is an amplification of expression suggesting a progesterone dependency (35). RTKs such as EGF-R were shown to be over expressed in leiomyoma (36).

1.9.2 Transcription factors

Transcription factors are defined as any protein required for the initiation or regulation of transcription. Regulatory proteins such as co activators, co repressors and chromatin remodeling complexes act directly on RNA-polymerase or via a mediator protein at the promoter complex. The promoter is the DNA sequence where general transcription factors and the polymerase assemble at the start site of transcription. Gene regulatory proteins act by binding to general transcription factors or polymerase. Whereas general transcription factors and mediators are the same for all polymerase transcribed genes, the gene regulatory proteins and their binding sites relative to the promoter differs between genes, modulating the cell specific response (32).

1.9.3 Nuclear receptors

Nuclear receptors comprise a super family of transcription factors, involved in several physiological functions, as control of cell differentiation and homeostasis. More than twenty receptors are classified as "orphan" receptors since the ligand is not yet known (37). The N-terminal DNA binding domain (DBD) contains activation function-1 (AF-1) and regulates the ligand independent activity of proteins necessary for transcription. AF-2 is located in the C-terminal Ligand Binding Domain (LBD) and regulates the ligand and co regulator recognition (38). All nuclear receptors ligate to DNA at hormone response elements (HREs), after dimerization of homo or heterodimers, during release of inhibitory HSPs. Impact from auto or paracrine co activators or co repressor proteins as well as additional transcription factors are needed for induction of transcription. This is followed by further downstream signaling, towards mediation of the biological response of the hormone in terms of cascades of enzymatic protein activity modulating expressions of downstream genes (39). The cellular response to receptor modulation, in terms of agonist or antagonist effect, is determined by the cellular relative balance of co activators and co repressors, as well as impact from other sub cellular signaling (40).

1.9.4 Progesterone receptor (PR)

Nuclear receptors for progesterone are present in the female genital tract, breast, and brain. The expression is induced by estrogen. The DNA promoter site for PR

transcription, involves a dedicated estrogen response element (ERE). The abundance is higher in leiomyomas than in adjacent myometrium (3) (41). PR exists as at least two isoforms, PRA (94kDa) and PRB (99 kDa). PRA (769 a.a.) is a subset of PRB (933 a.a.), lacking 164 amino acids at the N-terminal. The two isoforms are transcribed from a single gene by initiation of transcription from separate promoters and act selectively on different sets of responsive genes. The transcription start site for the PRA isoform has not yet been determined. For the PRB gene, the transcription start site is located at chromosome 11q22.1.

While the two forms of PR have similar DNA- and ligand-binding affinities they have opposite transcriptional activities. PRB functions as an activator of progesterone-responsive genes, while PRA is inhibitory. PRA functions as a strong repressor of PRB and ER activity in the presence of PR agonists and antagonists (42).

PRA is transcriptionally inactive because of an inhibitory domain, blocked by a PRB upstream activating factor AF-3, abundant only in the PRB isoform. Interestingly, mifepristone binding induced PRB_{mutant} mediated transcription of β-galaktosidas in an agonist mode, indicating that there are separate ligation sites for P4 and mifepristone in the utmost C-terminal end of the PRB (43).

1.9.5 Estrogen receptor (ER)

Estrogens are involved in cellular processes such as growth, differentiation and function of the reproductive system. In females, estrogen principal targets are the ovaries, uterus, vagina and mammary glands as well as the brain. The promoter for PR is targeted by ER, thus inducing a response in PR synthesis. The ER alfa (ESR1) and beta (ESR2) proteins are abundant in the cell nucleus where they persist as homo or heterodimers associated with HSPs. By analogy with other nuclear receptors, hormone ligation is followed by dissociation of HSP and activation of co activators or co repressors, like SRC-1 (NCOA1) (44).

1.9.6 Cyclicity and mutation

The risk for development of breast cancer is over all around 10% during a lifetime, pro primo related to inheritance and secondly deduced from the cyclic pattern of hormone exposure. Estrogens are known to enhance the rate of cell proliferation in glandular tissue of the breast, tentatively contributing to mutation, initiation and further promotion of breast cancer. Early menarche, nulliparity, high age at first delivery and late menopause are factors that increase the number of exposures to cyclic periods of proliferative enhancements, suggesting a higher risk for mutagenicity (45).

2   AIMS OF THE STUDY

<u>**To study the effect of 12 weeks treatment with low dose mifepristone on:**</u>

.

➢ Leiomyoma volume, endometrium, bleeding pattern, and side effects.

➢ Cell proliferation in normal breast of premenopausal women.

➢ Gene expression in leiomyoma.

➢ The molecular basis for the difference in leiomyoma volume regression in response to treatment.

3 MATERIALS AND METHODS

3.1   PATIENTS

A prospective, randomized, placebo controlled study was conducted at the Karolinska University Hospital, Stockholm, Sweden between November 2004 and June 2007. Approval from the Ethics committee (02-410) at the Karolinska Institutet and from the Medical Products Agency was sought and given. The study involved women who were referred to the hospital for surgery due to uterine leiomyoma in this period. Among eligible women (N=72) 42 declined participation while 30 were included in the study after giving their written informed consent. Participation criteria included premenopausal women with indication for leiomyoma surgery in terms of HMB or mechanical pressure or infertility issues, who were otherwise healthy, had not undergone steroid hormonal treatment during at least 3 months prior to start of the study medication. Normal blood biochemistry profile of the group included haematology, hormonal and parenchymatous organs, with verification of premenopausal hormonal status. Mammography was offered, if not already done during the 12 months before start of the study. The women underwent endometrial biopsy, and routine gynaecological examination including PAP smear and the results were evaluated as normal before their inclusion in the study. Ultrasonographic examination was also carried out in order to exclude signs of present gynaecologic or other malignancy. Criteria for excluding individuals from the study included the development of uterovaginal bleeding not possible to control by treatment with tranexamic acid and iron supplementation.

3.1.1 Sample size calculation
Assuming a standard deviation of 10% in the percentage volume change of leiomyoma, 18 subjects per group were considered to be required to detect a difference of at least 10% in percentual volume change between the treated and the placebo group with 90% power, using a one-sided 5% level test. Allowing for a 10% drop outs, 20 subjects per group, or a total of 40 women needed to be recruited into the trial.

3.1.2 Treatment
Eligible patients were randomized into two treatment groups using sequentially numbered, opaque sealed envelopes prepared according to a computer-generated randomization list by the Karolinska University Hospital Pharmacy. The patients received either mifepristone 50 mg (one quarter of 200 mg, Mifegyne[®], Exelgyn, Paris, France) as the active substance or visually identical B-vitamin tablets (one quarter of TrioBe[®]Recip, Stockholm, Sweden) as an inactive comparator, every other day for 12 weeks starting on cycle day one.

3.2   CLINICAL PROCEDURES

3.2.1 Ultrasonography and Doppler
A general assessment, utilizing TVS (Trans Vaginal Sonography) of pelvic, urinary organs, adnexae and uterus with leiomyoma diameters and endometrial thickness was performed at baseline, to rule out signs of coexisting pathology. Subsequently measurements with four week interval were conducted utilizing aVoluson730 Expert (General Electric, Zipt, Austria) with high frequency (7-9 MHz) transvaginal probe. All leiomyomas were localized and the diameter in three perpendicular planes was measured, the volume was then calculated by the formula describing an ellipsoid: $=0,523 \times d_1 \times d_2 \times d_3$. Pulsatile index (PI) = S-D (systolic-diastolic flow) / time average

maximal flow (TAMAX) and peak flow were measured in the uterine vessels and from the feeding vessels at leiomyoma periphery and in the center of the tumor.

3.2.2 Blood biochemistry

Routine blood biochemistry included haematological and parenchymatous organ functional parameters. Hormonal status included FSH, LH, estrogen, progesterone, testosterone, androstenedione, DHEAS, SHBG and prolactin, as well as 24 hour urinary cortisol assessment. Safety parameters for blood and liver status were repeated monthly. Hormonal status was assessed prior to initiation of medication and at the end of study. Concentrations of free testosterone were calculated from values for total testosterone, SHBG and a fixed albumin concentration of 40 g/l (46).

3.2.3 Questionnaires

3.2.3.1 Diary

Patients were asked to keep daily records on any vaginal bleeding, pelvic pain or pelvic pressure symptoms, without further estimation of the degree of symptoms. Instructions and follow-up was provided by the research nurses. Patients were followed weekly in order to check for compliance.

3.2.3.2 Likert scale

The Likert scale was used for weekly grading of pelvic symptoms like pain, pressure, dysuria or general symptoms like nausea, hot flushes or intestinal dysregulation. Changes in mood, headache, and libido were monitored. The scores were determined on a 5 point (0-4) Likert scale as; no, weak, moderate, severe or very severe symptoms. The scores were reported weekly and summarized in four week blocks, thus possible summarized scores were ranging from 0-16 for every treatment month.

3.2.3.3 Breast symptom index

A tool was used to assess the patient´s breast symptoms, BSI (Breast Symptom Index), by using monthly self registration, monitoring and recording of breast symptoms on an 11 point scale, from zero to ten. Symptoms, during the preceding four weeks, such as soreness, "needle like pains", pain, swelling and change in size were registered. Recordings were collected from baseline and on three consecutive occasions after 4, 8 and 12 weeks duration of the study.

3.2.4 Endometrial biopsy

Biopsy material was sent for histologic evaluation at baseline in order to rule out any preexisting atypia, before inclusion in the study. Endometrial biopsies were obtained from the fundus of the uterine cavity with a Randall® curette (Stille, Sweden), before starting medication and after the treatment period during surgery. In some cases we experienced difficulties in obtaining a baseline biopsy due to leiomyoma interference with the cervical canal or dislocation of the cervix or corpus.

3.2.4.1 Routine assessment and for IHC (immunohistochemistry)

Biopsies were fixed in formalin and embedded in paraffin blocks, prior to sectioning and mounted on slides for IHC.

3.2.4.2 Expert evaluation of endometrial morphology

Slides were also prepared with hematoxylin and eosin, and sent for a blinded assessment for any endometrial morphological changes due to mifepristone treatment by Professor Alistair Williams at Edinburgh University.

3.2.5 Surgery

The staff and surgeon were blinded to treatment allocation. The decision of the route of surgery was defined at the preoperative evaluation and summary of clinical data.

Abdominal myomectomy (N=7_{mif}+10_{contr}), abdominal hysterectomy (N=4_{mif}+6_{contr}), or vaginal hysterectomy (N=3_{mif} + 0_{contr}) were performed.

3.2.5.1 *Leiomyoma biopsy at surgery*

Leiomyoma biopsies perpendicular to the dissected leiomyoma surface were obtained using a dermal punch. Biopsies were fixed in formalin for embedding in paraffin blocks, prior to sectioning and mounting on slides in duplicates for IHC. Biopsies were also separately collected and snap frozen in liquid nitrogen for later isolation of total RNA.

3.3 RESEARCH LABORATORY INVESTIGATIONS

3.3.1 Breast biopsy and Immunocytochemistry analysis

Percutanous FNA from the upper outer quadrant of the left breast were performed at baseline, during the luteal phase and repeated at the end of treatment, close to the surgical intervention. FNA biopsies were performed using a needle with an outer diameter of 0.6 mm as previously described. A fine needle was used in order to minimize the trauma for patients and to allow a repeat biopsy after 3 months. By palpation of the breast it is often possible to localize the mammary gland for optimal procurement of MECs (mammary epithelial cells). In order to produce multiple identical slides, aspirated cells were suspended in 1.0 ml 4% buffered (pH 7.4) formalin. Cell suspensions of 110 µl were centrifugated in a cytocentrifuge at 700 rpm for 3 min at room temperature, and the cells were collected on to glass slides. Slides were probed for the nuclear antigen Ki-67 with the MIB-1 monoclonal antibody (Immunotech ®, Marseilles, France). Ki-67 is present in proliferating cells but absent in quiescent cells. Immunostained epithelial cells were quantified by cell counting at 200-fold magnification, simultaneously by two independent observers. An index for proliferativity in breast tissue, Ki-67 index, was calculated by dividing the Ki-67 positive cells with the number of unstained cells, not in the process of proliferation. The Ki-67 index was considered valid only if >50 unstained cells were obtained. As the usual percentage is around 2%, one stained cell among 50 not stained is expected. Thus, 50 MECs validates the Ki-67 index for premenopausal normal breast tissue.

3.3.2 Immunohistochemistry (IHC)

3.3.2.1 *IHC endometrium*

Both steroid hormone receptor expressions and Ki-67 were analysed by IHC in the luminal, glandular and stromal compartments of the endometrium.

The tissue sections were deparaffinized hydrated and quenched with hydrogen peroxide to inactivate the tissue peroxidase activity. The primary antibody was added targeting the receptor protein of interest. For procedure details, please see papers II and IV. Sections were evaluated by two independent investigators, blinded to treatment, using the IRS (Immuno Reactive Scoring system), a semi-quantitative subjective scoring system based on both percentual positive (PP) and staining intensity (SI) as follows. IRS = SI * PP, mean of 10 visual fields. SI was visually graded as 0= no staining; 1= weak staining; 2:=moderate staining and 3=strong staining and PP as the percentage of cells stained positive. The PP was estimated by counting approximately 200 background cells, and was scored as 0= no staining, 1= <10%, 2= >11<50%, 3= <80% and 4>81% stained (47). With regard to Ki-67, immuno reactivity was heterogeneous with no grading of intensity. Fewer cells were stained compared to the background. The scoring template was therefore modified as follows: 0=no cells stained; 1=<5%, 2=> 5<10%, 3=>10<20% and 4=>20% of cells stained, compared to background count. All expressions were evaluated by two independent researchers, blinded to treatment

allocation. Inter observer correlations for the significantly upregulated PRB and PRA+B was good, r=0.7 (p=0.005) and r= 0.9 (p<0.001) respectively.

3.3.2.2   *IHC leiomyoma*

The expression of steroid hormone receptors (ERα, ERβ, PR, growth factors -EGF, FGF), caspase-3 and proliferation marker Ki-67 was assessed by IHC. The tissue sections were treated as described in paper IV. A multiplicative modified "Quick"scoring was used, with the staining intensity 0-3 (no staining, weak, intermediate or strong staining) multiplied with a score for percentage (1-4) between (1) <25, (2) <50, (3) <75, (4) <100% of cells stained, with a calculation of the mean for ten visual fields. In the case of steroid receptors (PR and ER), growth factors and caspase-3 we scored the cross cut and oblique bundles separately. The scores correlated less well between observers. In general the cross cut scores were 1.4 fold higher than scores from the obliquely cut cells. From 10 HPFs scores were determined by two independent assessors. The mean of scores from two blinded assessors gave the immunoreactive score (IRS) for the protein analyzed in two categories, cross cut or oblique bundles.

For Ki-67 a modified scoring system was applied, counting the number of Ki-67 positive cells per visual field (x400 magnification) in ten HPFs. The mean value for 10 HPFs was used as an assessor specific index value for the case. Evaluations were performed by two independent researchers blinded to the treatment allocation. The inter observer scores correlated (r=0.52, p=0.049). The mean of scores from two observers represent the proliferative index for the case.

3.3.3 RNA extraction

Samples were homogenized in a Retsch® tissue mill at a shaking frequency of 30/sec according to the supplier's instruction. Total RNA was isolated using Trizol ® reagent (Life technoclogies; Invitrogen Corp. & Applied biosystems Inc., Carlsbad, California). The RNA extracts were stored in a freezer at -70 degrees C until reverse transcription into cDNA was carried out. The quality of RNA was checked using NanoDrop (Thermo scientific Waltham, MA, USA) as well as by running it on agarose gel. All samples had an OD260/280 ratio > 1.8.

3.3.3.1   *cDNA synthesis*

One microgram of total RNA was reverse transcribed to cDNA using Superscript II™ Reverse Transcription (Invitrogen) with Ribonuclease Inhibitor (RNasin, Promega Corp., Masden, WI) in a 20 microliter reaction volume. The synthesised cDNAs were diluted 1:2.5 in Millipore™ purified, autoclaved sterilised H_2O kept as 'stock' solutions in a freezer at -70 degrees C until used.

3.3.4 Microarray

3.3.4.1   *Control versus Mifepristone*

The total RNA from three cases per category was reverse transcribed to cDNA and labelled with probes and was hybridized onto the microarray chip.

 The microarray on an Applied Biosystems Human Genome survey Microarray V2.0 was processed at core facility - Karolinska Biomics Center. The data from the microarray analysis was normalized against each other and > ±1.5 fold, and significant (p<0.05) change in the expression levels were calculated using Analysis of Variance. The filtered set of significantly different genes with fold changes were uploaded to IPA (Ingenuity Pathway Analysis) software (48).

3.3.4.2   *Good versus poor responders*

A subgroup of mifepristone treated cases, were subjected to comparison of the gene expressions in one group responding with growth regression (N=4), compared to a

group of refractory cases, also treated with mifepristone (N=4). The objective was to study the molecular background for the observed difference regarding growth regression response. Total RNAs were reverse transcribed to double stranded DNA, which was transcribed into cRNA labeled with probes using T7 promoter and was hybridized onto the microarray chip. Microarray on an Affymetrix 46.000 genes platform (GeneChip Human Gene 1.0 ST Array) was processed at Bioinformatics and Expression Analysis core facility (BEA). The data from the microarray analysis was normalized against each other and $> \pm 1.5$ fold, and significant (p<0.05) change in the expression levels were calculated using Analysis of Variance. The filtered sets of significantly different genes with fold changes were uploaded to IPA.

3.3.5 Real Time PCR
Real time PCR was performed to evaluate and, when possible, confirm the findings from microarray analysed by IPA.

3.3.5.1   Real time PCR; control versus mifepristone group
For details of Real time PCR analyses, (please see paper IV). cDNAs from seven women from the control group and 8 from the mifepristone group were subjected to Real time PCR amplification of target genes as evident from IPA analysis and microarray. Seven target genes (microarray fold change) in the Ephrin pathway: GRIN2A (-3.3), CREB5 (-2.5), ROR1 (+3.6), ITGA3 (-2.4), PIK3C2G (-3.9), EFNB2 (-2.8), PAK3 (-1.9), and eight in the Integrin pathway: ARHGAP26 (+3.3), ITGB5 (+2.5), ITGA3 (-2.4), ITGAD (+2.0), PAK3 (-1.9), ROR1 (+3.6), PIK3C2G (-3.9), PIK3R1(-2.9). Olionucleotide primers and dye-labeled TaqMan probes, specific to seven target genes in Ephrin and eight in Integrin pathway were purchased from Applied Biosystems.
In addition MKI67 (-1.9), TP53 (+1.3) and CKDN1 (+1.6) were assessed for reference and correlations. Target gene Taq Man probes were FAM™ dye-labelled and 18S cDNA probes were VIC™ dye-labelled. The 18S and target gene were processed in the same well for optimal validation. Real time-PCR reactions were carried out on 96 well optical PCR plates with reaction volumes of 25μl in a 7300 Real Time PCR System (Applied Biosystems). Upon analysis after Real Time PCR, one case in the mifepristone group was excluded because of outlying low 18S content; the remaining 7 cases in each group were subjected to statistical analysis.

3.3.5.2   Real time PCR; good versus poor responders
The same procedure as for the previously described Real Time PCR was performed, (see paper III). Two subsets of cases from the mifepristone treated group, were categorized either as good (N=4) or poor (N=4) responders to mifepristone. Three cases from each group were subjected to microarray and IPA analysis. A strong difference of expression for genes in the Glutathione pathway was seen: Glutathione-s-transferase mu 1 (*GSTM1* +8.03), glutathione-s-transferase mu 3(*GSTM3* +2.26), glutathione-s-transferase mu- 5 (*GSTM5* +2.23), glutathione peroxidase2(G*PX2* -1.7). For the reference genes, progesterone receptor (*PGR +1.09*), estrogen-receptor alfa(*ESR1 +1.09*), p-21 activated kinase3 (*PAK3 -1.01*), the proliferation marker *MKI67(-1.02)* and the apoptosis marker *TP53 (+1.19)*. Glutathione and reference genes were assesed with Real time-PCR, for confirmation of differences. Primers and probes were purchased from Applied Biosystems and processed in the 7300 PCR system.

3.3.6   Primary cell culture study
Nine patients with myoma, otherwise healthy, without any preoperative hormonal medication, were recruited to the study. The cases were decoded and after consultation with the ethical committee it was decided that ethical permit was not necessary. All experiments were repeated using cells isolated from five different patients. The biopsies

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were collected during surgery and were placed in Ham F10 medium. The samples were finely minced in sterile petridishes at 4˚C, suspended in F10 medium and centrifuged at 460g for 1 min. In order to remove extracellular matrix, the tissue was digested with collagenase type 4, incubated at 37˚C for 4h with adequate shaking. DNase I 0.2mg/ml at final concentration was added after 2.5 h incubation. At the end of the 4h incubation, the tissue was resuspended gently using a Pasteur pipette. The cells were pelleted at 460g for 5min at room temperature and resuspended in fresh Ham F10. The cells were filtered using 40 μ cell strainer into petridishes. The cells were counted and frozen for further experiments. Cells were cultured in DMEM containing 10% FBS, 1% antibiotics in collagen coated petriplates at 37 ˚C with 5% CO_2 in air. Primary cells were used until passage five to avoid variations in phenotype and gene expression. The monolayer cell cultures, at about 70% confluence were treated with 2.5% charcoal activated serum overnight prior to treatment with 2.5% vehicle control (ethyl alcohol 0.01%), progesterone (P) (318nM), mifepristone (M) (318nM), and dexamethasone (D) (318nM) individually and/or in combinations P, P+M, P+D or P+M+D for 24 hours. Cells were harvested and lysed, total RNA was isolated to synthesize cDNA by reverse transcription following the same protocol as earlier described.

3.3.6.1 *Real time PCR*
 The integrin genes ARGHAP26, ITGB5, ITGA3, PAK3, ROR1, PIK3R1 and for correlations and reference TP53, MKI67 and CKDN1, were subsequently assessed with Real time PCR analysis, for assessments of the respective gene expressions during different conditions.

3.3.7 TUNEL
(Terminal deoxynucleotidyl transferase dUTP Nick End Labeling assay). In order to assess the degree of apoptosis, in situ end labeling of the 3'OH end of the DNA fragmentation was performed using the "In Situ Cell Death Detection kit, Fluorescein" (Roche Applied Science). Paraffin embedded leiomyoma biopsy sections were dewaxed by heating and serial washings in xylene, a graded series of alcohol and finally distilled water, for tissue rehydration. The tissue sections were permeabilised by treatment with proteinase K and incubated with the TUNEL reaction mixture (TUNEL labeling solution and enzyme) for 1h at 37 ˚C in the dark. The slides were rinsed with phosphate-buffered saline and counter stained with DAPI (4', 6-diamidino-2-phenylindole). Positive controls were prepared by treating slides with DNAse I (1000U/ml) for 10min at 25˚C before incubating with TUNEL reaction mix. Negative controls were prepared by incubating the slide with TUNEL labeling solution without the enzyme. Analysis and scoring was performed by two independent investigators under a fluorescence microscope with excitation wavelength 450nm. The mean of 4 scores, apoptotic index (AI), was calculated for each case and statistically analysed. The correlation observed for inter individual scores was good (R=0. 78, p<0.001).

3.4 STATISTICAL METHODS

Mann-Whitney U rank analysis was applied in order to test for significance between groups of continuous or ordinal scale data (symptom scores). For differences within groups paired dependent values were analysed with the Wilcoxon signed rank test. Spearman rank analysis was used to show correlations. Non parametric analysis utilizing Friedman's model was used for analysis of Real time PCR assessed gene expressions in cell culture. The significant differences between the groups were subsequently analyzed with Wilcoxon paired tests. Analysis for group differences in gene expressions such as dCts were performed using the Mann Whitney U test. For microarray, T-test analysis was utilised in order to select items for further analysis in IPA. For Real time PCR the mean value of sample triplicates were used for

normalisation versus the mean dCt of the "housekeeping" gene 18S ribosomal RNA, to obtain a ΔCt (Δ cycle threshold) value. Differential expressions between the control and active groups were calculated for each target gene ($\Delta\Delta$Ct). A P-value of <0.05 was considered as significant.

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4 RESULTS

4.1 PATIENTS AND RANDOMIZATION

Two women were not included in the study in spite of eligibility and obtained patient consent for participation. One due to a baseline ultrasound evaluation indicating a leiomyosarcoma and the other due to psychiatric problems. Because of reorganization of the clinic, recruitment had to stop when 30 women had been included and randomized. Two patients from the control group were excluded after randomisation, one because of elevated FSH, and the other after six weeks, because of uterovaginal bleeding in which required surgery without further delay. With regard to ultrasonographic volumetric assessments we had two further patients drop out of the study, one due to difficulties in determining the leiomyoma borders the other due to absence of an operator for the ultrasonography equipment. Twelve mifepristone cases and 14 control cases remained per protocol. Data was analysed for myoma volume regression, $N=15_{ctr}$ and $N=14_{mif}$, per protocol.

In order for breast biopsies to be useful, it was essential to have a minimum of 50 mammary epithelial cells to evaluate. Eight cases in the mifepristone group and 6 in the control group were assessable at baseline biopsy as well as at the end of study.

Study medication was initiated at the first day of menstruation. The mean ($\pm$SEM) duration of treatment days were 85 ± 1 days in the mifepristone group and 83 ± 2 days in the control group up to the day prior to surgery.
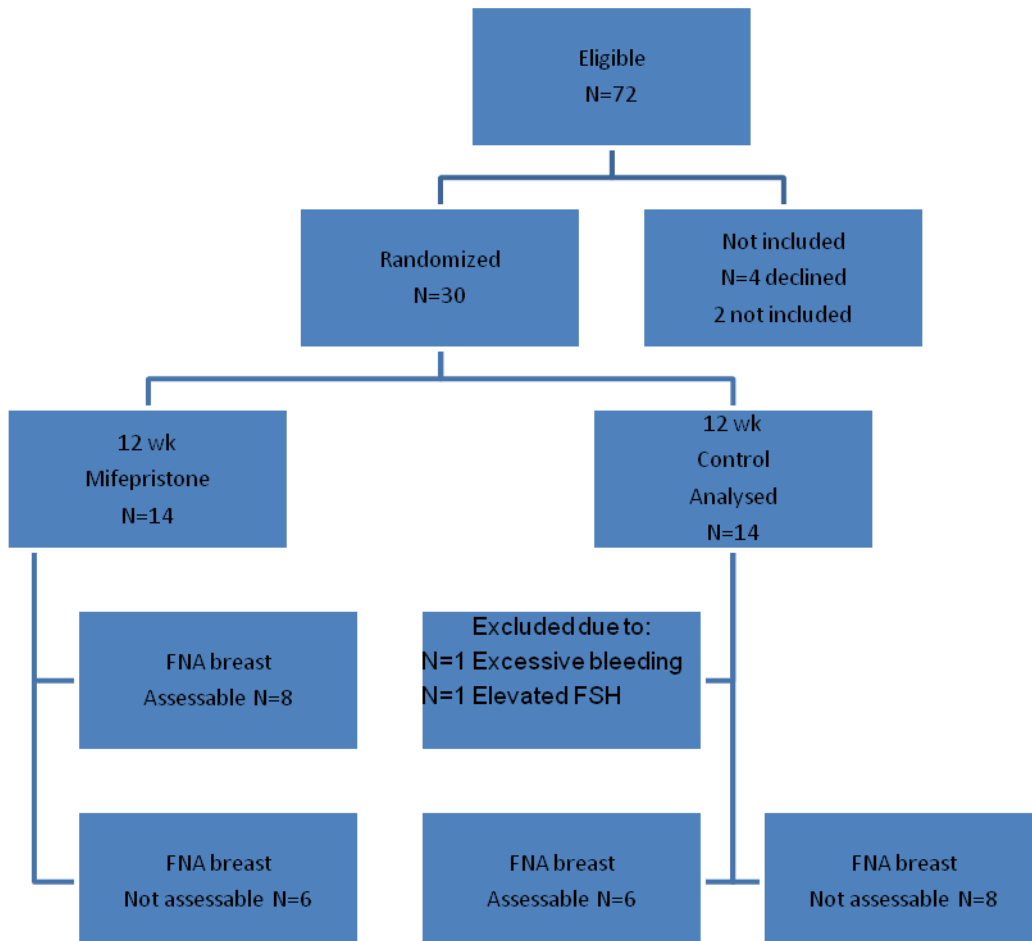
4.2 CLINICAL OUTCOME PARAMETERS

4.2.1 Leiomyoma volume and hemodynamic

The median (interquartile range) volume (ml) of the dominant leiomyoma at baseline was 97 (42–192) in the control group and 137 (111–163) in the mifepristone treated group. This difference was not significant. The volume of the dominant leiomyoma exceeded 375 ml in only one patient per treatment group at the end of study. The median (range) number of leiomyomas at baseline was 2.0 (1–4) in the control group and 1.5 (1–4) in the mifepristone group.

For the dominant leiomyoma, from baseline to the end of study, the mean percentual volume increase (± 95% confidence interval (CI)), was +8% (95% CI:-10 to 26) in the control group (N=15). The change was not significant within the control group.

The mean percentual change of the dominant leiomyoma at the end of study in the mifepristone group (N=12) was -27% (±95% CI: -47 to -8), (p=0.028 within group), this represents a significant change compared to the control group (p=0.014).

The total myoma volume % change, in the control group was +6% (95% CI -13% to 25%), which represents a significant difference when compared to the volume % change of -28% (95% CI -48% to -8%), (p=0.021) in the mifepristone group at the end of study. See table I.

No differences were seen for uterine and leiomyoma vascular impedance (PI) and peak flow measures. There was a pronounced variability among the obtained hemodynamic measures.

Table I

Leiomyoma volume change of the dominant and total leiomyoma volume expressed as median and IQR (interquartile range). The percentual change of volume is described as mean (±0.95 confidence interval).

Volume (ml)	Control			Mifepristone		
	Baseline (N=16)	End of study (N=15)	P=	Baseline (N=14)	End of study (N=12)	P=
Dominant myoma median (IQR)	97 (42-192)	85 (68-160)	n.s.	137(111-163)	96 (42-129)	0.028[a]
% change mean (±0.95 confidence interval)		8 (-10 to 26)	0.014[b]		-27 (-47 to -8)	0.014[b]
Total myoma median (IQR)	134 (53-196)	118 (80 -160)	n.s.	161 (111-209)	106 (52-205)	0.023[a]
% change total mean (±0.95 confidence interval)		6 (-13 to 25)	0.021[b]		-28 (-48 to -8)	0.021[b]

[a] p-value within group from baseline to the end of study
[b] p-value between the groups at the end of study
The percentual change (%) was distributed with skewness < ±1 permitting report as means and 95% confidence interval.
P-values were calculated by the use of non parametric Mann Whitney U-test between groups and Wilcoxon signed rank analysis for paired values over time.

Fig 3.

Distribution of leiomyoma volumes (ml) over time, during 12 wk treatment. The spread of volumes is visualized for both groups, the trend of regression, is prominent within the mifepristone treated group (p=0.028). One case in the control group was extremely outlying and not included in this graph. Due to heavy bleeding she was excluded after 6 weeks.
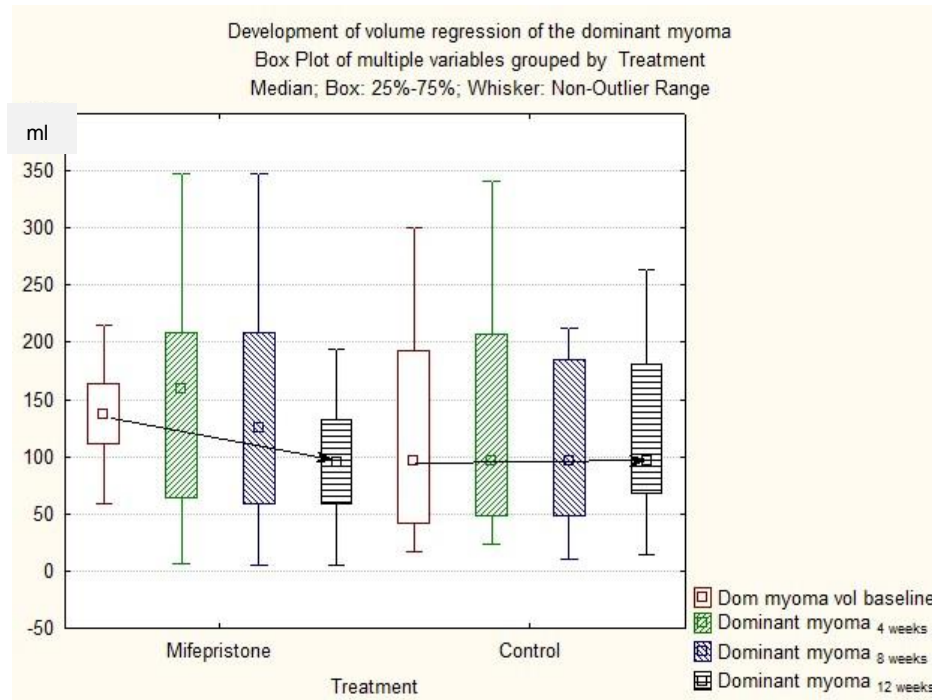
4.2.2 Uterovaginal bleeding

There was a similar distribution of age, menstrual days and cycle length in the treatment groups, as well as the indication for myoma surgery. During the first 4 weeks of treatment the control group bleeding day count for bleeding was median (range) 7.5 (2-19), and for the mifepristone group 7 (4–10). A highly significant reduction (p = 0.001) in bleeding days occurred during week 5–8 compared to the first 4 weeks of treatment within the mifepristone group. The bleeding days were 0 (0–2). In contrast, the count for the control group remained 7.5 (3–26). The difference between treatment groups was highly significant (p < 0.001). Only two women in the mifepristone group reported bleeding for 1 and 2 days respectively during treatment weeks 5–8. Only one of these patients reported spotting on one occasion during treatment week 9–12, while the control group had 3 (0–27), days of bleeding. Thus 86% of patients in the mifepristone treated group were completely free from vaginal bleeding during treatment week 5-8, and almost 100% during the last four weeks of treatment.

4.2.3 Local and general symptoms

4.2.3.1 Diary data

No significant changes between or within the groups were observed in daily self reports of pain, pressure and sense of heaviness in the pelvis.

4.2.3.2 Local and general symptoms

Likert scale scores were evaluated weekly by the participants for local and general symptoms like pelvic pain, backache, abdominal pain, bowel dysfunction, bladder dysfunction, dyspareunia as well as general symptoms like change of mood, headache, nausea or vomiting, decreased libido, hot flushes or loss of general energy. For the control group the score for hot flushes was median (range) at baseline 0 (0-2), and at the end of study 0(0). For the mifepristone group score at baseline was 0.5 (0-7) and at

the end of study 3.5 (0-16), p=0.002 within the group and between treatment groups, p=0.012 at the end of study.

4.2.3.3 Effect on breast symptoms

Significant differences between the groups, was found for swelling and pain (p=0.038). No difference was found between the groups for needle like pains, soreness, or sense of increased volume at the end of study. The parameters reaching significant change within the mifepristone group (mean±SEM) were soreness, 2.43±0.70, at baseline against 0.71±0.58, at the end of study (p=0.035). Swelling 1.93±0.78 at baseline versus 0.0±0.0 at the end of study (p=0.028). Increased breast volume scored mean 2.14±0. 90 at baseline and 0.36±0.36 at the end of study (p=0.043). In summary, all symptoms of breast soreness, swelling, pain or sense of increased volume at baseline faded out almost completely towards the end of study.

4.2.4 Hematology parameters

The difference in hemoglobin values between the two groups was significant after 12 weeks treatment (p=0.046). Within the mifepristone group hemoglobin significantly increased from baseline (mean±SEM) 121±5 g/l, to 133±3 at the end of study, p= 0.003. For the control group hemoglobin from baseline to the end of study was unchanged (mean±SEM) 124±16 to 122±17 g/l.
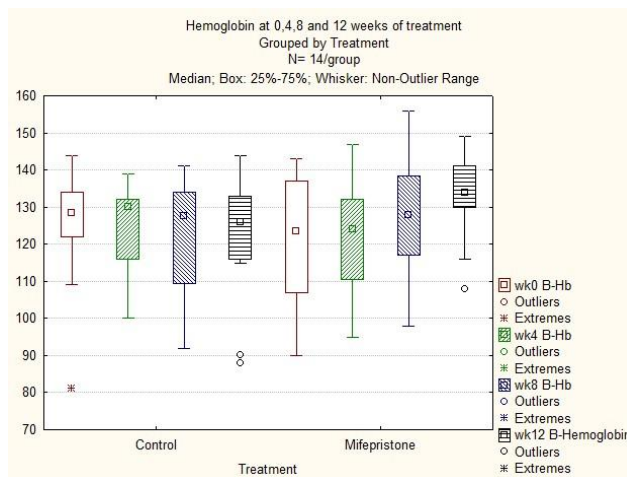

Fig 4.
Hemoglobin levels during mifepristone treatment, significantly improved from baseline, 4, and 8 weeks compared to the end of study. Hemoglobin was significantly higher in the mifepristone treated group after 12 weeks of treatment (p=0.048).

4.2.5 Serum steroid levels

For details, see table I in paper I. A significant increase in androgens and their precursor, androstenedione was observed in the mifepristone group compared with controls/baseline. Estradiol was significantly lower at the end of treatment compared with baseline (p=0.004), but still in the range of proliferative phase levels. Urinary 24 hour cortisol did not change during treatment within or between treatment groups.

4.2.6 Endometrial histology

No atypia or complex hyperplasia was seen among the patients included in the study. Histological analysis was done blinded to treatment group. In 8 out of 12 samples in the mifepristone group and 11 out of 14 samples in the control group it was possible to evaluate the morphologic structure and mitotic index (0-3). None of the biopsies showed any evidence of hyperplasia or malignancy. Seven out of eight mifepristone cases (for definition see Paper II) and 4 from 11 controls were classified as presenting a non physiological histology pattern. Cystic glandular dilatation occurred almost exclusively in the endometrium of mifepristone treated women and was present in 5 out of 8 cases compared to 1 out of 11 control cases (p=0.041, as calculated by Fisher´s exact two tailed test in contingence table). No differences between groups were observed in abnormal vessels or mitotic index.

4.2.7 Breast epithelial proliferation

Fine needle aspiration biopsy (FNA) was conducted on average 6 days before the start of study medication and was repeated preoperatively at the end of study. Steroid hormone blood levels were similar in both groups at baseline. In total, 58 breast biopsies were obtained, 30 at the baseline and 28 at the end of treatment (see paper I). The remaining 28 women were those who completed the study. Menstrual and hormonal data were used to verify luteal phase at the time of the baseline biopsy. Progesterone levels showed no significant differences between the groups at baseline or at the end of treatment. A total of 56 (28 pairs) biopsies were analysed. 14 (50%) had assessable paired samples, 8 from the mifepristone group and 6 controls. Previous studies show comparable dropout frequency for repeat biopsies, where MECs from both occasions must meet the assessability criteria (49) (50). For the control group Ki-67 index at baseline median (range) was 2.2 (1.3-21.1) and at the end of the study 4 .1 (0.0-28.5). For the mifepristone group Ki-67 index at baseline was 5.9 (0.4-10.9) and at the end of study 0.8 (0.0-1.86). The reduction of Ki-67 index within the mifepristone group was strongly significant (p= 0.012).

Fig 5.
FNA biopsies from the left breast of the same patient, at baseline and after 12 weeks treatment with mifepristone.
Brownish coloured nuclei => Ki-67 immunoreactive, proliferative MECs.

<u>**A: Before treatment; B: After 12 weeks mifepristone exposure.**</u>
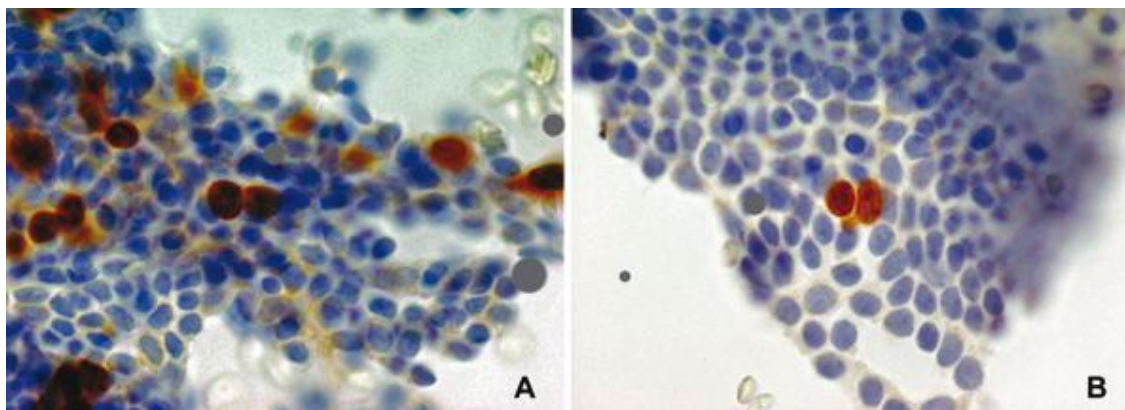
4.3   IMMUNOHISTOCHEMISTRY

4.3.1 Endometrium

4.3.1.1   Steroid receptors

On surgery endometrial biopsies were obtained, fixed in formalin and paraffin embedded. Sections were mounted on slides. Staining was conducted for the nuclear receptors ER-α, ER-β, PR-B and PR A+B, androgen receptor (AR) and Ki-67. Mifepristone increased the expression of ER-α (p=0.012), PR-B (p=0.017) PR (A+B) (p=0.039) and AR (p=0.04) for the *glandular* endometrial compartment. In luminal epithelial cell*s* PR (A+B) increased (P = 0.03) compared to the control group. No differences for steroid receptors were observed in the stromal compartment.

4.3.1.2   *Ki-67 immunoreactivity*

At the end of study mifepristone significantly down-regulated the expression of Ki-67 in the stromal compartment compared with controls (P =0.026).

4.3.2 Leiomyoma

4.3.2.1   *Steroid receptors and growth factors*

For mifepristone treated cases immunohistochemistry showed IRS up regulation for ERα in the diagonally cut category, median (range) 6 (3-10.5) p=0.035 compared to 4 (3-8) for controls, (p=0,059 for the cross cut category). There were no significantly different IRS scores for ER β, PR-B, PR A+B, Caspase-3, FGF or EGF in any of the categories.

4.3.2.2   *Ki-67 immunoreactivity*

For Ki-67, there was a significant reduction of proliferative index in the mifepristone treated group (N=9) mean (range) 5.0 (1.0-15.5), and for the controls (N=8) 15.3 (2.5-57), p=0.049.

4.4   GENE EXPRESSION: MIFEPRISTONE VERSUS CONTROL GROUP

4.4.1 Microarray

In the mifepristone group 3624 genes were changed by 2-fold or more in comparison with the control group. Among these, 113 genes were upregulated more than fivefold, and 62 were downregulated more than five fold upon mifepristone treatment. Ingenuity pathway analysis (IPA) showed changes in eighteen canonical pathways. The most significant pathway upon IPA analysis was the Integrin pathway (p=0.0007). Upon IPA processing, the EGF pathway was the second most significant pathway (p=0.002). The third most significant pathway was the Nrf2-mediated oxidative stress response pathway (p=0.005). The Ephrin pathway (p<0.007), was the 4th most significant pathway.

4.4.2 Real time PCR

- Gene expression, significant results from the Integrin pathway:

- ROR1, receptor tyrosine kinase-like orphan receptor 1, (PCR fold change: +3.1, p=0.013 (microarray fold change + 3.6)).
- PIK3R1, phosphoinositide-3-kinase, regulatory subunit 1 (PCR fold change -1.8 , p=0.038 (microarray fold change -2.9)).

- Gene expression, significant results from from the Efrin pathway:

- GRIN2A, Glutamate receptor ionotropic N-methyl D-aspartate 2A, (PCR fold change -5,6, p=0.041 (microarray fold change -3.3).
- CREB5, cAMP response element binding protein 5, (PCR fold change -2,3, p=0,030, (microarray fold change -2.5)).
- ROR1 receptor tyrosine kinase-like orphan receptor 1, (PCR fold change +2,5, p=0,021, (microarray fold change + 3.6)).
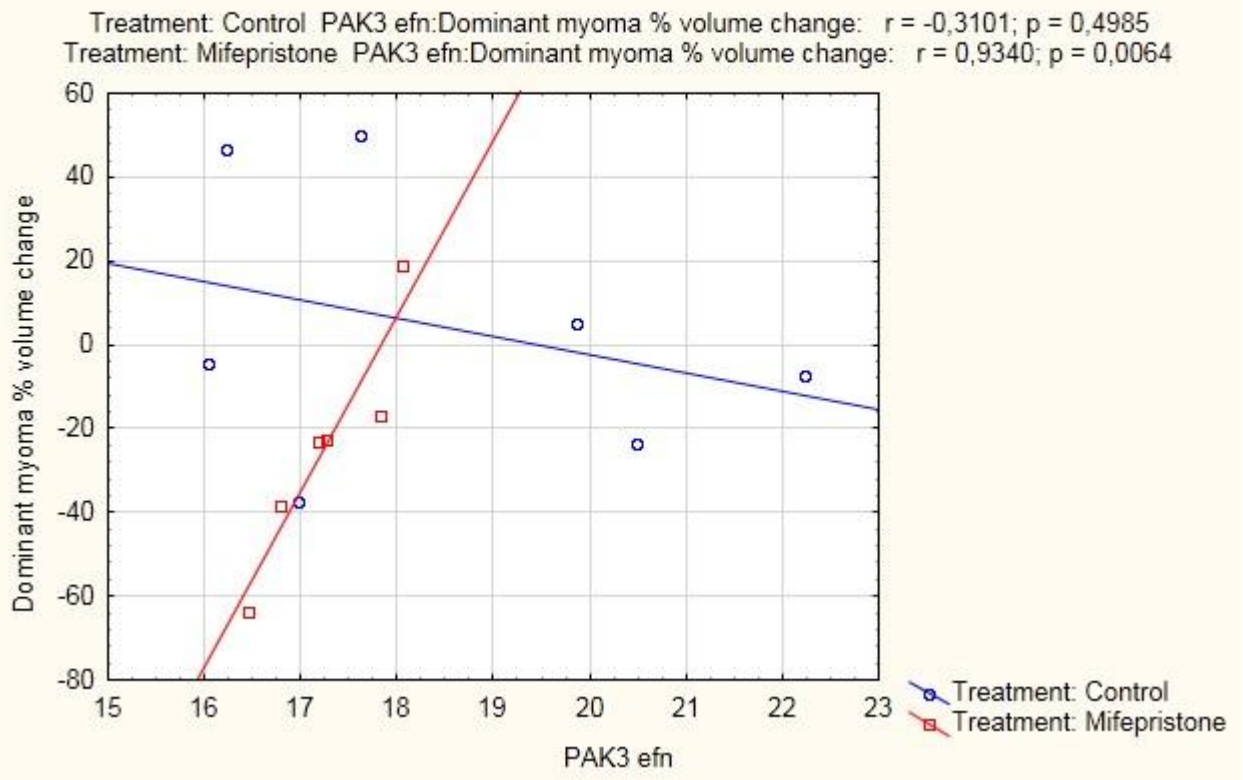
Confirmation of the microarray screening and IPA analysis was performed in order to verify the fold changes found in significant pathways by further analysis utilizing Real time PCR. Ten out of fourteen genes were confirmed by Real time PCR as similarly changed compared to microarray. Seven controls and 8 samples from the mifepristone

group were subjected to Real Time PCR amplification of Integrin and Ephrin pathway target genes, and the cell-cycle related genes: TP53, MKI67, and CDKN1A were also checked for correlations. Seven cases in each group were used for statistical analysis. One gene expression, PAK3, p21-activated kinase 3, showed linear correlation (p=0.006) to the clinical percentual volume regression of the dominant leiomyoma in the mifepristone treated group. The slope is significantly different for the mifepristone treated group compared to the controls. The difference as analysed by ANCOVA, between groups in degree of association between PAK3 expression and percentual myoma volume regression is significant (p=0.048).

Fig 6.
PAK3 dCts are correlating to percentual volume regression of the dominant myoma. The difference between treatment groups was significant upon ANCOVA, analysis of slopes for correlation curves (p=0.048)


Scatterplot of Dominant myoma % volume change against PAK3 efn; categorized by Treatment

Treatment: Control PAK3 efn:Dominant myoma % volume change: r = -0,3101; p = 0,4985
Treatment: Mifepristone PAK3 efn:Dominant myoma % volume change: r = 0,9340; p = 0,0064

4.5   GENE EXPRESSION: GOOD VERSUS POOR RESPONDERS

Fig 7.
A marked variation in myoma volume regression was observed in both groups. Two mifepristone treated cases had almost 20% volume increase; they were categorized as poor responders to mifepristone. The two strongest responders showed a volume reduction of -60 and -80% respectively. The population standard deviation was 30% (33% for controls, and 30% for the mifepristone treated group).
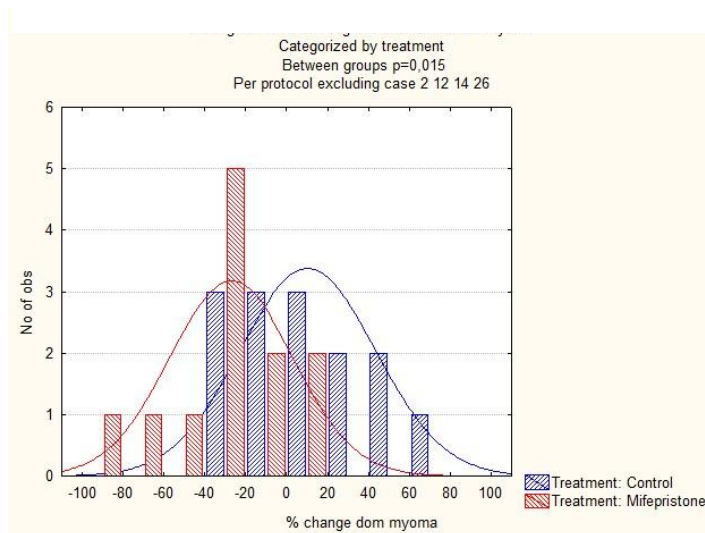
4.5.1 Microarray
The mean signaling values for the good and poor responders were analysed (utilizing IPA). Twentyone canonical pathways showed significantly different expressions (p <0.05) for good and poor responders to mifepristone treatment. The most differently expressed pathway was Metabolism of Xenobiotics by Cytochrome P450 pathway (p=$1.9*10^{-7}$). Participating glutathione s-tranferases have an impact on the cell-cycle and aldo-ketoreduktases are involved in the metabolism of sex steroids and the neutralisation of ROS (reactive oxygen species).
The second most significant pathway was the Glutathione pathway harboring glutathione-s tranferases (p=0.0001). The pathway includes: GSTM1, glutathione S-transferase mu 1 (fold change +8.0); GSTM2, glutathione S-transferase mu 2 (fold change +1.5 fold); GSTM3, glutathione S-transferase mu 3 (fold change +2.3 fold); GSTM5 glutathione S-transferase mu 5 (fold change +2.2). Gene expressions in the glutathione pathway overlapped in the Nrf-2 mediated antioxidant pathway. The rationale for choosing the glutathione pathway for further Real time PCR analysis, was the occurrence of an optional biomarker molecule such as GSTM1 and also the participation in the Nrf-2 mediated antioxidant pathway.

4.5.2 Real time PCR
Taq Man primers and probes were purchased for Real Time PCR analysis for the specific genes: GSTM1, GSTM3, GSTM5, and as reference genes ESR1,PGR,and PAK3,MKI67 and TP53. Among the glutathione genes GSTM1, GSTM3, GSTM5 and glutathione-peroxidase-2 (GPX2), only GSTM1 was differently expressed in the good responder group as compared to the poor responders. GSTM1 was not expressed at all among the poor responders.

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4.6   TUNEL ANALYSIS

Terminal deoxynucleotidyl transferase dUTP Nick End Labeling assay was done on deparaffinized tissue sections.There was no significant difference between the treatment groups in apoptotic index (AI). For the mifepristone treated group (N=12): the median (min, max) was 7.3 (1.4 -56.7), and for controls (N=9) 17.4 (1.8-130.0). Furthermore, there was no significant difference in AI between the good (N=4): 2.0 (1.4-8.5) and poor responders (N=4): 5.6 (5.1- 28.6). Higher ranks and more variation was seen among the poor responders. The interobserver correlation was good (R=0.78.p<0.001).

4.7   PRIMARY CELL CULTURE

It was possible to analyse five biopsies out of nine.Total RNA was isolated and cDNA was reversely transcribed. Real time PCR was conducted and the significant genes by Friedman analysis were tested for significance between exposure groups utilizing the Wilcoxon signed rank test. We found significant anti glucocorticoid responsiveness for PIK3R1 and PAK3. Addition of dexamethasone reversed the gene expression for PIK3R1. A down regulative response to dexamethasone on PAK3 expression was quenched by mifepristone (See details in Table VI paper IV).
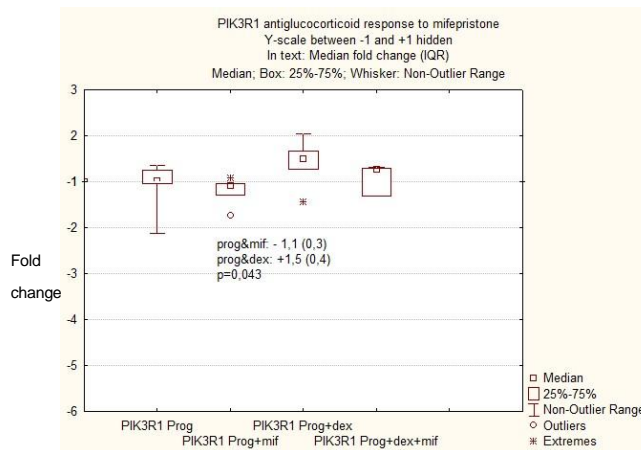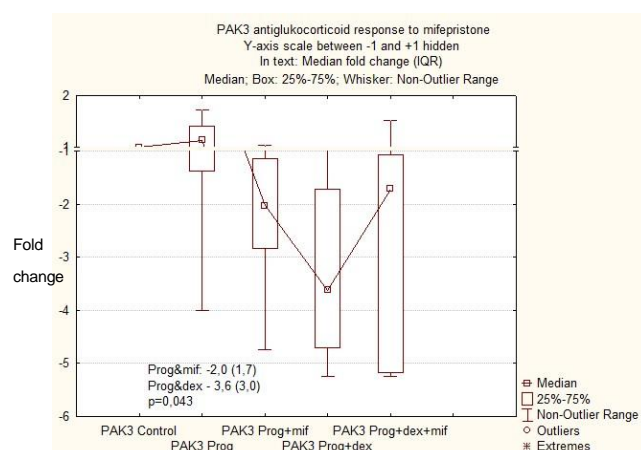

Fig 8.

Prog: Progesterone (P)
Mif: Mifepristone (M)
Dex: Dexamethasone (D)

PIK3R1: P+M was reversed with P+D (p=0.043).

PAK3: P+M+D group completely quenched the negative gene regulation induced by P+D, and therefore is considered as antiglucocorticoid responsive.

5 DISCUSSION

5.1 SUMMARY

The present thesis gives support for the implementation of mifepristone for preoperative treatment before leiomyoma surgery. In the study mifepristone treatment resulted in a rapid and significant reduction in vaginal bleeding and amenorrhea with subsequent improvement in hemoglobin level. A reduction in leiomyoma volume was seen without any dysplastic changes in the endometrium or negative effects on cortisol levels.

For the first time in vivo, we report that proliferation in normal breast tissue is reduced by mifepristone treatment.

Adverse effects from treatment were sparse, except for, mostly low intensity hot flushes. Breast discomfort in terms of soreness, swelling and a sense of increased volume declined almost completely in the mifepristone treated group.

Comparing the two groups, leiomyoma tissue gene expression changes, are seen most significantly in the Integrin and Ephrin pathways, as evaluated by microarray analysis processed in IPA. Gene expressions were correlated to percentual regression of leiomyoma volume as assessed with ultrasonography, with a positive correlation between p21-activated kinase 3 (PAK3) and volume regression. Furthermore, leiomyoma growth reduction was dependent of the expression of GSTM1. Since there is a marked variation in leiomyoma regression in response to mifepristone treatment, GSTM1 determination could provide useful information for preoperative selection of cases for this treatment. We propose GSTM1 as a prospective novel biomarker for leiomyoma regression during mifepristone treatment.

The antiproliferative and thus potentially protective effect on the normal breast is a finding of great interest for further studies of PRMs in treatment regimens with longer duration. Mifepristone has been shown to be highly effective for contraceptive use either by inhibiting endometrial receptivity or by blocking ovulation (51) (52) (53). The PRM Ulipristal was recently approved by the FDA for emergency contraceptive use. Given the substantial incidence of breast cancer, PRMs may confer major health benefits when used for indications including leiomyoma, or contraception.

5.2 COMMENTS ON MATERIALS AND METHODOLOGY

5.2.1 Study design
Only three previously published studies on leiomyoma regression by mifepristone treatment were placebo controlled. The lack of placebo control is rather understandable, as 3 months-or longer, without relief from bleeding and other symptoms with a placebo treatment, could limit the number of patients available to participate in clinical trials. Although recruitment was slow in the present study, only one patient in the placebo group discontinued the study for prompt surgical treatment due to unacceptable bleeding.

At the end of the study, some patients in the mifepristone treated group asked for continuation of the medication instead of surgery. It would be interesting to include such an option for the patients to choose in the protocol for future studies.

5.2.2 Breast biopsies
In the present and many previous studies on normal breast tissue, FNA is the method of choice. Repeat biopsies were frequently obtained without excessive discomfort for the patients. However, in about 50% of repeated biopsies the procuration of MECs were not obtained in sufficient number (>50 cells) to obtain a valid index for breast cell

proliferation. The 50% reduction in valid outcomes should be considered in future studies when calculating sample size.

5.2.3 Tools for symptom registration and monitoring

In the present study registration of leiomyoma derived symptoms did not disclose any significant findings with the exception of complete cessation of vaginal bleeding. Among general effects there was a small increase in the number of hot flushes recorded. No impact was found on pain, pelvic sense of heaviness or dysuria elicited by bladder pressure. However, patients with severe mechanical symptoms were less likely to accept participation in this study. Moreover, obvious risks of serious discomfort for the patient, or threat to pelvic organ function were criteria for exclusion of patients from the study. The patients not included due to severe symptoms at baseline reduced the inherent potential for dramatic reduction of mechanical symptom scores during the way of the study.A less blunt tool for evaluation of symptoms could add value for future studies. At the time of planning this study, to our knowledge, no validated tool for symptom self registration was available. Since then, the Uterine Fibroid Score-Quality Of Life questionnaire (UFS-QOL) has been developed. This questionnaire includes a number of validated questions which are actually similar to the questionnaire we used, (54).

5.2.4 Diagnostic issues with leiomyomas

Reduction of total uterine volume has been observed by other investigators. We agree with reports that ultrasound volumetric measurement has shown limitations in terms of accurately evaluating larger (>375 ml) and multiple (>4) leiomyomas (19, 55). In the present study the number of myomas was in the range of 1-4 for both treatment groups. The volume of all leiomyomas was calculated with the formula for an ellipsoid. However the total uterine volume cannot be considered ellipsoid, when multiple or larger leiomyomas were included. We therefore decided to exclude this parameter. Furthermore, it proved impossible to tag individual tumors. For this reason, in cases with multiple tumors, it was difficult to follow individual myomas between observations at 4 week intervals. Instead we restricted the measurements to the volume of the dominant leiomyoma and the sum volume of all others, up to 5, in each woman. With regard to the description of multiple leiomyomas, the lack of overview limits the accuracy of the vaginal probe compared to MRI. Dependence on observer experience is less with MRI than any of the ultrasound dependent investigation modalities, such as TVS or hydrosonography, as well as compared to hysteroscopy (55). MRI may be a better tool for reproducibility and mapping for scientific purposes. To study hemodynamic parameters, the use of ultrasound Doppler is considered superior, as no validated method for measuring blood flow and impedance is currently available with MRI. Our methods could not reveal any significant decrease in blood perfusion as an explanation for reduced bleeding and myoma regression. PI and peak flow was unchanged after mifepristone treatment within the group, or between groups at the end of study. The hemodynamic data showed pronounced variation within groups over time and between groups at the end of study.

5.2.5 Leiomyoma IHC

The cellular mitotic index is normally low in benign uterine leiomyomas. The PR receptor content is higher in leiomyoma than in the adjacent myometrium. The degree of proliferation is dependent on the menstrual cycle, with a marked increase of Ki-67 during the secretory phase; markers for apoptosis are normally absent (56). By analogy, in the present study a significantly reduced proliferation as measured by Ki-67 expression was found, and there was no increase of the apoptotic index as assessed by TUNEL analysis following exposure to mifepristone.

5.2.5.1   *Steroid receptors and growth factors*

The IRS scores on myoma tissue sections did not correlate well between the observers as the appearance of staining intensity varied in the sections according the plan of cut through the whorled tissue bundles. There was no difference in immunoreactivity between treatments, with the exception of ERα in the obliquely cut category. Nuclear receptors may be suitable for evaluation, but this is still difficult because of the multi directional arrangement of muscle and fibroblast bundles. Some cells are cross cut, potentially leaving behind a larger portion of nuclear material for staining and other tissue bundles exclude more of the nuclear substance by an oblique or tangential cut line. A 1.4 fold IRS over reactivity for cross cut bundles was seen similarly by both assessors.

5.2.6 Mifepristone dosage

We used a dose of 50 mg every other day which, due to the long half life of mifepristone should correspond to approximately 25 mg per day. It is likely that the dose could be reduced by 2-5 times and still retain the positive effects on bleeding and leiomyoma volume regression (57) (58). A lower dose may give even fewer vasomotor effects. The dose required for an antiproliferative effect on the breast and endometrium is not known. Lower doses of 2-5 mg daily have recently been applied to contraception and leiomyoma treatment.

- ❖ A 12 month study on 10 mg and 5 mg, gave 50% volume reduction and 60% amenorrhea after 6 months.
- ❖ A 10 % occurrence of what was reported as "endometrial hyperplasia" was present only in the 10 mg cohort.
- ❖ No further change occurred during the following 6 months.
- ❖ Follow up, during 6 months after cessation of treatment, showed a maintenance of uterine volume reduction of 40% less than baseline, 8 out of nine cases had resumed menstrual bleeding, all had normal FSH values (58).

Studies on daily doses of 2.5-5 mg of mifepristone, report 7% flushes and around 50% presence of endometrial cystic glandular dilatation without atypia (29, 59).

To what degree patients would prefer amenorrhea is not known. If bleeding were regarded as acceptable, an even lower dose would be likely to marginalize the side effects.

A higher dose, 50 mg daily, was used for a 6 month treatment of endometriosis and conferred endometrial changes with over expression of mitotic activity in stroma (60). In a case report from treatment of Mb Cushing, mifepristone 200 mg daily was administered. It was reported that the woman developed massive endometrial hyperplasia which was though reversible (61). It appears, thus, that the dose is crucial for the endometrium effect.

5.3   PREVIOUS REPORTS

Merging data from 384 cases, (Table II), a mean dose of 14 mg would give:
- ❖ 40% reduction of leiomyoma volume and amenorrhea in 90 % of cases after three months, and in 50% of cases after 6 months.
- ❖ Pain relief would be accomplished in 6/10 cases.
- ❖ Occurrence of hot flushes in 3/10 cases accompanied by endometrial "non physiological secretory changes" would be seen in 10% of cases.

15 reports 18 studies	Table I — Mifepristone treated cases (N=384), proportions after 3 and 6 months treatment, sorted after dosage. Eisinger reports a 12 month study with follow up. 18 studies, 15 reports, 2 Chinese reports, from abstract in English. Sorted according to dose. Three studies are placebo controlled *.								
	Year	N (Total 384)	Daily dose (mg)	Residual leiomyoma volume	Amenorrhea after 3 month	Amenorrhea after 6 month	Pain relief	Hot flashes	Endomet Hyperpl
Murphy	1995	9	50						
Murphy	1993	10	50	0.5	1.0			0.4	
Engman *	2009	14	25	0.7	1.0		0.0	0.4	0.0
Murphy	1995	11	25	0.4	1.0				
Reinsch	1994	8	25	0.7				0.0	
Yang(in Chinese)	1996	15	20	0.4	1.0		1.0	0.7	
Zeng(in Chinese)	1998	45	12,5	0.8	1.0		1.0	0.1	
Bagaria *	2009	19	10	0.7	0.8		0.3		0.6
Carbonell	2008	49	10	0.8	0.9			0.2	0.0
Eisinger ut vol	2003	20	10	0.5	0.9	0.7	0.3	0.1	0.3
Yang	1998	28	10	0.4	1.0		1.0	0.7	
Murphy	1995	9	5	0.7					
Carbonell	2008	50	5	0.6	0.9			0.1	0.0
Fiscella ut vol*	2006	20	5	0.5		0.4			0.0
Eisinger ut vol	2003	20	5	0.5	0.9	0.6	0.3	0.1	0.2
Murphy	1995	9	5	0.7	1.0			0.1	
Eisinger 12 months	2005	20	10	0.5		0.6			0.1
Eisinger 12 months	2005	20	5	0.5		0.6			0
Eisinger	2009	17	2,5	0.9	0.7	0.3			0.0
Mean			13	0.6	0.9	0.5	0.6	0.3	0.1
STDDEV				0.1	0.1	0.1	0.4	0.3	0.2

5.4   MIFEPRISTONE AND THE BREAST

5.4.1 The normal breast

Studies on the clinical effects in normal breast tissue during long-term treatment with mifepristone are of interest. They contribute to the knowledgebase necessary for expanding the clinical applications of antiprogestins to contraception and in the treatment of leiomyoma and endometriosis. The lack of clinical data further justified an effort to gather information about the proliferation in MECs during mifepristone exposure. A significant reduction in MEC proliferation was found in this study.

It has previously been shown that it is feasible to evaluate cell proliferation in FNA biopsy material from the breasts of post-menopausal women as well as from young, healthy women during the menstrual cycle, or from women on oral contraceptives. Data from these studies did not reveal any significant influence of age on breast

epithelial proliferation. Furthermore, parity had no influence on the expression of Ki-67. In previous studies on breast epithelium among naturally cycling women, the Ki-67 index was 2.04% (range 0–6%) during the luteal phase. (50). A 4-fold up regulation during HT correlated to the estrogen levels, not the progestins used (49). In the context of oral contraception, a 2-fold increase in Ki-67 index premenopausal among combined oral contraceptive (COC) pill users was seen. The Ki-67 index correlated to the serum progesterone level of non users and to the level of levonorgestrel in COC users. Interestingly some women had a very high response rate of up to 50% positive for the Ki-67 index, and marked individual variation was registered among the oral contraceptive users (62).

5.4.2 Cohort studies

The Women´s Health Initiative study was closed prematurely as it was considered to disclose evidence for an elevated risk for breast cancer associated with the addition of progestins for HT. Furthermore, increased density on mammary X-rays was called into question our diagnostic tools for breast malignancy (63).

Participants in the Breast Cancer Detection Demonstration Project (BCDDP) conducted between 1973 and 1980 were followed up until 1995 and 2082 breast cancers were identified among 46355 women. The relative risk was determined as1.2 for estrogen only and 1.4 for estrogen+progestin regimens. The yearly risk increase was 1 % for E2 only, compared to 8% for E2+P4 regimens (64).

5.4.3 In vitro studies

Currently there is a lack of human *in vivo* clinical studies for treatment of breast cancer with mifepristone (65). In MCF-7 cell lines mifepristone induced caspase activation and G_1 arrest with or without combination with TMX, for TMX resistant or TMX responsive cell lines (66). On BRCA1 deleted mice, there is impaired metabolism of PR, conferring increased proliferation in MECs. This increase in proliferation was cancelled by mifepristone addition (67). For receptor negative MCF-7 cells the combination of TMX and mifepristone gave an additive effect compared to single therapy. An elevation of TGFβ was seen upon such combination treatment. TGFβ is a molecule which has potential importance for the treatment of receptor negative breast cancer (68). In summary, mifepristone mediates a protective, down regulatory effect on breast tissue proliferation.

5.5   MIFEPRISTONE AND ENDOMETRIUM

5.5.1   Progesterone receptor modulator Associated Endometrial Changes (PAECs)

Endometrial effects of PRMs previously which have often previously been reported as "endometrial hyperplasia" have recently been modified and reviewed. The diagnostic criteria for PAEC are currently under development (69). Specific endometrial changes occur on treatment with PRMs. A mixed degree of proliferation and apoptotic changes are seen in specific, dilated cystic endometrial glands, with condensed stroma and perivascular thickening. In agreement with previous studies, specific cystic glandular dilatation and PAECs were observed in the present study. Earlier studies have reported that hyperplastic changes, seen in the endometrium, are reversible on discontinuation of treatment (61). Progesterone and its receptor have a key position in determination of tissue and cell-cycle response to activated estrogen receptor; however mifepristone does not bind to ER. Continuous progesterone addition to estrogen is reported to exert a protective effect on the endometrium in a HT context (70). For PRMs a concern is directed towards the tentative endometrial proliferative effect. There is at present no evidence that bone demineralization or thrombembolism need to be taken into account

with PRM treatment. Nevertheless endometrial proliferation remains a concern, as blocking of the progesterone receptor leaves estrogen exposure unopposed.

5.5.2 Antiproliferative effect in the endometrium

Despite the potential of the therapy, concern has been raised about the effect of mifepristone, and other progesterone receptor antagonists, on the endometrium. Hypothetically, long-term treatment blocks the biological effects of progesterone, and could result in unopposed estrogenicity leading to endometrial hyperplasia and atypical changes. There is a growing body of evidence that PRMs exert an antiproliferative effect on the endometrium during the formation of specific cystic glandular dilatation. Reports describe suppression of ovulation and in the majority of cycles and asynchrony between endometrial and ovarian activity. After 120 days of treatment with two or 5 mg mifepristone daily no endometrial hyperplasia was seen, and markers of endometrial proliferation had decreased. Mitotic activity in glands and stroma was suppressed (71). In monkey studies, 30 days of daily 2 mg mifepristone, expression of Ki-67 and PCNA (Proliferating Cell Nuclear Antigen) indicated a cell cycle arrest in G1 as no mitoses were seen. Ki-67 and PCNA do not discriminate between cells arrested in G1 and cells actually included in proliferation (72). The present study may provide a basis for reduced concern about proliferation in the endometrium. It provides support for a protective effect of mifepristone on the endometrium.

5.5.3 Androgen mediated effects

The slight but significant increased levels of testosterone and androstenedione seen in the present study may be relevant to observed changes in endometrial morphology and induction of amenorrhea. Androgens inhibit endometrial proliferation. Increase of endometrial AR is seen with mifepristone treatment. Flutamide blocks AR and proliferation is no longer inhibited as shown in monkey studies. Treatment with the anti-androgen flutamide reversed several of the effects of progesterone antagonists on the endometrium which is strong evidence that AR is involved in the antiproliferative effect (73). In primates and women an up regulation in ARs was reported and a possible antiestrogen effect is thereby induced, leading to down regulation of endometrial proliferation as measured by an H3 (phosphorylated histone) mitosis marker (74) (75). Earlier studies showed that mifepristone bind to AR (Moguilewsky and Philibert, 1985). This was confirmed recently by studies on RBA (Relative Binding Affinity) to several steroid hormone receptors (76). The mechanism for increasing the androgen expression could be via a discrete activation of the pituitary-adrenal axis without obvious effect on the cortisol 24 h urinary excretion or alternatively by blocking endometrial aromatase activity. A direct inhibitory effect of mifepristone on endometrial aromatase activity in primary cell culture has been reported (77) and may have a role in the AR associated antiproliferative effect of mifepristone in the endometrium.

5.5.4 Antioxidant effect

In cell culture, a direct anti proliferative effect of mifepristone by inhibition of ^{3}H-thymidin incorporation was shown. However, this impact was dependent on the antioxidant properties of the molecule, at the 11-di- methyl amino-phenyl side chain, not the progesterone receptor targeting 17-prop-1-ynyl region of the mifepristone molecule (78). Reduction of the mifepristone molecule at C17 deletes its antiprogesterone but not its antioxidant properties.

5.5.5 Effect on Uterovaginal bleeding

The most promising and directly clinically useful effect of mifepristone may be the ability to promptly induce amenorrhea. HMB is often the reason for considering

surgical treatment. In the event of successful cessation of bleeding, the indication for surgery eventually declines. In our study no change in resistance or uterine blood flow was seen. A significant up regulation of micro vessels and GR (Glucocorticoid Receptors) in endometrial glands, and luminal epithelium was previously reported. Furthermore a down regulation of VEGF (Vascular Endothelial Growth Factor) in endometrial stroma was observed (79).

5.6   GROWTH REGULATION OF LEIOMYOMAS

In the present study the volume of the largest leiomyoma and the sum of all coexisting leiomyoma volumes were significantly reduced. Mifepristone treatment reduces the volume of uterine leiomyoma, but with pronounced individual variations. The strength of the present study is the the correlation of the in vivo treatment response in volume regression of uterine leiomyomas, to the gene expressions.

Expression of the cytoplasmatic enzyme GSTM1 could hypothetically increase the effect of mifepristone for leiomyoma volume reduction, by reducing the expression of Reactive Oxygen Species (ROS), and increased metabolism of estrogens.

In our study 8/12 women that had a leiomyoma volume regression of more than -20% were categorized as good responders while 4/12 with between -17 to +19% volume change were categorized as poor responders. We found genes which significantly correlated to clinical myoma volume regression: PAK3, EFNB2 and PIK3C2G. Only PAK3 was significantly different between treatment groups in correlation to the percentual leiomyoma volume regression.

The RTK ROR1 (+2, 5 fold) was positively correlated to TP53 (R=0, 89, p=0,019) which is associated with cellular down tuning and senescence, although the mechanism of action by which ROR1 exerts down tuning of the cell-cycle is not clear (see table VII and Fig.4 in paper IV). ROR1 is established as a Wnt protein frizzled receptor, and as such is tentatively involved in the Wnt pathway with tumor repressor activity (80) (81). Real time PCR confirmation showed for the mifepristone group, a + 3, 1 fold up regulation of the Wnt receptor tyrosine kinase ROR1.

In a previous report, TP53 over expression, indicating apoptosis, led to senescence (82). In the present study, the effect of mifepristone treatment appears to be down regulated proliferation rather than up graded apoptosis, as indicated by results from TUNEL analysis.

Down regulation of MKI67 gene expression, and decreased immunoreactivity of Ki-67 in this study, could reflect leiomyoma cellular senescence (83). Significant down regulations were seen for PIK31(-2,9 fold), GRIN2A(-5,6 fold) and CREB5(-2,3 fold) which are involved in submembranous induction of kinases, inducing Ras mediated cytoplasmatic MAPK cascades.

5.6.1 Cytokines

Most growth factor receptors are cytokine RTKs. The transduction of extracellular signaling to induction of intracellular signaling is activated through IGF-1/IGF-1R, FGF/FGFR, EGF/EGFR, PDGF/PDGF-R gene families that are involved in growth of uterine leiomyomas. Interestingly it was shown that P4 upregulated EGF and E2 EGF-R. The expression of both hormones in cooperation was essential to mediate growth of leiomyoma cells in culture. Furthermore an up regulation of bcl-2 (apoptosis protector), and down tuning of TNF-alfa (apoptosis mediator), is consistent with a proliferative net response in leiomyoma to progesterone exposure (34). Also an inhibitory effect on IGF-1 m- RNA by P4 but not E2 is seen, down tuning the proliferative activity, indicating a dual effect of P4, depending on the local tissue composition and menstrual phase. The IGF-1R expression was unaffected by either P4 or E2 exposure. Multiple mutually dependent inputs on timing and tissue abundance, for the induction of

proliferation may give clues as to why certain leiomyomas grow during pregnancy and others do not.

5.6.2 Treatment selectivity

The PRM CDB2914 was observed to increase apoptosis and inhibit proliferation in leiomyoma cell cultures, but not in cells originating from adjacent myometrium. Cleaved Caspase 3 expression was upregulated and bcl-2 was downregulated in leiomyoma cells by CDB2914 exposure. Furthermore the expression of VEGF (Vascular Endothelial Growth Factor) was down regulated in myoma tissue, but not in adjacent myometrium (84), indicating a useful tumor vascular selectivity of CDB 2914.

5.7   MEDICAL OR SURGICAL MANAGEMENT OF LEIOMYOMA

Problems related to uterine leiomyoma cause a huge request for treatment and demand on resources which involve both the individual women affected and society as a whole.

5.7.1 Surgical treatment

Surgical treatment of uterine leiomyomas, whether more or less invasive, have for more than a century offered an option to address clinical problems related to uterine leiomyoma and have the advantages both of tradition and established methodology. For hysterectomy with benign indication, a report was compiled from 42 different clinics in Sweden. The proportion of severe complications reported by all participating clinics was mean (interclinic variation); 5% (0-13%) and mild complications was 23% (13-47) (85). Thus, this demonstrates that there is room for a shift in the paradigm towards medical options.

5.7.2 Medical treatment

Uterine leiomyoma growth is dependent on response evoking signaling from steroid hormones, mainly estrogen and progesterone. Manipulation of cellular receptors for steroid hormones is therefore a logical target for pharmacological interaction. PRMs are suggested in the context of several gynecological applications with hormonal dependence, such as fertility regulation, contraception, uterine leiomyoma and endometriosis. Clinical investigations in women confirming the potential benefits for long term treatment with no adverse effects on bone mineral density and with preserved level of serum estradiol were reported since 1993 (51, 86, 87) as well as research on cynomologous maquaques(88). When the present RCT was planned no placebo controlled studies were available.

In the present study we limited the study duration to 12 weeks in order to provide a background for further studies with options for dose reduction and extended time periods as required for the treatment of uterine leiomyoma and endometriosis. With the aim of avoiding surgical treatment during the premenopausal years when increasing symptomatic prevalence develops, symptomatic neutralization of uterine leiomyoma may be indicated between the ages of 40- 50, until natural menopause. Routine gynecological examination, cervical PAP smear, ultrasonographic examination, mammography and endometrial biopsy should be regarded as indispensable before initiation of treatment, with yearly follow up.

5.8 FUTURE PROSPECTS

➢ PRMs are considered useful for the treatment of leiomyoma and endometriosis in premenopausal women apart from their usage for contraception. Application in postmenopausal HT could also be foreseen.

➢ Further monitoring and research concerning the effects of mifepristone on endometrium and breast tissue is needed, although available data suggests a protective effect on breast tissue and an absence of harmful effects in the endometrium.

➢ In order to study the molecular mechanism of action of mifepristone at protein level in leiomyoma, highly sensitive quantitation techniques are necessary. We plan to apply proximity ligase protein assay for this task.

➢ Protein correlations to leiomyoma volume change in vivo could be relevant and helpful for understanding the molecular regulation of leiomyoma senescence. This could be explored in order to identify future therapeutic targets for leiomyoma.

➢ Development of GSTM1 as a biomarker for the tailoring of medical treatment of uterine leiomyoma would include clinical trials and investigation of the possibility of developing a blood biochemical test suitable for the detection of GSTM1 expression.

6 CONCLUSIONS

➢ Mifepristone treatment is appropriate for preoperative treatment of leiomyomas. During the treatment patients were free from vaginal bleeding, significantly avoiding the disadvantage of anemia at surgery.

➢ A novel and important finding is the absence of increased proliferative development in breast tissue during treatment. Whether this effect would indeed lower the risk for breast cancer development during antiprogestin treatment remains to be shown.

➢ Endometrial changes like PAEC are expected with low dose PRMs, though signs of proliferation are absent.

➢ A down regulation of the phosphoinositide kinase regulating protein PIK3R1, involved in the initiation of Ras signaling and MAPK cascade activation, was seen following mifepristone exposure.

➢ Involvement of PAK3 gene expression correlated to the clinical leiomyoma volume regression. PAK3 was characterized as a responder to antiglucocorticoid properties of mifepristone by cell culture experiment.

➢ Determinants of the leiomyoma volume regression response to treatment may be associated to glutathione and Nrf pathways which are involved in quenching the effects of ROS, inducing down regulation of cell cycle progression in leiomyomas.

➢ The objective of the medical community should be to provide effective and safe long term neutralization of leiomyoma symptoms, especially uterovaginal bleeding, although not necessarily removal of leiomyomas.

➢ The introduction of PRMs for treatment of leiomyomas which interfere with the quality of life for women in their fertile age, may have substantial importance for the improvement of women's health in the near future.

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