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HORMONAL REGULATION OF THE PRIMATE UTERUS - EFFECTS OF HORMONE TREATMENT AND LIFESTYLE INTERVENTION

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Stockholm 2015

Published by Karolinska Institutet.
Printed by E-Print AB 2015

ISBN 978-91-7549-836-2

Hormonal Regulation of the Primate Uterus - Effects of Hormone Treatment and Lifestyle Intervention

Thesis for doctoral degree (Ph.D.)

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“If we knew what it was we were doing,
it would not be called research,
would it?”
Albert Einstein

To my family and my country, **Ukraine**

ABSTRACT

The uterus is a major hormone-responsive reproductive organ in primates. Hormones promote their effect via different pathways. Receptors involved in steroid hormone, thyroid hormone and insulin signaling have been found in the endometrium. Several lines of evidence suggest that endocrine and metabolic disturbances may affect the endometrium adversely, leading to abnormalities and reproductive failure. This has stimulated research on factors and potential mechanisms that are related to endometrial dysfunction. The overall aim of this project was to increase the understanding of hormone receptor regulation of the primate uterus, and in particular to investigate the effects of different hormone treatments and lifestyle intervention on endometrial receptor expression.

In paper I and II, 88 ovariectomized cynomolgus macaques were treated with conjugated equine estrogens (CEE), a combination of CEE + medroxyprogesterone acetate or tibolone (TIB), a compound with estrogenic, progestogenic and androgenic activity. At the end of the treatment period uterine tissues were collected and the presence and localization of receptors for steroid hormone and thyroid hormone signaling were investigated.

In contrast to conventional hormone treatment, TIB treatment did not decrease the immunostaining of estrogen receptors in the endometrium of macaque uterus. However, androgen receptor expression in endometrial stroma in this group was higher as compared to controls, which could be one mechanism to explain the endometrial atrophy and beneficial bleeding profile that is associated with TIB treatment.

Immunostaining of thyrotropin-releasing hormone (TRH) receptor, thyroid-stimulating hormone (TSH) receptor and thyroid hormone receptors was detected in all compartments of macaque uterus. Their expression was affected in a specific way by long-term sex hormone treatment. Thus, TRH, TSH and thyroid hormones may have direct effects on the endometrium and thereby influence its function.

In paper III and IV, 20 overweight/obese women with polycystic ovary syndrome (OB-PCOS) were recruited to a three months lifestyle intervention, aiming at weight reduction. Endometrial biopsies were collected at mid-proliferative and mid-secretory phases of the menstrual cycle. The endometrial expression of receptors involved in insulin and estrogen signaling was evaluated.

Gene and protein expression of molecules involved in insulin signaling was up-regulated following lifestyle intervention, indicating improvement of glucose homeostasis and thereby endometrial functioning in these women. Moreover, lifestyle intervention influenced estrogen receptor expression towards normalization in the follicular phase of the menstrual cycle. In six OB-PCOS women ovulation was confirmed. Despite restored ovulation, endometrial expression of the proliferation marker Ki67 was higher in the secretory phase of OB-PCOS women as compared to controls. The elevated endometrial expression of the non-genomic estrogen receptor, G-protein coupled estrogen receptor-1, may be proposed as one mechanism behind the high proliferation activity in PCOS women.

Key words: endometrium, hormone treatment, polycystic ovary syndrome, lifestyle intervention

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Hulchiy M *, Zhang H*, Cline M, Hirschberg A.L, Sahlin L
Effects of long-term tibolone treatment on nuclear sex steroid hormone receptors and G-protein-coupled estrogen receptor-1 expression in macaque uterus
Menopause.2012;19:332-338

II. Hulchiy M , Zhang H, Cline M, Hirschberg A.L, Sahlin L
Receptors for thyrotropin-releasing hormone, thyroid-stimulating hormone, and thyroid hormones in the macaque uterus: effects of long-term sex hormone treatment
Menopause.2012;19:1253-1259

III. Ujvari D, Hulchiy M, Calaby A, Nybacka Å, Byström B, Hirschberg A.L
Lifestyle intervention up-regulates gene and protein levels of molecules involved in insulin signaling in the enometrium of overweight/obese women with polycystic ovary syndrome
Human reproduction.2014;29:1526-1535

IV. Hulchiy M, Nybacka Å, Sahlin L, Hirschberg A.L
Endometrial expression of nuclear estrogen receptors and non-genomic estrogen receptor α36 and G-protein coupled estrogen receptor-1 in women with polycystic ovary syndrome
In manuscript

*- shared first authorship

CONTENTS

LIST OF ABBREVIATIONS

AP-1	activator protein 1
AR	androgen receptor
BMI	body mass index
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CEE	conjugated equine estrogens
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	dehydrotestosterone
DIO1	type I iodothyronine deiodinase
DIO2	type II iodothyronine deiodinase
DIO3	type III iodothyronine deiodinase
dNTP	deoxynucleoside triphosphate
EIA	chemiluminescent enzyme immunometric assay
ER	estrogen receptor
ERα	estrogen receptor α
ERα36	estrogen receptor α36
ERβ	estrogen receptor β
FaR®	physical activity on prescription
FSH	follicular stimulating hormone
GLUT	glucose transporter
GnRH	gonadotropin releasing hormone
GPER	G-protein coupled estrogen receptor-1
HR	hormone receptor
HRE	hormone response element
IGFBP-1	insulin-like growth factor binding protein-1
IR	insulin receptor
IRMA	immunoradiometric assay

IRS1	insulin receptor substrate 1
LH	luteinizing hormone
LS-MS/MS	liquid chromatography-tandem mass spectrometry
MPA	medroxyprogesterone acetate
mRNA	messenger ribonucleic acid
NHS	normal horse serum
NW-C	normal weight controls
NW-PCOS	normal weight women with polycystic ovary syndrome
OB-C	overweight/obese controls
OB-PCOS	overweight/obese women with polycystic ovary syndrome
OvxC	control group without hormone treatment
PCOS	polycystic ovary syndrome
PDK	3-phosphoinositide dependent protein kinase
PI3K	phosphatidylinositide 3 – kinase
PKB	serine/threonine kinase Act/protein kinase B
PRA	progesterone receptor A
PRB	progesterone receptor B
QUICKI	quantitative insulin sensitivity check index
RIA	radioimmunoassay
RPL13A	60S ribosomal L13a
SHBG	sex hormone-binding globulin
SHR	steroid hormone receptor
SP1	specificity protein 1
T3	triiodothyronine
T4	tetraiodotyrosine
TBG	thyroxin-binding globulin
THRα	thyroid hormone receptor α
THRβ	thyroid hormone receptor β
TIB	tibolone
TRH	thyrotropin releasing hormone
TRHR	thyrotropin releasing hormone receptor

TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
UPLC	ultra performance liquid chromatography

1 INTRODUCTION

The uterus is the major hormone-responsive reproductive organ in primates. The first knowledge about the uterus is coming from Egyptian papyrus texts. It took a long time before the anatomy of the uterus was accurately described by Soranus of Ephesus (98-138 A.D). The uterus is a flattened, pear-shaped organ. It is located in the pelvis and it is within the uterus that the full fetal development takes place during gestation. The uterus is built up of three layers. The outer thin layer is known as ***serosa***. The middle layer of the uterus is represented by a thick smooth muscle wall, the ***myometrium***. The uterus expands during pregnancy, providing protection for the fetus, and later during parturition the myometrium serves as a mechanism for fetal expulsion. The inner lining of the uterus is called ***endometrium*** and can be considered as one of the most complex tissues in the body because of the cyclical changes in response to sex steroid hormones produced by the ovaries (Figure 1).

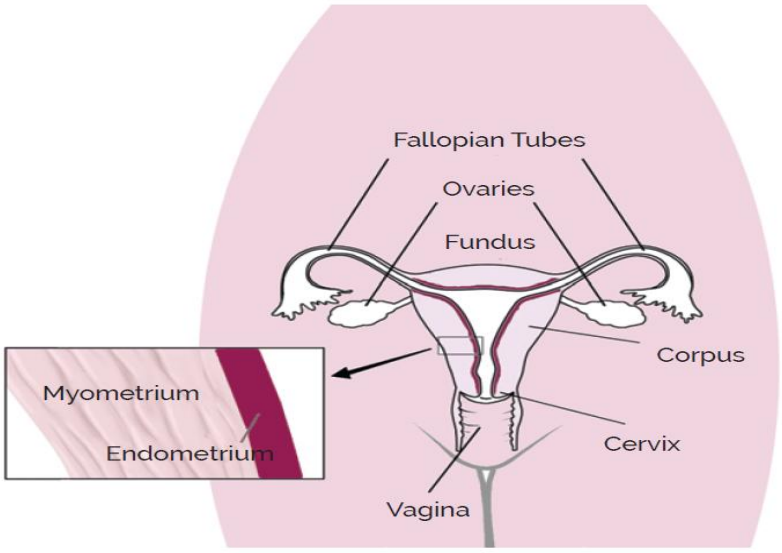

Figure 1. Female reproductive organs.

1.1 THE MENSTRUAL CYCLE

The normal menstrual cycle in women could be described depending on the organ of interest. The ovarian cycle consists of follicular, ovulatory and luteal phases while the endometrial changes are denoted as proliferative phase, secretory phase and menstruation (Figure 2).

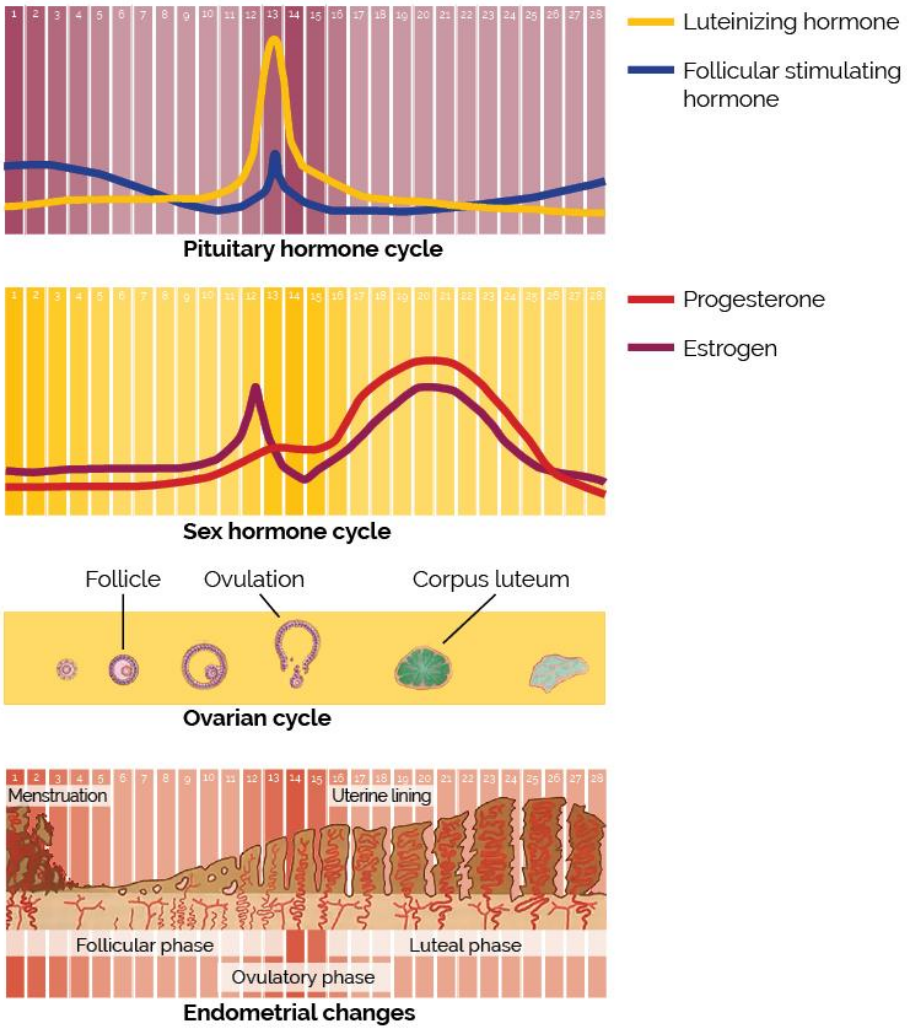

Figure 2. The menstrual cycle (adopted from Wheater's Functional Histology, 5th ed. Elsevier Limited, 2006)

1.1.1 Ovarian cycle

1.1.1.1 The follicular phase

The first day of the menstruation marks the beginning of the new menstrual cycle. During the follicular phase, a cohort of ovarian follicles is recruited and starts to grow under the stimulation of follicular stimulating hormone (FSH) from the pituitary. Circulating levels of sex steroid hormones are low during the first days of the follicular phase but growing follicles start to produce estradiol. The estradiol concentration in the blood increases by the mid-follicular phase and reaches its maximum on the day before ovulation. Usually a single follicle will be selected to become fully mature and ready to rupture, releasing the ovum that is ready for fertilization at the time of ovulation.

1.1.1.2 The ovulatory phase

The rise in estradiol concentration stimulates the secretion of luteinizing hormone (LH) but inhibits FSH secretion from the pituitary. The blood concentration of LH peaks around mid-cycle, the so called LH surge, and induces ovulation about 12 hours later. Androgen production at ovulation enhances atresia of the smaller follicles and stimulates libido. After the LH surge, estradiol levels decrease.

1.1.1.3 The luteal phase

After ovulation, the empty follicle is transformed into another structure, called the corpus luteum. Corpus luteum is producing both estrogen and progesterone. Levels of estradiol and progesterone increase after ovulation, peak in the mid-luteal phase and remain high during the lifespan of the corpus luteum. In the absence of fertilization, corpus luteum degeneration takes place and the production of sex steroid hormones decline. Sex steroid hormone withdrawal leads to endometrial breakdown and menstruation.

1.1.2 Endometrial changes

The human endometrium consists of two layers: the transient ***stratum functionalis*** (upper two-thirds) and the permanent ***stratum basalis*** (lower one third). The ***stratum functionalis*** is built up of a superficial zone, the stratum compactum and an intermediate zone, the stratum spongiosum. Both of them undergo degeneration and breakdown every month of a normal menstrual cycle. The ***stratum basalis*** does not respond to sex steroid hormone fluctuations; instead, it is the source of endometrial regeneration after each menstruation (Figure 3).

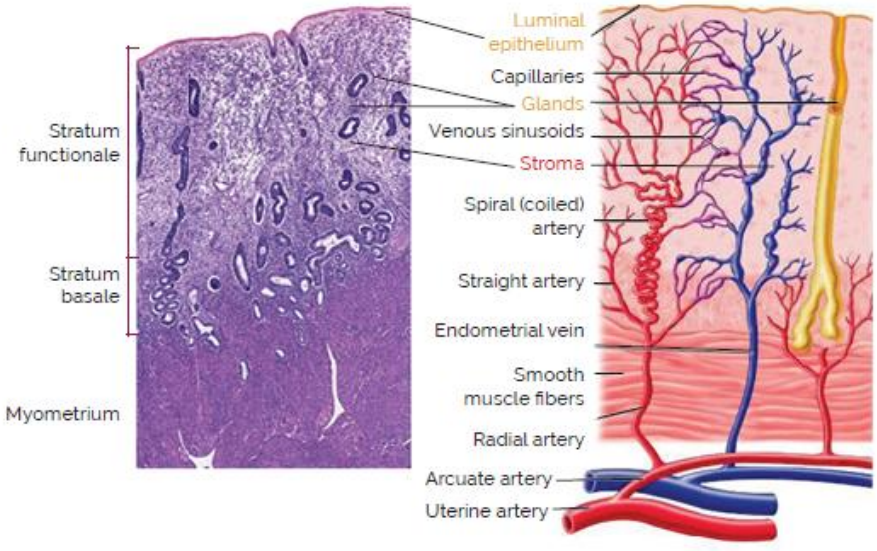

Figure 3. Endometrial morphology (adopted from Pearson Education, Inc., publishing as Benjamin Cummings)

The functional layer consists of different cell types such as luminal epithelium, glandular epithelium and stromal cells (Figure 3). Epithelial and stromal compartments of the endometrium are changing in a dynamic fashion during each menstrual cycle in order to create an appropriate environment for implantation. The different phases of the endometrium are regulated by cyclic production of sex steroid hormones by the ovaries (Speroff and Fritz, 2005).

1.1.2.1 The proliferative phase

During the proliferative phase, the endometrium is growing and proliferating under the rising secretion of estradiol from the ovaries. The endometrium is growing from approximately 2 mm after menses to 10-12 mm in the periovulatory phase. Endometrial glands are becoming coiled and more closely packed and mitotic figures can be seen in glandular epithelium and stroma (Young *et al.*, 2006).

1.1.2.2 The secretory phase

During the secretory phase, after ovulation, progesterone counteracts estradiol stimulation and the endometrium undergoes further transformation, known as cellular differentiation. At this stage the glands are containing glycogen vacuoles, while mitotic figures are not seen anymore. Secretory activity of the glands reaches its maximum around postovulatory day 6 to 7 in preparation for implantation. At the same time, the stroma is beginning to show signs of edema which is continuously progressing until the late secretory phase (Young *et al.*, 2006). Two days before menstruation, a massive leukocyte infiltration of the endometrium takes place, indicating its collapse. Regression of corpus luteum and subsequent decrease in sex steroid hormone production leads to the breakdown and shedding of ***stratum functionalis***.

1.1.2.3 Menstruation

In the absence of fertilization and implantation, corpus luteum regression takes place and estradiol and progesterone levels decrease. The sex steroid hormones withdrawal leads to a profound vascular spasm of the spiral arteries, causing endometrial ischemia as well as a breakdown of lysosomes and a release of proteolytic enzymes. All these events are promoting endometrial destruction and shedding. Simultaneously, myometrial contractions increase and serve to physically discharge destroyed endometrium from the uterus.

1.2 ENDOMETRIUM

1.2.1 Sex hormone receptors in the endometrium

Sex steroid hormones promote their effect on the endometrium via genomic activation by binding to nuclear receptors (functional effect within hours) or more rapid non-genomic mechanisms via membrane-bound receptors (functional effect within minutes) (Figure 4). Classical genomic activation includes the presence of intracellular receptors. After simple diffusion through the cell membrane, sex steroid hormones bind to the respective steroid hormone receptor (SHR) and the hormone receptor complex that is formed interacts with a hormone response element (HRE) on the DNA which results in synthesis of messenger RNA (mRNA), its transportation to the ribosomes and protein synthesis. Many hormone responsive genes lack the HRE and interact with SHR via transcriptional factors, e.g. the specificity protein 1 (SP1) and activator protein 1 (AP-1). In addition to ligand dependent-pathways, ligand-independent pathways, as a response to signaling via growth factor receptors (so called cross-talk), have been described. Non-genomic activation involves membrane bound receptors, such as G protein coupled receptors, which are represented by a polypeptide chain that spans across the membrane. Interaction between receptor and hormone leads to the activation of second messengers.

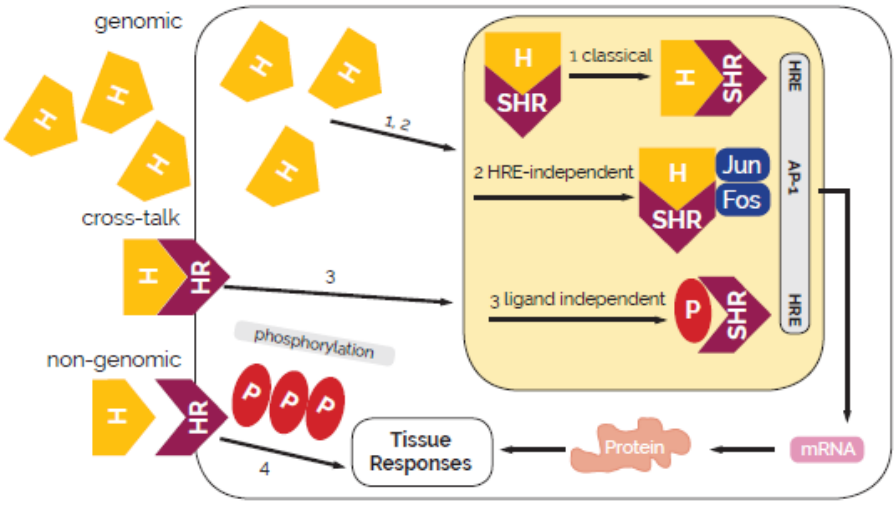

Figure 4. Mechanisms of steroid hormone action. Classical (**1**) and HRE (hormone response element) –independent (**2**) pathways mediate the genomic action via nuclear steroid hormone receptors (SHR). The hormone-receptor (HR) complex either binds DNA via the hormone response element (HRE) or via transcriptional factors, e.g. the specificity protein 1 (SP1) and activator protein 1 (AP-1). Activation of the HRE results in transcription and protein synthesis. Some estrogen receptor-mediated transcription can take place in the absence of a ligand (ligand independent pathway) (**3**) when receptors are activated by phosphorylation in response to signaling via growth factor receptors (s.c. cross-talk). By the non-genomic pathway (**4**), a rapid hormone action is mediated via binding to membrane-bound receptors and inducing a subsequent signaling cascade.

Steroid hormone receptors share a common structure with the thyroid hormone receptors. They all belong to the steroid/thyroid hormone super family of nuclear receptors (Figure 5).

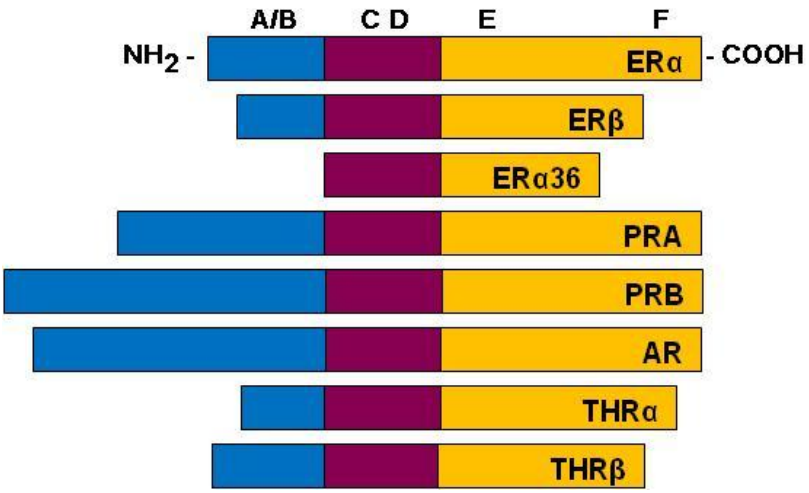

Figure 5. The common structure of the nuclear steroid/thyroid hormone superfamily. The amino terminal (**A/B**), the DNA-binding domain (**C/D**) and ligand-binding domain (**E/F**). **ERα** – estrogen receptor α, **ERβ** – estrogen receptor β, **ERα36** – estrogen receptor α36, **PRA** – progesterone receptor A, **PRB** – progesterone receptor B, **AR** – androgen receptor, **THRα** – thyroid hormone receptor α, **THRβ** – thyroid hormone receptor β.

1.2.1.1 Estrogen receptors

Estrogen is crucial for endometrial proliferation and vascularization and exerts its function via specific estrogen receptors (Smith, 2001; Giudice, 2006). In 1986 the first estrogen receptor (ERα) was cloned (Green *et al.*, 1986; Greene *et al.*, 1986). For a long time it remained the only known ER. However, in 1996 a second estrogen receptor (ERβ) was identified (Kuiper *et al.*, 1996). Both receptors, ERα and ERβ, can bind estradiol with similar affinity and have been shown to be present in the human endometrium. Their expression is cycle-dependent and sex steroid hormone regulated (Giudice, 2006). Expression of ERα and ERβ in the epithelial cells increases during the proliferative phase, reaches its peak in the late proliferative phase and gradually decreases during the secretory phase in preparation for embryo implantation (Giudice, 2006). In the mid-secretory phase, expression of ERα in the stroma decreases under the regulatory effect of progesterone while the expression of ERβ is maintained, making ERβ a predominant receptor of the late secretory phase in the stromal compartment of the endometrium (Hapangama *et al.*, 2014) (Figure 6).

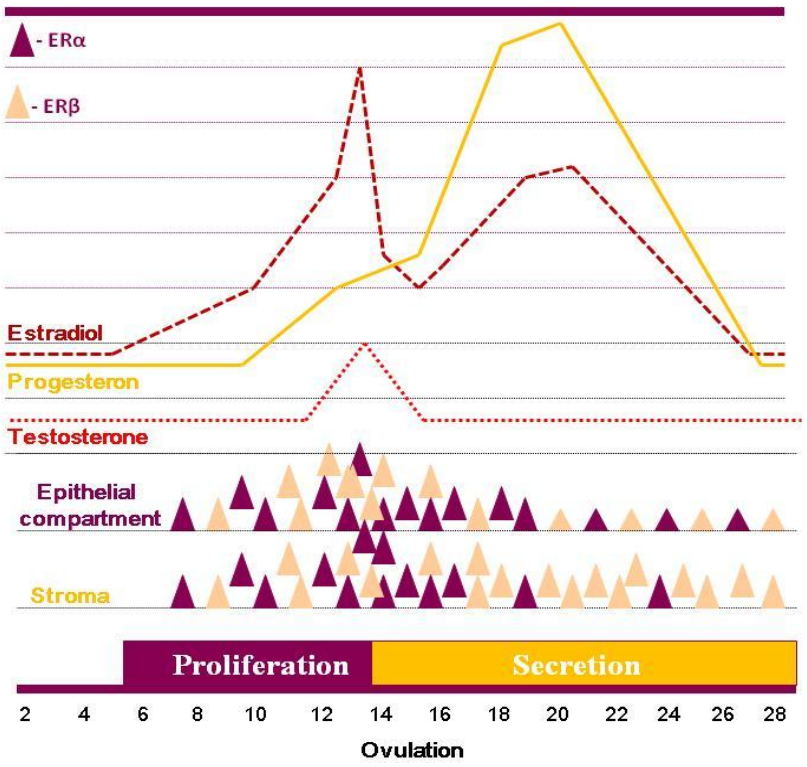

Figure 6. Dynamics of estrogen receptor α (ERα) and estrogen receptor β (ERβ) expression in the epithelial and the stromal compartments of the endometrium during the menstrual cycle.

Although ERα and ERβ share a common structure, their function in the endometrium is distinct in terms of gene regulation and consequently in biological response. While ERα promotes proliferation of the endometrium, ERβ counteracts estrogen mediated effects, providing protection against ERα induced hyperstimulation (Hapangama *et al.*, 2014). Loss of ERβ have been suggested to be an important step in estrogen-dependent tumor progression (Bardin *et al.*, 2004). An increase of the ERα/ERβ ratio was shown in several cancers as compared to normal tissue, including breast and ovary cancers (Bardin *et al.*, 2004). Therefore an imbalance in the expression of ERα and ERβ may play a role in endometrial proliferative abnormalities and should be of special interest to study.

1.2.1.2 G protein coupled estrogen receptor-1

While nuclear ERs have been widely investigated in the human endometrium, the role and regulation of the receptors involved in the immediate, non-genomic, responses are largely unknown. Nearly 50 years ago, it was reported that the intracellular concentration of cAMP increases immediately after estrogen stimulation (Szego and Davis, 1967). The discovery of the membrane bound receptors that bind estrogens opened new possibilities to explain these rapid non-genomic effects. G-protein coupled estrogen receptor-1 (GPER), formerly referred to as GPR30, was first described in 1996 (Owman *et al.*, 1996), however its ER properties

were demonstrated later in 2005 (Revankar *et al.*, 2005; Thomas *et al.*, 2005). GPER belongs to the family of 7-transmembrane G protein – coupled receptors and is localized to the cell membrane and endoplasmic reticulum (Thomas *et al.*, 2005; Funakoshi *et al.*, 2006; Otto *et al.*, 2008) (Figure 4). Its expression is cycle dependent and proposed to be regulated by ovarian steroid hormones in a similar way as ERα and ERβ, with up-regulation in the proliferative phase as a response to the rising levels of estradiol and down-regulation in the secretory phase due to increasing levels of progesterone (Kolkova *et al.*, 2010; Prossnitz and Barton, 2011; Plante *et al.*, 2012) (Figure 7).

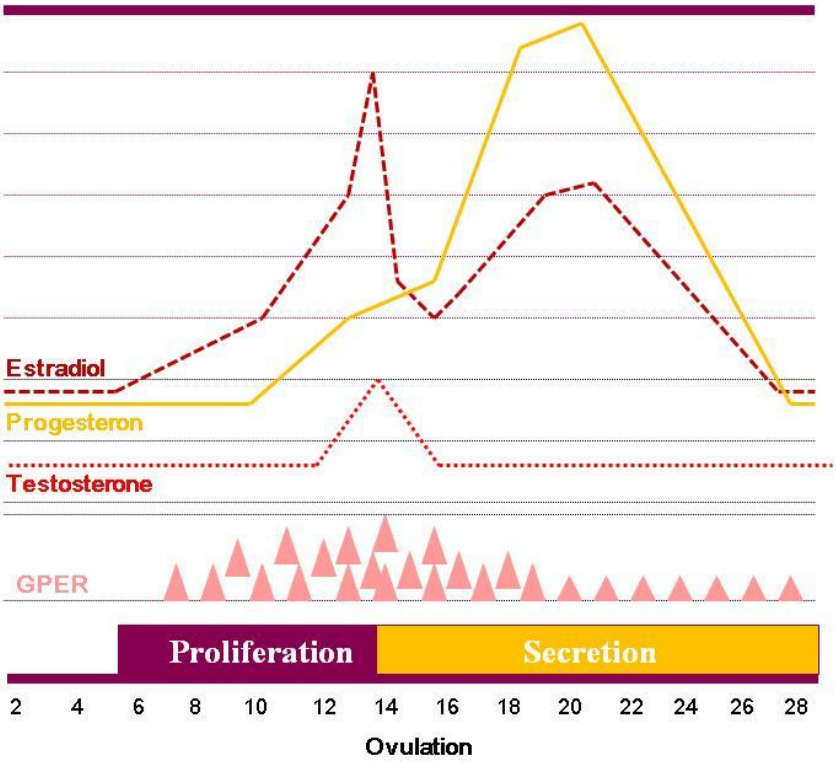

Figure 7. Dynamics of GPER expression during the normal menstrual cycle.

GPER is proposed to regulate cell growth and proliferation through functional cross-talk with ERα. Both receptors have been shown to be co-expressed in the epithelial and stromal compartments of the normal human endometrium (Kolkova *et al.*, 2010). Functional cross-talk between these two receptors was demonstrated in the studies where GPER expression together with ERα was required for estrogen-induced activity in cancer cells (Albanito *et al.*, 2007) and inhibition of ERα mediated effects in endometrial epithelial cells (Gao *et al.*, 2011). Thus, the identification of GPER opens new approaches in the understanding of estrogen mediated effects in the endometrium and the possible abnormalities that is associated with estrogen hyperstimulation.

1.2.1.3 Estrogen receptor α36

ERα36 is a newly described variant of ERα which is lacking the transcriptional activation domains (AF-1 and AF-2) but maintains DNA-binding and partially, ligand-binding domains (Gu *et al.*, 2014) (Figure 5). It was cloned and found to be localized in both cell membrane and cytoplasm mediating non-genomic estrogen signaling (Wang *et al.*, 2006). The GPER specific agonist G1 was shown to bind ERα36 and to promote its activities (Kang *et al.*, 2010). The expression of ERα36 in the endometrium during the menstrual cycle is largely unknown. However, the expression of ERα36 in endometrial cancer tissue was found to be lower as compared to normal tissues (Sun *et al.*, 2013). Moreover, lower expression of ERα36 was associated with endometrial hyperplasia in women with polycystic ovary syndrome (PCOS) (Lin *et al.*, 2013). The higher proliferation activity associated with lower expression of ERα36 can be explained by inhibition of the transcriptional activity promoted by AF-1 and AF-2 domains of ERα (Wang *et al.*, 2006). Thus, low expression of ERα36 may be associated with endometrial hyperplasia and further studies are needed to clarify its function and role in the endometrium (Lin *et al.*, 2013).

1.2.1.4 Progesterone receptors

Progesterone actions are mediated via two nuclear receptors, PRA and PRB. These two isoforms have been found to be expressed in the epithelial and stromal cells of the endometrium (Critchley and Saunders, 2009). Several studies suggest that PRA and PRB display different functions. PRA was proposed to be a transcriptional inhibitor of PR-B when the two receptors are co-expressed (Giangrande and McDonnell, 1999). Moreover, animal studies have demonstrated that mice lacking PRA, but not PRB, fail to inhibit estrogen-induced proliferation (Conneely *et al.*, 2002), which may suggest a key role for PRA in endometrial function. Both isoforms are expressed in the endometrium in a cyclic manner.

During the proliferative phase, expression of PRA and PRB increases in response to the rising levels of estradiol. Importantly, PRA is the predominant PR in the stromal compartment of the proliferative endometrium (Li *et al.*, 2014). During the secretory phase, PRA and PRB expression gradually decreases in the epithelial compartment of the endometrium in response to increased progesterone levels (Li *et al.*, 2014). However, a different situation is observed in the stromal compartment where expression of PRA remains unchanged while PRB is decreasing (Li *et al.*, 2014) (Figure 8).

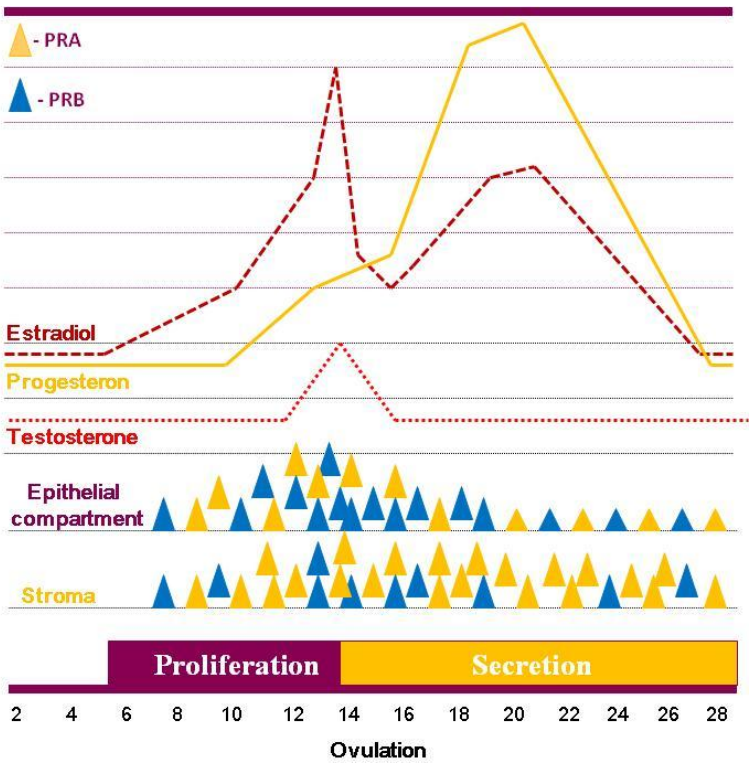

Figure 8. Dynamics of progesterone receptor A (PRA) and B (PRB) expression during the normal menstrual cycle.

1.2.1.5 Androgen receptor

The main androgens that can be found in the blood circulation of women are: testosterone, androstenedione, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) and dehydrotestosterone (DHT). DHT and testosterone are the major ligands for the androgen receptor (AR), while other androgens act as prohormones (Cloke and Christian, 2012). DHT has a higher affinity for AR than testosterone. Endometrial levels of testosterone, androstenedione and DHEA can be higher as compared to blood levels and are increasing in the secretory phase of the menstrual cycle (Cloke and Christian, 2012). Interestingly, expression levels of different enzymes that are important for conversion of androgens are also higher in the endometrium during the secretory phase of the menstrual cycle, leading to the subsequent production of testosterone and DHT that can bind AR (Cloke and Christian, 2012).

AR has been found in the stromal and epithelial compartments of the human endometrium (Mertens *et al.*, 2001). Endometrial AR shows variation in expression during the menstrual cycle with a gradual decrease from the early proliferative phase to the mid-secretory phase (Mertens *et al.*, 1996) (Figure 9).

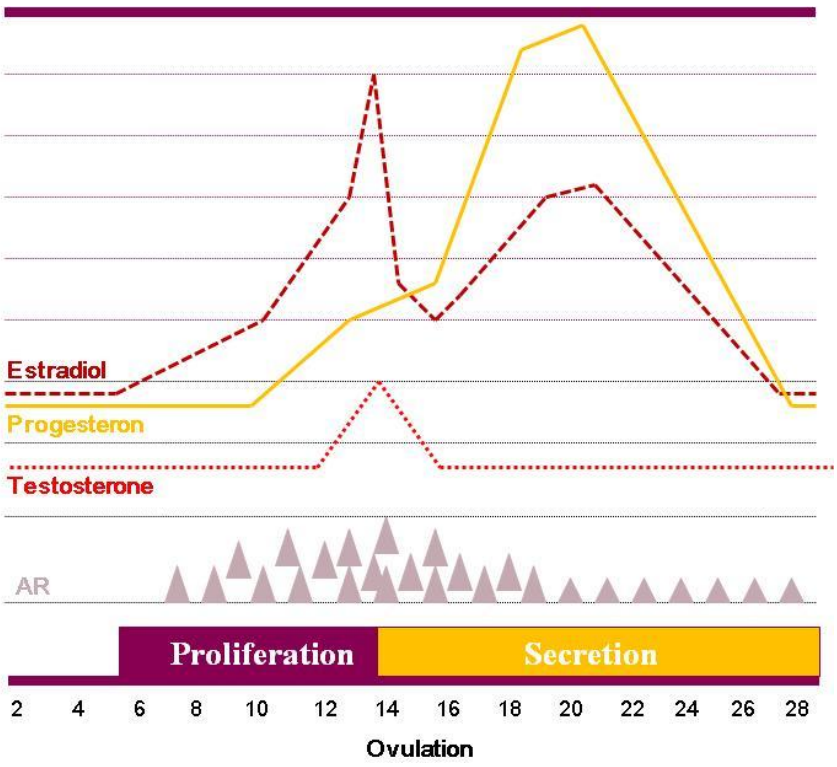

Figure 9. Dynamics of androgen receptor (AR) expression during the normal menstrual cycle.

In *vitro* studies of stromal and epithelial cells have revealed that AR is positively regulated by estrogens and androgens, but down-regulated by progesterone (Apparao *et al.*, 2002). Furthermore, AR expression increases following the administration of the anti-progestin mifepristone, in both human and primate endometrium (Slayden *et al.*, 2001). There is an increasing body of evidence suggesting that androgens can counteract trophic effects of estrogens. After androgen administration endometrial atrophy was observed in female transsexuals (Miller *et al.*, 1986). Moreover, our group has previously demonstrated that testosterone administration to postmenopausal women does not stimulate proliferation of the endometrium but rather counteracts estrogen-induced effects (Zang *et al.*, 2007).

1.2.2 Thyroid hormone receptors in human endometrium

Production of thyroid hormones is controlled by the thyroid-stimulating-hormone (TSH) which is released from the pituitary gland in response to production of thyrotropin-releasing hormone (TRH) from the hypothalamus (Speroff and A.Fritz, 2005) (Figure 10). TSH is regulating iodine uptake by the thyroid gland and coupling iodine to thyroglobulin (Speroff and A.Fritz, 2005) (Figure 10).

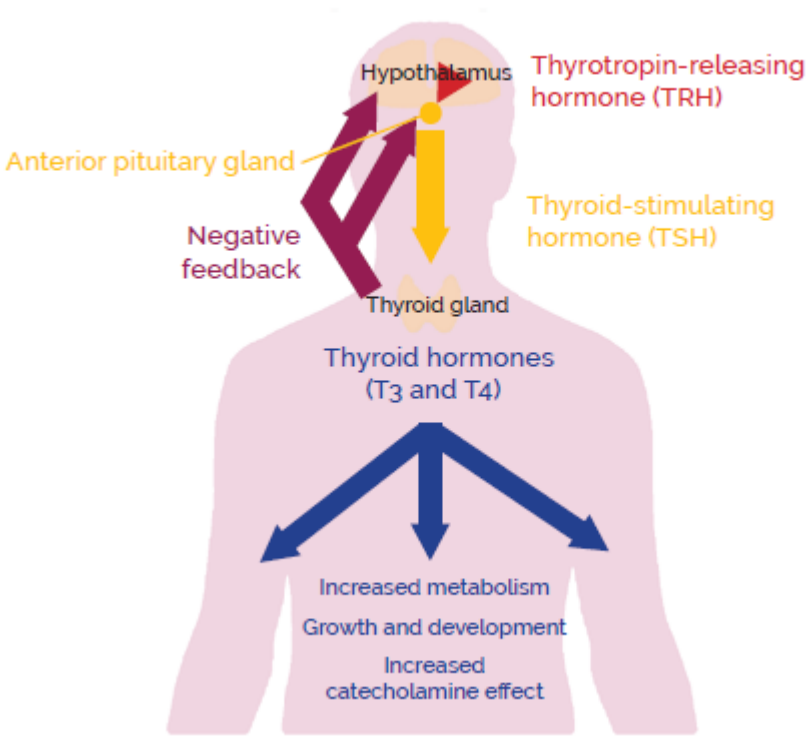

Figure 10. Regulation of thyroid hormone production.

The thyroid gland is a butterfly shaped organ that is situated on the front of the neck and is producing thyroxin (tetraiodotyrosine, T4) and triiodothyronine (T3). Thyroid hormones are important regulators of growth and metabolism (Yen, 2001). Thyroid dysfunction is a condition known to be associated with abnormal pubertal development, menstrual irregularities, infertility and increased frequency of miscarriages, which suggest an important role for thyroid hormones in reproductive function (Poppe and Velkeniers, 2004). Moreover, thyroid dysfunction is more common in women as compared to men. However, the reason behind this sex difference is still unknown.

In the circulation, approximately 70% of thyroid hormones are bound to thyroxin-binding globulin (TBG) which is produced in the liver (Speroff and A.Fritz, 2005). The secretion of TBG can be increased by estrogens. Although T4 is the principle hormone that is produced by the thyroid gland, T3 is much more portent and all thyroid actions in the body can be attributed to its activity (Speroff and A.Fritz, 2005).

In peripheral tissues, T4 can be deiodinated to T3 by peroxidase enzymes, called iodothyronine deiodinases. Several types of iodothyronine deiodinase have been identified: type I iodothyronine deiodinase (DIO1), type II iodothyronine deiodinase (DIO2) and type III iodothyronine deiodinase (DIO3) (Yen, 2001). While DIO1 and DIO2 promote the activation

of the prohormone T4 to T3, DIO3 catalyzes inactivation of T3 and T4 (Yen, 2001; Huang *et al.*, 2003). All three enzymes were found to be present in the human endometrium, raising the possibility of thyroid hormone conversion in this tissue (Aghajanova *et al.*, 2011). Moreover, mRNA expression of the deiodinases was lower in the mid-secretory phase compared to the other phases of the menstrual cycle (Aghajanova *et al.*, 2011). Lower expression in the luteal phase may be attributed to the rising levels of progesterone.

1.2.2.1 Thyroid hormone receptors

The genomic actions of thyroid hormones are mediated via the nuclear thyroid hormone receptors (THRα1, THRα2, THRβ1 and THRβ2) (Zhang and Lazar, 2000) (Figure 5). In contrast to other thyroid hormone receptors, THRα2 is not able to bind T3 and its role is largely unknown (Cheng, 2005).

Thyroid hormone receptors have been found to be present in human endometrial and ovarian tissues (Aghajanova *et al.*, 2011). The immunostaining of THRα1 and THRβ1 in the epithelial compartment of the human endometrium have been found to be significantly higher in the mid-secretory phase of the menstrual cycle as compared to the early secretory phase (Aghajanova *et al.*, 2011). This result is further supported by the study where the administration of mifepristone resulted in down-regulation of THRα1 and THRα2 mRNA expressions and up-regulation of THRβ1 mRNA expression in the human endometrium (Catalano *et al.*, 2007). These findings suggest a possible role of progesterone in the regulation of THR expression in the human endometrium (Catalano *et al.*, 2007).

In an *in vitro* study where endometrial and Ishikawa cells were used, the authors raised the suggestion that T3 and T4 can be produced by endometrial cells (Aghajanova *et al.*, 2011). Taking into account that the different factors required for thyroid hormone synthesis have been found to be present in the human endometrium, it is likely that thyroid hormones are produced locally in this tissue. Moreover, the stimulation of Ishikawa and endometrial cells by TSH resulted in an increase of T3 and T4 levels (Aghajanova *et al.*, 2011).

The role of thyroid hormones in the endometrium is largely unknown. However, several *in vitro* studies propose the possible role of TSH and thyroid hormones in the regulation of endometrial glucose transport via glucose transporters, in particularly glucose transporter 1 (GLUT1) (Hosaka *et al.*, 1992; Weinstein and Haber, 1993; Aghajanova *et al.*, 2009). GLUT1 expression in the human endometrium is higher compared to the other glucose transporters and might be essential for decidualization of stromal cells (Frolova and Moley, 2011).

1.2.2.2 Thyroid stimulating hormone receptor and thyrotropin-releasing hormone receptor

The respective receptors for TSH and TRH belong to the G - protein coupled receptor family. The thyroid stimulating hormone receptor (TSHR) has been shown to be present in human endometrium (Aghajanova *et al.*, 2011). Its immunostaining in the glandular epithelium was found to be higher during the proliferative phase of the menstrual cycle as compared to the secretory phase (Aghajanova *et al.*, 2011). However, in the luminal epithelium a more intense staining was observed in the mid-secretory phase as compared to the early secretory phase (Aghajanova *et al.*, 2011).

The role of TSH and TRH in the endometrium has not been thoroughly investigated. However the stimulation of Ishikawa cells and endometrial cells by TSH resulted in secretion of thyroid hormones. This may indicate functionality of TSHR and its possible involvement in endometrial physiology (Aghajanova *et al.*, 2011).

1.2.3 Insulin signaling in human endometrium

Cyclical changes in the endometrium are dependent on glucose metabolism and adequate glucose supply (Schulte *et al.*, 2015). Specifically, the endometrium needs to accumulate glycogen in the epithelial cells in preparation for embryo implantation.

Insulin is a key player in the regulation of energy metabolism. It can modify glucose homeostasis by stimulation of glucose uptake (Diamanti-Kandarakis and Dunaif, 2012). However, only physiological levels of insulin may have a beneficial effect on the endometrial energy homeostasis. *In vitro*, insulin was shown to inhibit the insulin like growth factor binding protein I (IGFBP-1) which is produced by endometrial stromal cells and a well-recognized biomarker of decidualization (Giudice *et al.*, 1992). Thus, hyperinsulinemic conditions may adversely affect the metabolic state of the endometrium.

The human endometrium expresses molecules that are required for insulin-signaling, including the insulin receptor (IR), insulin receptor substrate (IRS) and insulin glucose transporters, GLUT1, GLUT3 and GLUT4 (Mioni *et al.*, 2012; Shang *et al.*, 2012).

1.2.3.1 Insulin receptor

The insulin receptor consists of two linked dimers: the extra-cellularly located α-subunit with a ligand-binding domain and the β subunit, which crosses the membrane and contains an intrinsic protein kinase activity (Diamanti-Kandarakis and Dunaif, 2012). After insulin binding, the β subunit of the receptor undergoes auto-phosphorylation which leads to its

activation and further phosphorylation of different substrates, including IRS1. Activated IRS can recruit a number of signaling molecules that promote GLUT4 translocation from the intracellular vesicles to the cell surface (Diamanti-Kandarakis and Dunaif, 2012) (Figure 11).

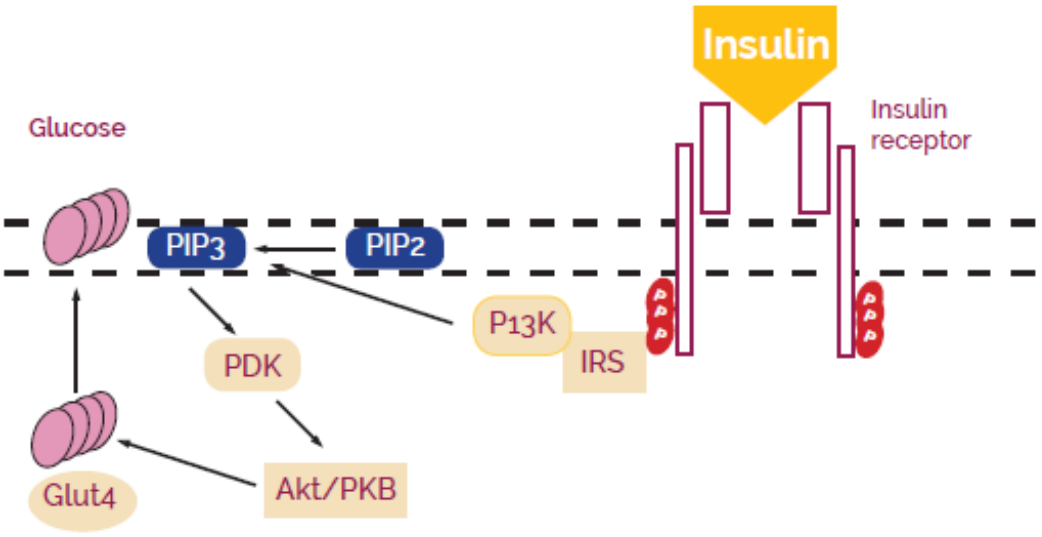

Figure 11. The insulin receptor pathway. Activation of phophatidylinositide 3- kinases (PI3K) results in the phosphorylation of membrane phospholipids and further recruitment of the 3-phosphoinositide-dependent protein kinases (PDK). These kinases activate the serine/threonine kinases Akt/protein kinase B (PKB) and further mediate GLUT4 translocation to the membrane.

Although all molecules that are important for insulin signaling have been demonstrated in the endometrium, the understanding of their regulation is far from complete. The insulin receptor was found in all endometrial compartments with a higher expression in the secretory phase of the menstrual cycle as compared to the proliferative phase which may indicate progesterone dependent regulation (Giudice, 2006; Mioni *et al.*, 2012). Results from *in vitro* studies have shown that both estrogen and progesterone can increase IR mRNA expression in cultured stroma cells (Strowitzki *et al.*, 1993). Moreover, this reaction can be amplified when progesterone is added after estrogen priming (Strowitzki *et al.*, 1993) (Figure 12).

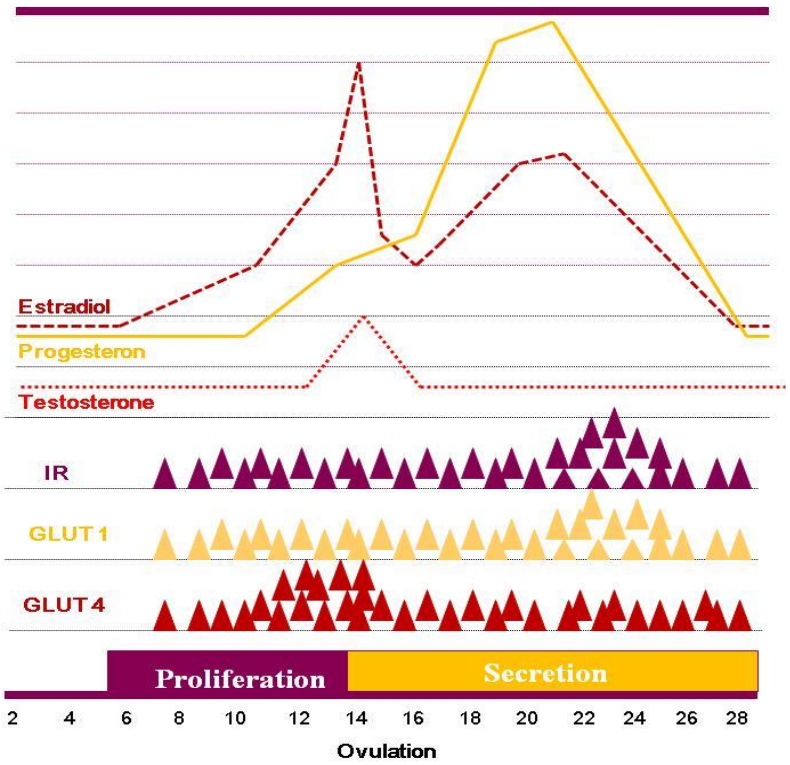

Figure 12. Dynamics of the expression of insulin receptor (IR), glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) during the normal menstrual cycle as described by Mioni et al. (Mioni *et al.*, 2012).

Several glucose transporters, both insulin-dependent and -independent, have been reported to be present in the human endometrium. GLUT4 belongs to the insulin-dependent transporters, while GLUT1 located on the surface and inside the cell, represents insulin-independent glucose transporters (Mioni *et al.*, 2012). GLUT4 immunostaining was mainly localized in the epithelial compartment of the endometrium with the expression higher during the proliferative phase as compared to the secretory phase (Mioni *et al.*, 2012) (Figure 12). Moreover, testosterone was found to down-regulate GLUT4 mRNA expression in the endometrium (Zhang and Liao, 2010).

GLUT 1 plays an important role in basal glucose uptake and storage. It was proposed to be the main glucose transporter in stromal cells of human endometrium (Frolova and Moley, 2011). GLUT1 expression increases during the secretory phase and reaches maximum in the decidualized endometrium (Frolova and Moley, 2011; Mioni *et al.*, 2012) (Figure 12).

1.2.4 Hormonal treatment and the endometrium

In everyday clinical practice, a great range of hormonal preparations are used for contraception, and treatment of menstrual irregularities and climacteric symptoms. However,

they all are different and therefore the effect on the endometrium is largely dependent on the type, dose and regimen used.

1.2.4.1 Estrogens

There are several types of estrogens used for hormonal treatment, such as estradiol, estriol and ethinyl estradiol. It is well established that long-term estrogen treatment alone increases the risk of endometrial hyperplasia and carcinoma (Grady *et al.*, 1995). To avoid the stimulatory effect of estrogen on the endometrium, progesterone addition is compulsory to all women with intact uterus.

1.2.4.2 Progestogens

Besides natural progesterone, there is a great variety of synthetic progesterone preparations (progestogens) available, such as medroxyprogesterone acetate. Depending on their structure and binding capacity, synthetic progestogens can promote progesterone-like, anti-androgenic or androgen-like effects. However, all of them can induce secretory transformation of the endometrium and thereby exert anti-proliferative effects on the endometrium (Pike and Ross, 2000).

1.2.4.3 Tibolone

Tibolone is a synthetic steroid, which was developed in the interest of optimizing the beneficial effects and safety of hormone treatment. In the body it is converted to three major metabolites: two have estrogenic effects (3α and 3β-hydroxy-TIB) and one promotes progestogenic and androgenic effects (Δ4 isomer) (Hammar *et al.*, 2007). Lower bleeding incidence with tibolone as compared to conventional hormone treatments has been reported (Hammar *et al.*, 2007).

1.2.5 An animal model to study primate endometrium

Studies on the endometrium are not always possible to perform in women. Therefore it is important to use as appropriate animal model as possible. The rodent model is widely used, however there are fundamental structural and functional differences of the endometrium between the species that makes this model hard to translate and validate to humans. For example, rodents can have eight to twelve ovulations per cycle while humans have only one. Hence, the primate model seems to be more relevant to humans when it comes to studying the reproductive system (Cline *et al.*, 2008).

The cynomolgus macaque (*Macaca fasicularis*) (Figure 13) is a non-human primate with well described similarities to humans in terms of female reproductive biology. Female

macaques have the similar peripheral steroid hormone metabolism, structure of the endometrium, endometrial response to exogenous estrogens and sex steroid hormone receptor expression as humans (West and Brenner, 1983; Brenner *et al.*, 1990). They have 28 to 30 day long menstrual cycles, single-egg ovulation and single off-spring delivery, distinct menarche and menopause, at 3 and 20 years of age respectively (Cline *et al.*, 2008). Their similarity to humans makes the non-human primates a suitable model for endometrial studies.

Figure 13. The cynomolgus macaque.

1.3 ENDOMETRIAL DYSFUNCTION

Human endometrium undergoes specific changes during each menstrual cycle. All those changes are hormone dependent and metabolically demanding (Schulte *et al.*, 2015). Thus, any hormone or metabolic disturbance may affect the endometrium adversely leading to abnormalities and poor reproductive outcome. PCOS and thyroid gland dysfunction are associated with hormonal and metabolic abnormalities that may affect the endometrium adversely, leading to alterations in its functions and receptivity.

1.3.1 Polycystic ovary syndrome

PCOS is one of the most common endocrine disorders in women of reproductive age, with a reported prevalence as high as 15% (Fauser *et al.*, 2012). PCOS is commonly associated with infertility and metabolic disorders**.** Its clinical manifestations may include menstrual irregularities, signs of androgen excess (hirsutism), overweight and obesity. Independent of

obesity, women with PCOS often present insulin resistance and hyperinsulinemia (Diamanti-Kandarakis and Dunaif, 2012).

Ovarian hyperandrogenism is the common feature and the biochemical hallmark of the syndrome (Azziz *et al.*, 2009). Androgen excess may produce abnormalities in the feedback control of pulsatile gonadotropin releasing hormone (GnRH) secretion from the hypothalamus. In women with PCOS, relatively low levels of progesterone, resulting from infrequent ovulations, may play a role in the persistently rapid GnRH pulse frequency, elevated levels of LH and relative FSH deficiency. LH stimulates the ovarian theca cells, leading to an increase in androgen levels which establishes a vicious circle. Moreover, hyperinsulinemia, resulting from insulin resistance, can increase circulating androgen levels (Micic *et al.*, 1988), without affecting gonadotropin (FSH and LH) secretion (Dunaif and Graf, 1989). Insulin was shown to affect androgen production directly by stimulating theca cells (Nestler *et al.*, 1998) and indirectly by inhibiting the hepatic production of sex hormone-binding globulin (SHBG) (Giallauria *et al.*, 2009; Dumesic and Richards, 2013).

The etiology of PCOS is largely unknown, but a multi-factorial background including genetic factors (Legro *et al.*, 1998; Kahsar-Miller *et al.*, 2001), androgen stimulation/programming *in utero* (Sir-Petermann *et al.*, 2002), as well as lifestyle factors (Harwood *et al.*, 2007) has been suggested.

Reproductive disorders in women with PCOS have mostly been explained by anovulation but there is increasing evidence that the endocrine and metabolic abnormalities that are present in PCOS may have complex effects also on the endometrium, as revealed by altered receptivity, implantation failure and increased incidence of endometrial cancer in these women (Giudice, 2006; Shang *et al.*, 2012).

Insulin resistance and subsequent hyperinsulinemia that is present in PCOS was proposed to have an adverse effect on the endometrium (Schulte *et al.*, 2015). Several molecules involved in the insulin pathway, including IRS1 and GLUT4 have been found to be reduced in the endometrium of obese women with hyperinsulinemia and PCOS (Mozzanega *et al.*, 2004; Fornes *et al.*, 2010; Kohan *et al.*, 2010; Rosas *et al.*, 2010; Mioni *et al.*, 2012). Moreover, insulin-resistant women with PCOS have a significant decrease in implantation and pregnancy rates as compared to non-hyperinsulinemic women with PCOS (Chang *et al.*, 2013). In women with PCOS, embryo development is not affected, indicating that it is the impaired endometrial receptivity that plays a role in the poor reproductive outcome in those women. Furthermore, the insulin-sensitizing drug, metformin was found to promote positive

effects on endometrial insulin signaling in women with PCOS (Zhai *et al.*, 2012; Carvajal *et al.*, 2013). However, the role of insulin signaling in the endometrium as well as the relation to reproductive function in women with PCOS needs further investigation.

In the absence of ovulation and the regulatory effects of progesterone, the endometrium does not undergo secretory transformation and is continuously exposed to the stimulatory and mitogenic effects of estradiol that can lead to endometrial overgrowth and hyperplasia (Elliott *et al.*, 2001). In women with PCOS, ERα expression in proliferative and secretory endometrium was found to be higher as compared to healthy controls (Gregory *et al.*, 2002; Maliqueo *et al.*, 2003; Quezada *et al.*, 2006; Villavicencio *et al.*, 2006). However, one study showed that expression of ERα, ERβ and GPER during the window of implantation was lower in PCOS women, as compared to controls (Wang *et al.*, 2011). Limited data is published regarding the role of ERα36 in the endometrium of patients with PCOS. In a recent study, ERα36 expression was shown to be lower in the proliferative endometrium of PCOS women as compared to the control group (Lin *et al.*, 2013). Considering limited and conflicting data regarding estrogen signaling in the endometrium of PCOS women, further studies are needed to clarify ER expression, distribution and importance for reproductive function.

Approximately 40-60% of PCOS women are obese (Ehrmann *et al.*, 2006). Obesity can aggravate insulin resistance and thereby worsen reproductive dysfunction in women with PCOS (Ehrmann *et al.*, 2006; Hirschberg, 2009). Several studies have shown that even a 5-10% loss in body weight can improve menstrual cyclicity and restore ovulation in PCOS women (Hoeger *et al.*, 2004; Tolino *et al.*, 2005; Palomba *et al.*, 2008; Thomson *et al.*, 2008; Moran *et al.*, 2009). Moreover, our group has demonstrated that diet and exercise alone or in combination can improve reproductive function in overweight/obese women with PCOS (Nybacka *et al.*, 2011). Thus, lifestyle modification aiming at weight loss is the first-line choice of treatment to improve reproductive and metabolic health in women with PCOS. However, the effect of lifestyle intervention on endometrial function has not been previously studied.

1.3.2 Thyroid dysfunction

Thyroid dysfunction is a condition known to reduce the likelihood of pregnancy (Poppe and Velkeniers, 2004). Both hyperthyroidism, with suppressed levels of TSH and increased T3 and T4 production, and hypothyroidism, with elevated levels of TSH, are associated with menstrual disturbances and reproductive dysfunction. The prevalence of hyperthyroidism in

the general population is approximately 1.5%, while hypothyroidism comprises from 2% - 4% in reproductive age women (Poppe and Velkeniers, 2004).

Hypothyroid women exhibit a number of reproductive disorders, including menstrual cycle aberration and infertility (Poppe and Velkeniers, 2004). The most common cause of hypothyroidism is autoimmune thyroiditis, which incidence was demonstrated to be threefold higher in women with PCOS (Janssen *et al.*, 2004). The increased prevalence of autoimmune thyroiditis in PCOS women was suggested to be in part due to some common genetic defect, however other factors cannot be excluded (Janssen *et al.*, 2004). Moreover, hypothyroidism was found to be associated with insulin resistance in muscle and adipose tissues (Dimitriadis *et al.*, 2006). An association between TSH levels and insulin resistance in PCOS women, independent of age and body mass index (BMI), have also been reported (Mueller *et al.*, 2009). In an animal study, thyroid hormone treatment of obese diabetic rodents increased insulin sensitivity and reduced hyperglycemia and hyperinsulinemia (Koritschoner *et al.*, 2001). Thus, evidence from animal and human studies proposes that insulin resistance in PCOS women could be enhanced in the presence of thyroid gland dysfunction.

The TSHR as well as THRs are expressed in the human ovary and endometrium (Aghajanova *et al.*, 2009; Aghajanova *et al.*, 2011) indicating that the corresponding hormones might exert direct effects on endometrial function. Their expression in the human endometrium has been sparsely studied and proposed to vary during the menstrual cycle, depending on sex hormone secretion (Catalano *et al.*, 2007; Aghajanova *et al.*, 2011). Thus, disturbed production of sex steroid hormones observed in women with PCOS may adversely affect thyroid hormone signalling in the endometrium, contributing to the reproductive dysfunction. However, the effects of sex steroid hormones on the regulation of factors involved in thyroid hormone action in the endometrium remains to be established.

2 AIMS

The overall aim of this thesis was to increase the understanding of hormonal receptor regulation of the primate uterus, and in particular to investigate the effects of different hormone treatments and lifestyle intervention on endometrial receptor expression.

Specific aims of the study:

I. To study the effects of long-term sex hormone treatment including tibolone on uterine expression and distribution of the estrogen receptors (ERα, ERβ, GPER), the progesterone receptors (PRA, PRB) and the androgen receptor (AR) in the cynomolgus macaque.

II. To investigate the effects of long-term sex hormone treatment on the expression, distribution and regulation of the receptors for TRH, TSH and thyroid hormones in monkey uterus.

III. To explore the effects of lifestyle intervention aiming at weight loss on the expression of molecules involved in insulin signaling in the endometrium of overweight/obese women with PCOS.

IV. To study receptors of genomic and non-genomic estrogen signaling in relation to proliferation activity in the endometrium of women with PCOS as compared to BMI-matched controls.

3 MATERIAL AND METHODS

The thesis includes methods from studies where an animal model was used (paper I and paper II) and clinical investigations were performed (paper III and IV).

3.1 PAPER I AND II

3.1.1 Animals in paper I and II

Female adult cynomolgus macaques (*Macaca fasciculais*) (Figure 13) ranging from 6 to 8 years of age were imported from Indonesia to the United States. Animals were housed in social groups of four to six monkeys each. Each animal underwent clinical assessment and determined to be healthy.

Three months before initiation of treatment all animals were ovariectomized. Each social group was randomly assigned to oral treatment for 24 months with conjugated equine estrogens (CEE) alone or in combination with medroxyprogesterone acetate (MPA) or with tibolone (TIB) (Figure 14). The control group received no hormonal treatment (OvxC). The animals were treated, with doses corresponding to those given to women and in relation to body weight. Thus, the daily dose was designed to correspond to 0.625 mg CEE, 2.5 mg MPA, and 3 mg TIB. The animals were treated twice daily with the dose split between feedings. At the end of the treatment uterine tissues were obtained from 88 monkeys (OvxC, n=20; CEE, n=20; CEE+MPA, n=20; TIB, n=28) (Figure 14).

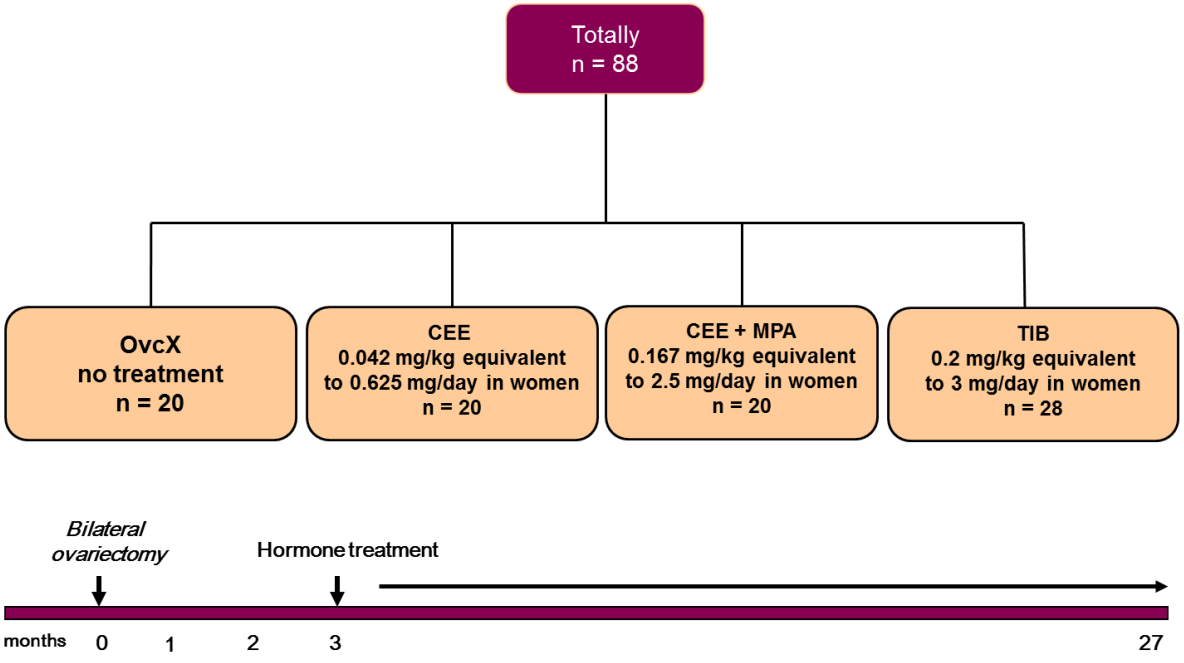

Figure 14. Schematic diagram of the study plan.

The study was approved by the Institutional Animal Care and Use Committee of Wake Forest University (A96-155; A93-116).

3.1.2 Tissue collection in paper I and II

Uterine tissues were fixed in 4% paraformaldehyde for 24 hours and transferred to 70% ethanol for further storage and paraffin embedding. The paraffin blocks of the uteri were sectioned at 5 µm for immunostaining.

3.1.3 Determination of hormone levels in paper II

Serum levels of TSH and T4 were evaluated using an immunoradiometric assay (IRMA) and radioimmunoassay (RIA) kit, respectively, from Diagnostic Products Corp. (Los Angeles, CA); TSH, Cat#IKTS1; and free T4, Cat#TKT41. The determinations of hormone levels were performed at Biomarkers Core Lab Yerkes (National Primate Research Center, Emory University, Atlanta, GA).

3.2 PAPER III AND IV

3.2.1 Subjects in paper III and IV

The clinical study was performed at the Women's Health Research Unit at Karolinska University Hospital, Stockholm, Sweden. Women of reproductive age (18-40 years) with PCOS, and their controls, were recruited between 2008-2012 by clinical referral or advertisements in the local newspapers. Initial screening was performed by a nurse using a standardized telephone questionnaire. Those women that seemed eligible were scheduled for a visit to a gynecologist. The PCOS diagnosis was based on the Rotterdam criteria, i.e. oligo- or anovulation, hyperandrogenism and polycystic ovaries on ultrasound (Rotterdam, 2004). Women were eligible if they met all these three criteria. Additional inclusion and exclusion criteria are shown in Table 1.

Table 1. Inclusion and exclusion criteria of PCOS women

Inclusion criteria	Exclusion criteria
PCOS diagnosis based on the three Rotterdam criteria	Hysterectomy
	Endometrial pathology at screening
Otherwise healthy	Intrauterine device
Age 18-40	Steroid hormone treatment less than 3 months prior to the study
	Pregnancy or lactation during the preceding 12 months
	Weight loss during the preceding 12 months
	Virgo
	Physical handicap
	Eating disorders
	Smoking, alcohol abuse, chemical dependence

In total 52 women, both those diagnosed with PCOS and their age- and BMI- matched regularly cycling controls, participated in the study. All women were divided into four groups according to PCOS diagnosis and BMI:

- PCOS women with a BMI > 27 (**OB-PCOS**), n=20
- Healthy control women with a BMI > 27 **(OB-C**), n=10
- PCOS women with a BMI between 19 and 25 (**NW-PCOS**), n=11
- Healthy control women with a BMI between 19 and 25 (**NW-C**), n=11

Informed consent was obtained from all participants. The study was approved by the local ethics committee (Dnr 2008/865-32).

3.2.2 The intervention in paper III and IV

Women in the OB-PCOS group underwent three months of an individually adapted lifestyle intervention program. The aim of the program was weight reduction. To do so participants in the OB-PCOS group were closely supervised by a dietician and received a membership of a local gym network (Figure 15).

The recommended diet was high in protein and low in carbohydrates (40E% carbohydrates, 30E% fat, and 30E% proteins). This diet has previously been shown to be efficient in women with PCOS and in maintenance of weight loss (Moran *et al.*, 2003; Larsen *et al.*, 2010). A strict regimen of three main meals and two or three snacks were introduced to all women in the OB-PCOS group. Participants recorded and reported their food intake which was corrected if required.

In order to increase physical activity all participants received a membership to a local gym network and a recipe of recommended physical activity (FaR® – physical activity on prescription).

FaR® is a public health initiative to improve physical activity and reduce obesity in Sweden. It allows licensed medical personnel to prescribe physical activity to the patient and is proven to be effective in clinical settings (Kallings *et al.*, 2008). In our study, type of training, its frequency and duration was recommended depending on each individual's preferences, interest, experience and situation. An average physical activity consisted of aerobic activity for 45 min 2 or 3 times per week. Participation was recorded by the gym staff (Figure 15).

Figure 15. Schematic graph of the intervention program.

3.2.3 Experimental design in paper III and IV

All participants in paper III and IV underwent a general health examination including assessment of blood pressure, weight, height, and waist/hip ratio before and at the end of the intervention.

Women in the OB-PCOS and NW-PCOS groups were initially anovulatory presenting either amenorrhea (no bleeding for the past 3 months) or oligomenorrhea (5-9 periods during the past year with intervals more than 6 weeks), while the women in the OB-C and NW-C groups had regular menstrual pattern.

Women in the OB-PCOS group were examined before and after 3 months of lifestyle intervention on cycle day 6-8 and on cycle day 21-23 of the menstrual cycle as determined by spontaneous menstruation or by induced menstruation after progestogen test.

A fasting blood sample was collected from a peripheral vein at 8.00 a.m. and stored at -70°C for analysis of hormones and binding proteins. In addition, gynecological examination, including transvaginal ultrasound was performed by the same investigator using Sonoline SL-250 equipment (Siemens Healthcare Diagnostics).

Endometrial biopsies were collected under local anesthesia using an endometrial suction curette (Pipet Curet, CooperSurgical, USA) for analysis of proteins and genes involved in insulin and steroid hormone signaling in the endometrium. The women in the OB-C, NW-PCOS and NW-C groups were examined once at cycle day 6-8 and on cycle day 21-23 of the menstrual cycle (Figure 16).

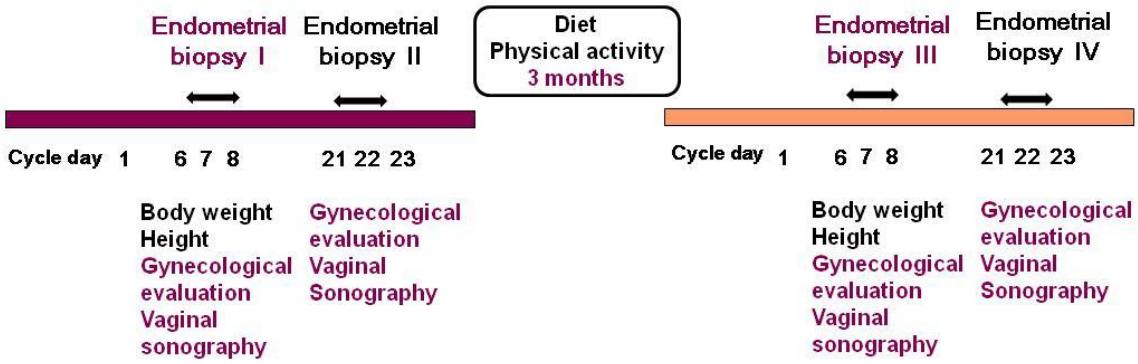

Figure 16. Schematic diagram of the study plan.

During the lifestyle intervention, the menstrual pattern of the women in the OB-PCOS group was recorded for further evaluation. Ovulation was confirmed on the bases of the elevated serum level of progesterone (>17nmol/l) in the luteal phase of the menstrual cycle. The menstrual pattern was considered to be improved when there was a shift from amenorrhea to oligomenorrhea/regular menstruation or from oligomenorrhea to regular menstruation.

3.2.4 Tissue collection in paper III and IV

All biopsies were divided into two pieces: one was preserved in RNA*later*® for further isolation of RNA and the other one was first fixed in 4% phosphate buffered formaldehyde for 9h and then transferred to 70% ethanol for later embedding in paraffin. The endometrium was sectioned at 5µm for immunostaining.

3.2.5 Determination of hormone levels in paper III and IV

Serum concentrations of LH, FSH and SHBG were analyzed using chemiluminescent enzyme immunometric assays (EIA) while estradiol was measured by RIA. Serum concentrations of testosterone were analyzed by liquid chromatography (UPLC)/tandem mass spectrometry (LS-MS/MS). Detection limits and coefficient of variations are presented in Table 2.

Table 2. Methods, detection limits, intra-and interassay coefficients of variation (CV) for hormones and SHBG.

Analyte	Method	Detection limit	Within assay CV	Between assay CV
LH	EIA	0.7 IU/l	6.0%	9.0%
FSH	EIA	0.1 U/l	8.0%	8.0%
Testosterone	LC-MS/MS	0.1 nmol/l		overall 9.2%
SHBG	EIA	0.2 nmol/l	6.5%	8.7%
Estradiol	RIA	5 pmol/l	3.0%	6.0%
Insulin	RIA	2.7 mIU/l	3.0%	4.0%

Free testosterone was calculated from the serum concentrations of total testosterone and SHBG, utilizing a fixed albumin concentration 40g/l and successive computerized approximation involving a system of equation derived from the law of mass action.

Glucose was assayed using the YSI 2300 STAT Plus ™ Glucose & Lactate Analyzer (YSI, Inc., Life Science, Yellow Springs, OH, USA).

Insulin resistance was assessed by the calculating the quantitative insulin sensitivity check index (QUICKI).

QUICKI = 1/(log(fasting insulin mIU/1) + log(fasting glucose, mmol/l)

Insulin resistance is defined when **QUICKI ≤ 0.33**

3.3 MOLECULAR BIOLOGY IN PAPER III-IV

3.3.1 RNA extraction in paper III and IV

Total RNA from endometrial biopsies were purified with RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance to the manufacture's protocol, including a DNase treatment step.

Reverse transcription was performed using 2 µg of total RNA from each sample and the reaction mixture (Qiaqen) containing 1 x RT buffer, dNTP mix (0.5 mM each dNTP), 600 ng random primers (Invitrogen, Paisley, UK), 30 units RNase inhibitor (Qiaqen), and 4U of Omniscript™ reverse transcriptase (Qiagen). A total volume of 20 µl was reverse transcribed at 37˚C for 60 min. The obtained cDNA was then stored at -70°C until further investigations.

3.3.2 Real time PCR analysis in paper III

Taqman® assays for INSR, IRS1, GLUT1, GLUT4 and housekeeping gene RPL13A were purchased from Applied Biosystems. Following assays were used: Hs00961554_ml for INSR, Hs00178563_ml for IRS**,** Hs00892681_ml for GLUT1, Hs01573827_ml GLUT4 and Hs01926559_ml for RPL13A. The real-time PCR was performed according to the manufacturer's standard protocol. All reactions were carried out in triplicates with Taqman Universal PCR Master Mix (Applied Biosystems) in StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). The housekeeping gene RPL13A was used to normalize the mRNA levels of the gene of interest.

3.3.3 Real time PCR analysis paper IV

The oligonucleotide primers for ERα, ERβ, GPER, ERα36 and RPL13A (housekeeping gene) are presented in paper IV. This housekeeping gene was chosen because of its expression and minimal fluctuation in the endometrium. Real time PCR was performed in an iCycler™ iQ Real Time PCR System (Bio-Rad Laboratories, Inc, CA, USA).

A reaction volume of 25 µl was used, containing cDNA corresponding to 50 ng RNA for ERα and ERα36 or 100 ng for ERβ and GPER, 12.5 µl of iQ™ SYBR® Green Supermix (Bio-Rad) and 0.3µM of each oligonucleotide primer. All measurements were performed in duplicates.

An RNA sample without reverse transcription was used as a negative control. The melting curve was used to control the purity of PCR products.

3.3.4 Immunohistochemistry in paper I, II and IV

Immunostaining was performed on 5µm thick sections using a standard immunohistochemical technique (avidin-biotin-peroxidase). All sections were deparaffinized in xylene and rehydrated in graded ethanol. All sections were subjected to the antigen retrieval except those to be stained for TSHR. Antigen retrieval was achieved by boiling in 0.01M citrate buffer (pH6.0) in a microwave oven. The endogenous peroxidase activity was blocked by treatment of 3% H_2O_2 in methanol. Before applying the primary antibody all slides were treated with 1.5% normal horse serum (NHS), 2% NHS or 5% NHS + 5% BSA. The primary antibodies and their dilutions are presented in Table 3. After the primary antibody the corresponding secondary antibody was applied, i.e. either horse anti-mouse IgG (BA-1400, Vector Laboratory) or horse anti-mouse/rabbit IgG (BS-1400, Vector Laboratory).

Immunostaining was performed using a Vectastain Elite ABC kit (Vector Laboratories Inc.) and diaminobenzidine (DAKOcytomation) as chromogen. The sections were counterstained with hematoxylin and dehydrated before mounted with Pertex (Histolab, Gothenburg, Sweden). The negative controls were obtained by replacing the primary antibody with the corresponding concentration of mouse or rabbit IgG.

Table 3. The primary antibodies and dilutions.

Protein	Antibody	Dilution	Manufacturer
ERα	monoclonal	1:5	Zymed, 08-1149
ERβ	monoclonal	1:20	Serotec, MCA 1974
GPER	polyclonal	1:250	Atlas, HPA027052
PRA	monoclonal	1:500	Novocastra, NCL-PgR312
PRB	monoclonal	1:100	ABR, MAI-411
AR	polyclonal	1:200	DAKO, M 3562
Syndecan-1	monoclonal	1:30	DAKO, M7228
TRHR	polyclonal	1:150	Gen Way, 18-461-10338
TSHR	monoclonal	1:500	MCA 1571, AbD Serotec
THRα1/α2	monoclonal	1:200	Santa Cruz, SC-56874
THRβ1	monoclonal	1:500	Santa Cruz, SC-737

3.3.5 Immunohistochemistry in paper III

Immunostaining was performed using paraffin sections, 5 µm thick. All sections were deparaffinized before antigen retrieval was performed in a 2100 autoclave (Prestige medical, Minworth, England) using Diva Decloaker™ (Biocare Medical) and Hot Rinse™ (Biocare medical) according to the manufacturer's instructions. Endogenous peroxidase activity was blocked using undiluted Peroxidazed™ (Biocare Medical) while non-specific binding was prevented by applying Background Sniper™ (Biocare Medical). Thereafter the primary antibody was applied using the concentrations that are shown in Table 4.

Table 4. The primary antibodies and dilutions.

Protein	Antibody	Dilution	Manufacturer
INSR α subunit	monoclonal	1:50	LifeSpan Biosciences, Seattle, WA, USA
INSR β subunit	monoclonal	1:100	LifeSpan Biosciences
IRS1	polyclonal	1:200	Santa Cruz Biotechnology, Santa Cruz,CA USA
GLUT1	monoclonal	1:400	LifeSpan Biosciences
GLUT4	monoclonal	1:500	LifeSpan Biosciences
pY612IRS1	polyclonal	1:2500	Abcam, Cambridge, UK
pS312IRS1	polyclonal	1:500	MyBio source, San Diego, CA, USA

After the primary antibody, the corresponding secondary antibody was applied. The immunohistochemistry was performed using diaminobenzidine (DAKOcytomation) as chromogen. At the final step, sections were counterstained with hematoxylin, dehydrated in ethanol, rinsed in xylene and mounted with Pertex. To obtain a negative control the primary antibody was replaced by the corresponding concentration of mouse or rabbit IgG.

3.3.6 Image analysis in paper II and IV

A Leica microscope and Sony video camera (Park Ridge, NJ) connected to a computer with an image analysis system (Leica Imaging system Ltd, Cambridge, UK) were used to perform quantitative evaluation of the nuclear immunostaining (Figure 17).

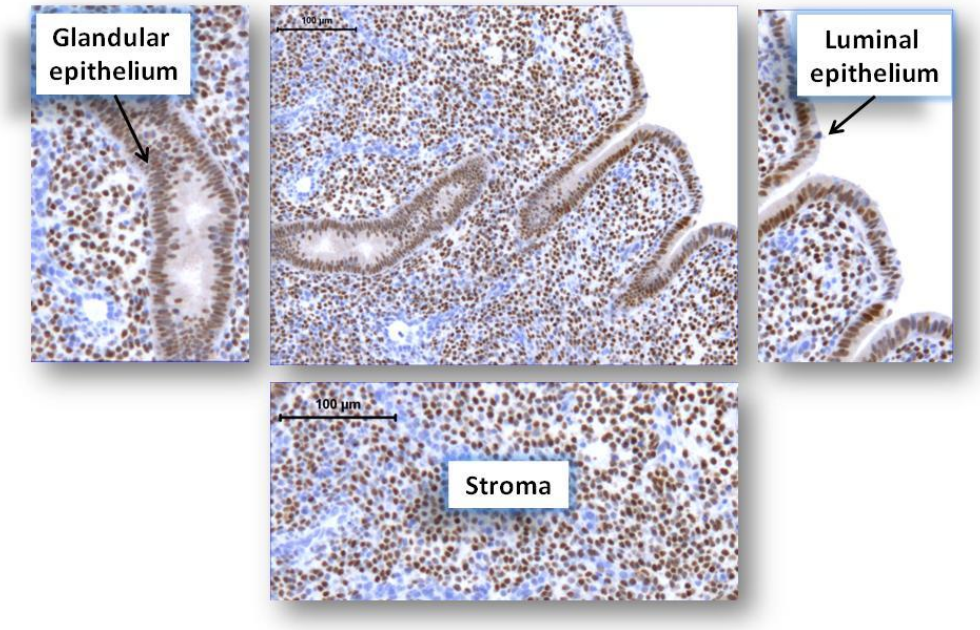

Figure 17. Representative image of ERα immunostainig in human endometrium.

The quantification of immunostaining was performed in 10 randomly chosen fields. When less than 10 fields where available, all epithelia or stromal cells were used for evaluation. In short, an image analysis system using color discrimination software was used to calculate the total area of positively stained nuclei (brown) expressed as a ratio of the total area of cell nuclei (brown + blue negative). Luminal epithelium, glandular epithelium and stroma were evaluated separately (Figure 17).

3.3.7 Manual scoring of immunohistochemical staining in paper I - IV.

The intensity and tissue distribution of cytosolic immunostaining were manually and independently evaluated by two investigators blinded to the group and order of sampling using a four-point grading scale: (-) negative, (+) faint, (++), moderate; and (+++) strong immunostaining. The correlation between the two observers with this method was 75% for the three tissue types (paper IV).

3.3.8 Statistical analysis in paper I-IV

All values are expressed as mean ± standard deviation (SD) or median and quartile range (P_{25}-P_{75}) depending on sample distribution. Differences within a group were analyzed with either paired t-test or the Wilcoxon matched pairs test depending on data distribution. Differences between more than two groups were evaluated by the Kruskal-Wallis test followed by Dunn's test, whereas differences between two groups were performed by the Mann-Whitney U test. To examine if menstrual pattern, ovulation and insulin sensitivity were changed following lifestyle intervention, we used the sign test.

Correlations between the variables were evaluated using the Spearman's rank order correlation test. The significant level was set at $p<0.05$.

4 RESULTS AND DISCUSSION

4.1 PAPER I AND II

4.1.1 Sex hormone treatment and steroid hormone signaling in monkey uterus

The primate endometrium is a sex hormone sensitive tissue. Sex hormones are regulating its transformation and function. In everyday clinical practice, different hormonal preparations are used to treat menstrual irregularities and climacteric symptoms. The endometrial response to treatment depends on the type and the dose of the regimen used. New synthetic steroids are developed in order to optimize the beneficial effects in relation to risks associated with hormone treatment. TIB is a synthetic steroid, which mediates estrogenic, progestogenic and androgenic effects. In an animal model, long-term treatment with TIB resulted in lower bleeding incidence and endometrial proliferation as compared to conventional hormone treatment (Cline *et al.*, 2002). This finding raised the hypothesis that TIB affects the endometrium differently from that observed by conventional treatment. However, the underlying mechanisms for the beneficial bleeding profile, as well as for the lower proliferation activity are largely unknown. In this study, we investigated the effects of conventional hormone treatment and TIB on the expression of sex steroid hormone receptors in monkey uterus.

Estrogen receptors are important regulators of endometrial proliferation. We found immunostaining of ERα, ERβ and GPER in all compartments of monkey endometrium (Figure 18).

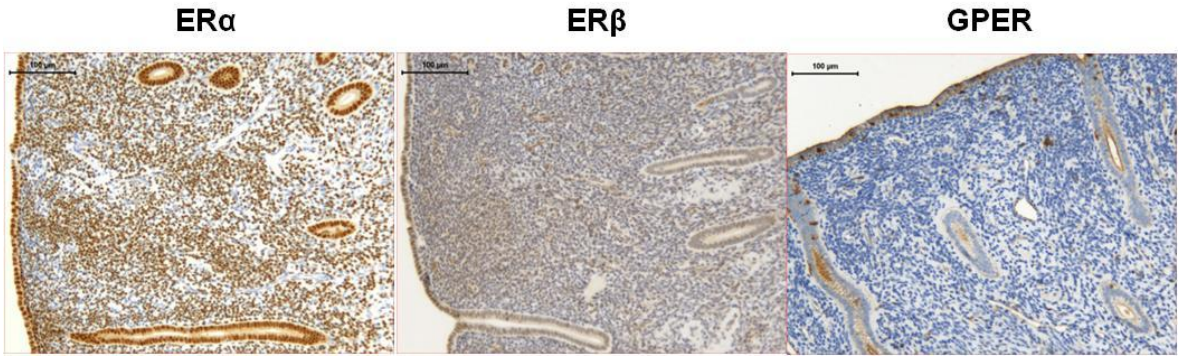

Figure 18. Representative images of estrogen receptor α (ERα), β (ERβ) and G-protein coupled estrogen receptor-1 (GPER) immunostaining in the endometrium of the cynomolgus macaque.

In the monkey uterus, the combined treatment with CEE+MPA resulted in down-regulation of ERα in luminal epithelium and in myometrium as compared to the TIB treated group and

controls (Figure 19). In the stromal cells, ERα immunostaining was lower in the TIB treated group as compared to the CEE treated group, but not different to the combined CEE+MPA treatment and the control group (Figure 19). Lower expression of ERα in the TIB group as compared to the CEE group can be attributed to the regulatory effect of its Δ4 isomer metabolite which has both progestogenic and androgenic effects. Loss of ERβ expression was proposed to be associated with estrogen-dependent tumor progression (Bardin *et al.*, 2004). Expression of ERβ in the epithelial compartment and myometrium was not different between the treatment groups. Overall, we have demonstrated that TIB treatment was not different from the control group in regard to ERα and ERβ expression, while combined CEE+MPA treatment affected ERα and ERβ immunostaining in the monkey endometrium.

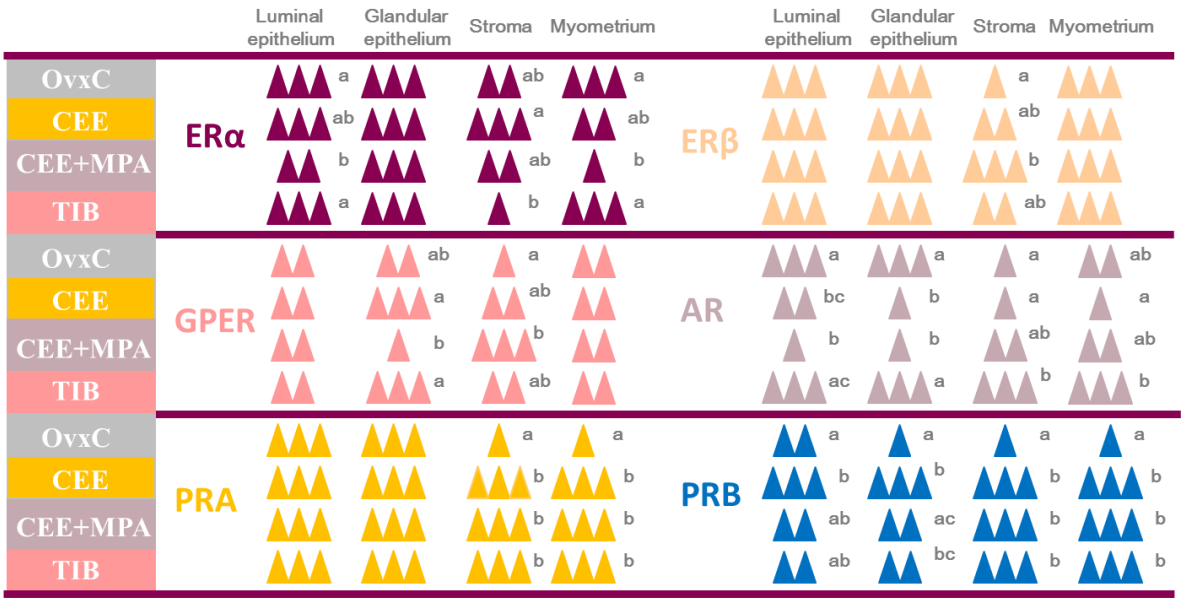

Figure 19. Expression of estrogen receptor α (**ERα**) and β (**ERβ**), G-protein-coupled estrogen receptor-1 (**GPER**), progesterone receptors A (**PRA**) and B (**PRB**) and androgen receptor (**AR**) in the monkey endometrium after long-term sex hormone treatment. Δ - weak staining, ΔΔ- moderate staining and ΔΔΔ- strong staining; as compared to the other groups. Results with different letter designations are significantly different ($p<0.05$). Those with a common letter are not significantly different.

GPER immunostaining in the luminal epithelium and myometrium was not affected by sex hormone treatment. However in glandular epithelium, treatment with CEE and TIB resulted in increased GPER immunostaining as compared to the combined treatment with CEE+MPA but not in comparison to the control group (Figure 19). In the stromal cells, combined treatment with CEE+MPA resulted in an increase of GPER immunoreactivity as compared to the control group (Figure 19). TIB treatment had no effect on GPER immunostaining in

monkey endometrium when compared to the controls (Figure 19). Our findings may suggest that unchanged protein expression of ERα, ERβ and GPER in the TIB group as compared to the controls, is a part of the mechanism behind the low endometrial proliferation associated with this treatment.

PRA and PRB promote anti-proliferative action on the human endometrium (Graham and Clarke, 1997). Thus, loss of PRs may lead to estrogen-mediated endometrial proliferation. We found that the hormone treatments used did not reduce PR expression in the monkey endometrium but rather increased its expression in stroma, as well as in the myometrium.

Several lines of evidence support an ability of androgens to counteract trophic effects of estrogens on the endometrium (Miller *et al.*, 1986; Brenner *et al.*, 1990; Zang *et al.*, 2007; Zhang and Liao, 2010). AR immunostaining in the epithelial compartment of monkey endometrium was down-regulated by CEE and CEE+MPA treatments (Figure 20). In contrast, TIB treatment had minimal effect on AR expression in luminal and glandular epithelia which was comparable to that found in the control group (Figure 20). In the stromal compartment, TIB treatment resulted in up-regulation of AR as compared to CEE alone or control treatment (Figure 20). Thus, enhanced expression of AR after TIB treatment may be a mechanism which explains the endometrial atrophy in TIB users.

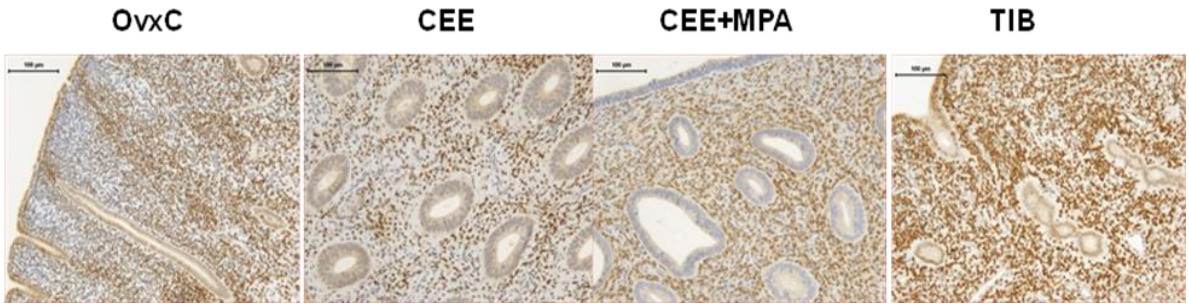

Figure 20. Representative images of androgen receptor (AR) immunostaining in the endometrium of cynomolgus macaque after long-term sex hormone treatment.

In conclusion, we have found that in contrast to CEE alone or combined CEE+MPA treatment, TIB has a minor effect on androgen signaling in the monkey endometrium. Moreover, TIB treatment resulted in up-regulation of AR in the stroma. Thus, lack of down-regulated AR expression may be of importance for endometrial atrophy and beneficial bleeding profile associated with this treatment in comparison to conventional hormone treatment.

4.1.2 Sex hormone treatment and thyroid hormone signaling in monkey uterus

Thyroid gland function is essential for reproduction. Disturbances in the thyroid hormone secretion are associated with menstrual irregularities, infertility, and increased frequency of miscarriage (Poppe and Velkeniers, 2002; Poppe and Velkeniers, 2004). Since all receptors of thyroid hormone signaling, except TRHR, have been shown to be present in the human endometrium (Aghajanova *et al.*, 2011), we hypothesized that the regulation of their expression can be sex steroid hormone dependent. We used an animal model to clarify the potential role of sex hormones in the regulation of the expression and distribution of TRHR, TSH, THRα1/α2 and THRβ1 in the primate uterus.

We found that all receptors of the thyroid hormone system, i.e. TRHR, TSHR and THRs were present in all compartments of the primate uterus (Figure 21).

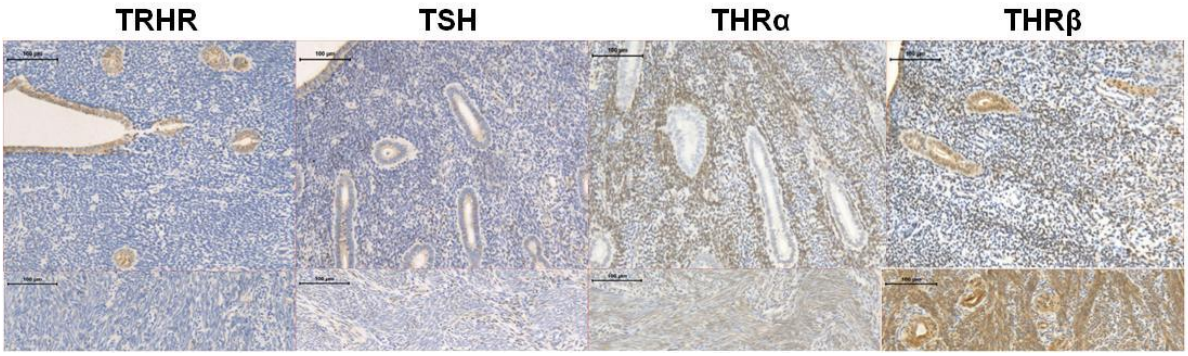

Figure 21. Representative images of thyrotropin-releasing hormone receptor (TRHR), thyroid-stimulating-hormone receptor (TSHR) and thyroid hormone receptor α (THRα) and β (THRβ) immunostaining in the endometrium (upper part) and myometrium (lower insert) of the cynomolgus macaque.

Although the expression of TSHR and THRs have been described previously in the human endometrium (Aghajanova *et al.*, 2011), we are the first to demonstrate that TRHR is also present in the primate endometrium. Thus, TRHR may have a direct effect on endometrial receptivity and function. TRHR expression in the primate endometrium was affected by long-term sex hormone treatment. Combined treatment with CEE+MPA resulted in down-regulation of TRHR in the luminal epithelium as compared to CEE alone treatment (Figure 22). TRHR expression in glandular epithelium after CEE+MPA treatment was lower as compared to all other groups (Figure 22). However, in the stromal compartment, CEE+MPA treatment resulted in an up-regulation of TRHR expression as compared to the TIB treatment and the control group, indicating a different treatment response. A study on myometrial strips

showed that TRH promotes a relaxant effect on the human myometrium (Potter *et al.*, 2004). Thus, TRH may have a direct effect on the primate uterus via its cognate receptor. The expression of TRHR was affected by sex hormone treatment. However, the role of TRH in the regulation of the primate endometrium is yet to be defined.

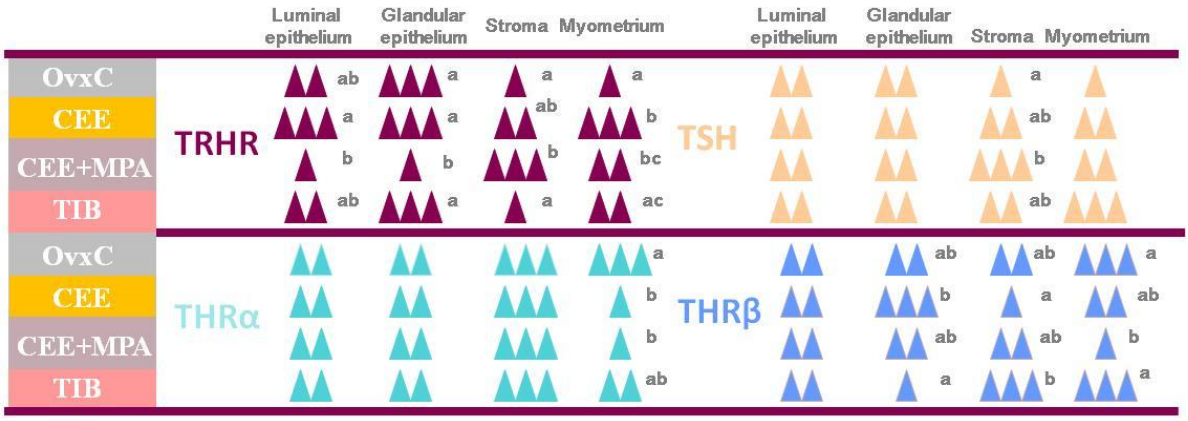

Figure 22. Expression of thyrotropin-releasing hormone receptor (TRHR), thyroid stimulating hormone receptor (TSHR), thyroid hormone receptor α (THRα) and thyroid hormone receptor β (THRβ) in the monkey uterus after long-term sex hormone treatment. Δ - weak staining, ΔΔ- moderate staining and ΔΔΔ- strong staining; as compared to the other groups. Results with different letter designations are significantly different ($p<0.05$). Those with a common letter are not significantly different.

TSHR immunostaining in the control group was higher in the epithelial compartment of the endometrium as compared to the stromal cells (Figure 22). This finding is in agreement with the data published on the human endometrium (Aghajanova *et al.*, 2011). Expression of TSHR in the epithelial compartment was not different between the groups while in the stromal cells, immunostaining was higher in the group treated with CEE+MPA as compared to the controls (Figure 22). Furthermore, TSHR was found to be present in the myometrium where its expression was up-regulated by the TIB treatment as compared to the controls (Figure 22). Since TIB mediates estrogenic, progestogenic and androgenic effects it is hard to draw a clear conclusion via which of the steroid hormone receptors this effect is promoted on the myometrium. Interestingly, TSH levels correlated negatively with immunostaining of TSHR in the glandular epithelium and the myometrium. An increased risk of miscarriage have been reported in women with hypothyroidism, who also are known to have high levels of TSH (Poppe and Velkeniers, 2002). We suggest that TSH can directly affect the endometrium and be involved in the mechanism for increased risk of miscarriage in women with thyroid gland dysfunction.

In the human endometrium, THRs have been found to be expressed in a cyclic dependent manner with an up-regulation in the secretory phase (Aghajanova *et al.*, 2011). We found no difference in the expression of THRs in the luminal epithelium of monkey endometrium (Figure 22). However in glandular epithelium, immunostaining of THRβ was higher in the CEE treated group as compared to the TIB group (Figure 22). In the stromal cells, THRα expression was not affected by sex hormone treatment while in the myometrium it was lower in the CEE and CEE+MPA groups as compared to controls (Figure 22). THRβ immunostaining was lower in stroma of the CEE as compared to the TIB group (Figure 22). In the myometrial compartment, THRβ immunostaining was lower in the CEE+MPA group as compared to the TIB group and controls (Figure 22). Overall, our findings suggest that both estrogen and progesterone are involved in the regulation of THRs expression in the primate uterus.

We have demonstrated that all receptors involved in thyroid hormone signaling are present in the primate uterus and that they are all regulated by sex hormone treatment. Our findings raise the possibility of direct actions of TRH, TSH and thyroid hormones on the function of the primate uterus.

4.2 PAPER III AND IV

4.2.1 Effects of lifestyle intervention on the expression of molecules involved in insulin signaling in the endometrium

The endometrium is an active metabolic tissue which requires an adequate glucose supply for its proper function (Schulte *et al.*, 2015). Insulin is regulating energy metabolism by stimulating glucose up-take in the cells (Diamanti-Kandarakis and Dunaif, 2012). Molecules that are required for insulin signaling, including IR, IRS, GLUT1, GLUT3 and GLUT4 are expressed in the human endometrium (Mioni *et al.*, 2012; Shang *et al.*, 2012). Their regulation has been proposed to be sex hormone dependent (Mioni *et al.*, 2012). Women with PCOS often display insulin resistance which aggravates hyperandrogenic symptoms and features of this syndrome. These hormonal disturbances may affect the expression of molecules that are required for insulin-signaling in the endometrium and thereby its function.

Several studies, including our own, have shown that lifestyle modification improves insulin sensitivity and ovulation rates (Moran *et al.*, 2003; Nybacka *et al.*, 2011). However, the effect of lifestyle modification strategies on the expression of the molecules involved in insulin signaling in the human endometrium is largely unknown. We hypothesized, that an

individualized dietary and exercise program will promote a favorably effect on the systemic insulin sensitivity, as well as on insulin signaling in the endometrium. This is the first study to investigate the effect of dietary intervention and increased physical activity on endometrial insulin signaling in PCOS women.

Clinical characteristics and endocrinological variables in OB-PCOS, OB-C, NW-PCOS and NW-C groups are presented in Table 5. Before intervention, OB-PCOS women had higher serum levels of total and free testosterone and lower FSH and SHBG as compared to the OB-C group.

Table 5. Clinical characteristics and endocrinological variables on cycle day 6-8 in overweight/ obese women with PCOS (OB-PCOS), obese controls (OB-C), normal weight PCOS (NW-PCOS) and normal-weight controls (NW-C).

	OB-PCOS# **n =18**	**OB-C** **n = 10**	**NW-PCOS** **n =11**	**NW-C** **n = 11**
Age	28.2 ± 4.9	33.5 ± 3.4	28.4 ± 5.5	27.2 ± 5.9
BMI (kg/ m^2)	37.4 ± 5.4	34.0 ± 5.1	21.9 ± 1.8	21.7 ± 1.8
Endometrial thickness (mm)	5.1 (3.7-7.5) [a]	5.0 (4.2-6.5) [a]	2.8 (2.1-4.7) [b]	3.0 (2.4-3.9) [b]
Endocrinological variables				
LH (IU/I)	4.6 ± 1.6 [a]	4.9 ± 2.0 [ab]	8.6 ± 3.1 [b]	4.7 ± 1.1 [a]
FSH (IU/I)	5.1 ± 1.1 [a]	7.3 ± 2.9 [b]	6.9 ± 2.1 [b]	6.7 ± 2.4 [b]
Total testosterone (nmol/l)	1.3 ± 0.6 [a]	0.7 ± 0.2 [b]	1.2 ± 0.3 [ab]	1.0 ± 0.3 [ab]
SHBG (nmol/l)	27.4 ± 11.3 [a]	41.9 ± 18.6 [b]	63.5 ± 25.7 [b]	68.4 ± 22.6 [b]
Free testosterone (pmol/l)	30.8 ± 17.3 [a]	12.4 ± 5.1 [bc]	18.1 ± 5.5 [ab]	12.8 ± 3.7 [c]
Estradiol (pmol/l)	148 ± 66	171 ± 70	108 ± 31	134 ± 70

The values are presented as means ± SD or medians with quartile range (25^{th}-75^{th} quartiles).
#Prior to the intervention.
Values with different letter designations (**a, b, c**) are significantly different ($p<0.05$).
BMI, body mass index; **FSH**, follicle stimulating hormone; **LH**, luteinizing hormone; **SHBG**, sex biding globulin.

Out of the 20 initially recruited overweight/obese women two dropped out for personal reasons and one was excluded from study III because the endometrial biopsy was too small, and all was used in study IV. Following three months of lifestyle intervention, 88% of OB-PCOS women reduced their body weight by 5% on average. In 65% of the women, menstrual pattern was improved and in 35% ovulation was confirmed. Fasting insulin levels were significantly reduced and insulin sensitivity as measured by QUICKI, was enhanced.

The endometrium from PCOS women and healthy BMI-matched controls was similar regarding expression of IR, on both mRNA and protein levels. Our findings are consistent with studies on other insulin-dependent tissues (fat and skeletal muscle), where the

expression of the IR in PCOS women did not differ from healthy controls (Diamanti-Kandarakis and Dunaif, 2012). Moreover, lifestyle intervention did not affect IR expression in the endometrium of overweight/obese women with PCOS. Impaired insulin sensitivity associated with this syndrome can be attributed to abnormalities of post-receptor signaling as has been proposed by several *in vitro* and *in vivo* studies (Dunaif *et al.*, 2001; Li *et al.*, 2002; Diamanti-Kandarakis and Dunaif, 2012).

Binding of insulin to the IR leads to its phosphorylation and activation of IRS1. The function of IRS1 can be determined by the levels of its activated (pY612IRS1) and inhibitory forms (pS312IRS1). We found that mRNA expression of IRS1 was lower in the combined (NW-PCOS + OB-PCOS) group of PCOS women as compared to the healthy controls (NW-C + OB-C) ($p<0.01$) (Figure 23).

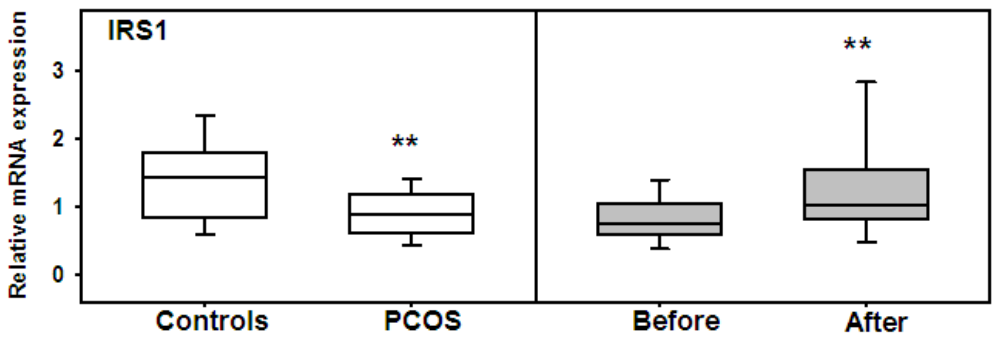

Figure 23. The left panel shows relative mRNA expression of the insulin receptor substrate 1 (IRS1) in proliferative endometrium of OB-PCOS women as compared to BMI matched controls. The right panel shows relative mRNA expression of IRS1 in proliferative endometrium of OB-PCOS women before and after lifestyle intervention.

Following life-style intervention, mRNA expression of IRS1 increased in the endometrium of overweight/obese women with PCOS (Figure 23). Moreover, immunostaining of both activated and inhibitory forms of IRS1 were up-regulated by lifestyle intervention.

Several glucose transporters have been found to be present in the human endometrium, among them GLUT4 which is located in intracellular vesicles. Insulin stimulation is required for its activation and translocation to the cell surface for glucose uptake. Lower mRNA and protein levels of GLUT4 have been found in the proliferative endometrium of hyperinsulinemic and obese PCOS women, while lean PCOS women displayed expression of

GLUT4 comparable to that seen in healthy controls (Mioni *et al.*, 2004; Mozzanega *et al.*, 2004; Fornes *et al.*, 2010). This finding is an agreement with the present study. We found lower GLUT4 mRNA expression in the overweight/obese PCOS women as compared to the BMI-matched controls. However, the expression of GLUT4 mRNA in lean PCOS women was comparable to the controls. The lower GLUT4 expression in the OB-PCOS women may be explained by the higher testosterone and insulin levels in this group. An *in vitro* study demonstrated that testosterone reduces expression of GLUT4 mRNA in endometrial glandular epithelial cells in culture (Zhang and Liao, 2010). Our lifestyle intervention had no major effect on GLUT4 expression. However, in the group of women with improved menstrual function, GLUT4 mRNA expression was higher, but did not reach significance (p=0.09). Thus, the effect of lifestyle intervention on the expression of GLUT4 in the endometrium of OB-PCOS is yet to be determined.

GLUT1 is responsible for basal glucose uptake and was shown to be of importance for endometrial decidualization (Frolova and Moley, 2011). Little is known about its role in proliferative endometrium. In our study, expression of GLUT1 mRNA increased after lifestyle intervention in the OB-PCOS group (Figure 24).

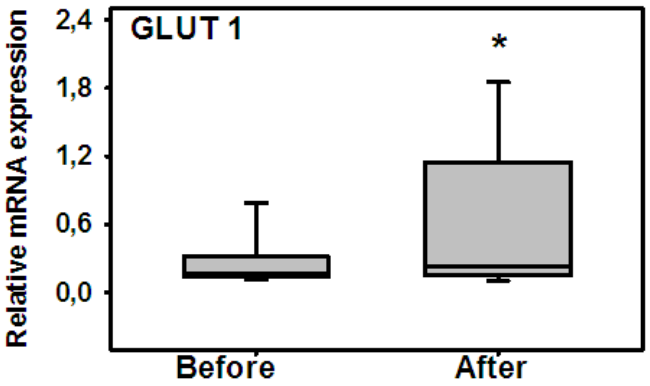

Figure 24. Relative mRNA expression of the insulin glucose transporter 1 (GLUT1) in proliferative endometrium of OB-PCOS women before and after lifestyle intervention.

Moreover, immunostaining of GLUT1 in the stromal compartment of the endometrium was also up-regulated by lifestyle intervention. Our findings may indicate that lifestyle intervention promotes a favorable effect on endometrial basal glucose up-take and thereby improves endometrial function.

Lower expression of IRS1 and GLUT1 in overweight/obese PCOS suggests altered insulin signaling in the endometrium as compared to the healthy controls. To our knowledge, this is

the first study to demonstrate that lifestyle intervention up-regulates endometrial expression of molecules involved in insulin signaling in overweight/obese women with PCOS. We suggest that this treatment improves endometrial glucose homeostasis and thereby endometrial function in these women.

4.2.2 Genomic and non-genomic estrogen signaling in the endometrium of PCOS women

PCOS is associated with disturbances of sex hormone production. It is characterized by androgen excess and relatively low levels of progesterone due to anovulatory cycles (Giudice, 2006; Azziz *et al.*, 2009). Endocrine and metabolic abnormalities that are present in PCOS may alter endometrial receptivity as revealed by implantation failure and increased incidence of endometrial hyperplasia and cancer in these women (Giudice, 2006; Shang *et al.*, 2012). An increased risk of endometrial hyperplasia can be attributed to the absence of the regulatory effects of progesterone on the endometrium (Giudice, 2006). Estrogens and their cognate receptors are important regulators of endometrial proliferation and differentiation (Giudice, 2006). However, little is known about their expression in the endometrium of PCOS women. In the present study, we investigated genomic and non-genomic estrogen receptors in the endometrium of PCOS women throughout the menstrual cycle and in relation to proliferation.

We found that obese women with PCOS have decreased mRNA expression of ERα and ERα36, as well as a lower ERα/ERβ mRNA ratio in proliferative endometrium as compared to healthy BMI-matched controls (Figure 25). These findings are not in agreement with other studies. Vega and collaborators demonstrated higher immunostaining of ERα and ERβ in proliferative endometrium as compared to controls (Maliqueo *et al.*, 2003; Villavicencio *et al.*, 2006). This discrepancy may be explained by the timing of biopsy collection and the BMI of the controls. In the above studies, biopsies were collected in anovulatory PCOS women but the duration of amenorrhea was not clear. Moreover, the controls were not BMI-matched. In our study, endometrial biopsies were collected on specific cycle days after spontaneous or induced menstruation and the controls were BMI-matched.

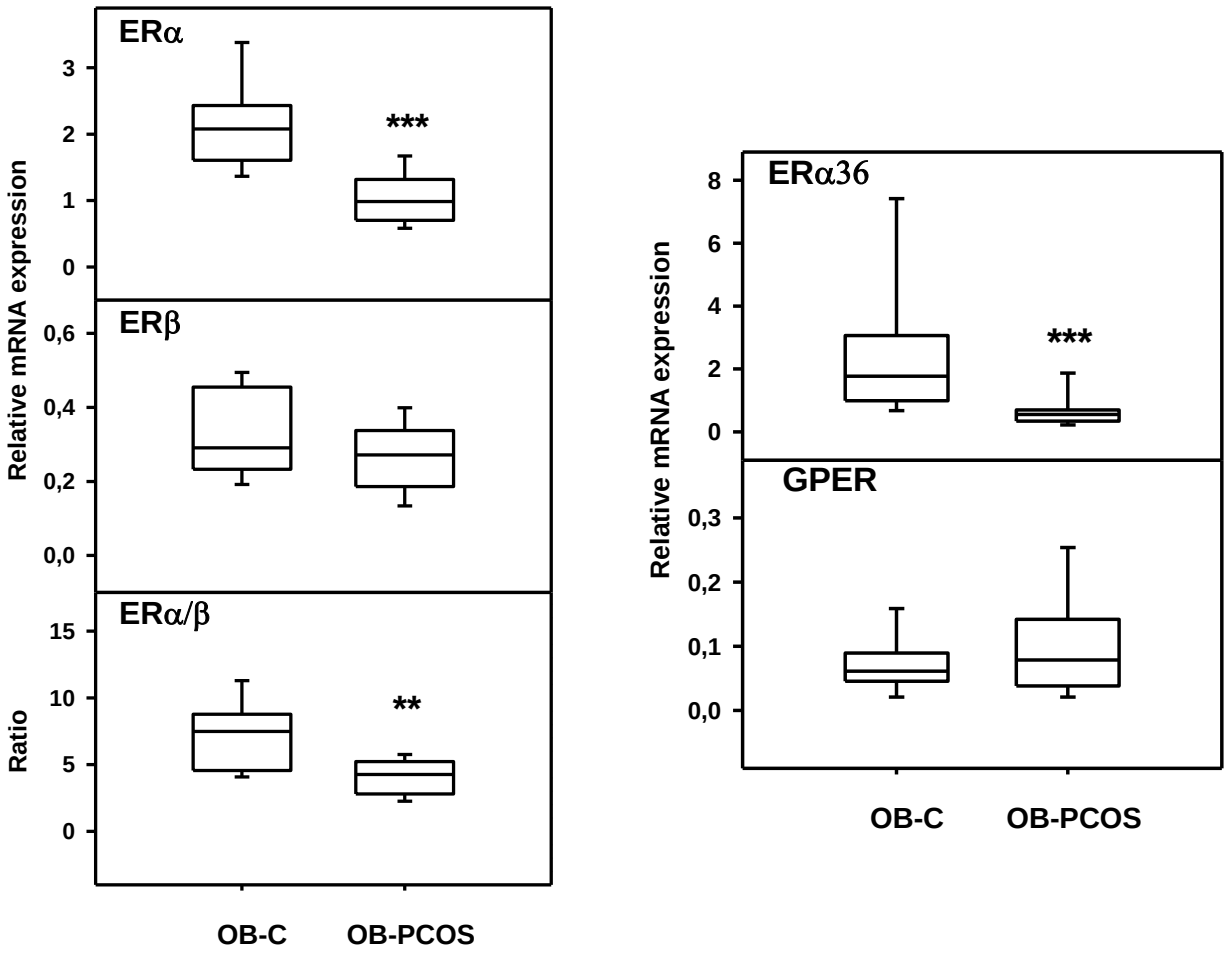

Figure 25. The left panel shows relative mRNA expression of the estrogen receptor α (ERα), β (ERβ), and their ratio in proliferative endometrium of OB-PCOS women as compared to BMI-matched controls. The right panel shows relative mRNA expression of the estrogen receptor α36 (ERα36) and G-protein coupled estrogen receptor-1(GPER) in proliferative endometrium of OB-PCOS women as compared to BMI-matched controls.

In the combined group of PCOS women (OB-PCOS + NW-PCOS), the mRNA expression of ERα, ERα36 and the ERα/ERβ ratio was also lower as compared to the controls. The lower mRNA level of ERα and ERα36 in PCOS women correlated negatively with free testosterone (r= - 0.45, r= - 0.46 respectively). Testosterone administration *in vitro* was shown to promote an inhibitory effect on human endometrial proliferation (Rose *et al.*, 1988). This effect of testosterone was proposed to be associated with the interaction between ER and AR pathways (Panet-Raymond *et al.*, 2000). Moreover, testosterone administration was found to down-regulate ER protein expression and reduce proliferation in vaginal tissue samples of young

women (Baldassarre *et al.*, 2013). Thus, testosterone may interact via ER pathways in gynecological tissues.

The ERα/ERβ mRNA ratio was also found to be lower in the combined group of overweight/obese women (OB-PCOS+OB-C) compared to the normal-weight women (NW-PCOS+NW-C). However, in the overweight/obese women, the ratio was associated with a higher ERβ mRNA level and not with a lower ERα mRNA expression as in the PCOS women. Although, the particular mechanism behind the increased mRNA expression of ERβ in the endometrium of overweight/obese women is not known, our data suggest that proliferative endometrium of women with PCOS exhibits a lower ERα/ERβ ratio which can be even lower when obesity is present.

Little information is available regarding ERα36 expression in the endometrium of PCOS women. In the present study, we observed a lower ERα36 mRNA expression in the endometrium of PCOS women compared with controls. This finding is in agreement with previous data where lower ERα36 mRNA expression in PCOS women was associated with endometrial hyperplasia (Lin *et al.*, 2013). Thus, ERα36 expression in the proliferative endometrium of PCOS women is altered and needs further investigation.

Following lifestyle intervention, the ERα/ERβ protein ratio increased in glandular epithelium and tended to increase in luminal epithelium in the proliferative endometrium of OB-PCOS women with improved menstrual function. A positive association between improved menstrual function and the ERα/ERβ protein ratio in stroma and glands in the endometrium was supported by correlation analyses ($r= 0.55$ and $r= 0.75$ respectively). A low ERα/ERβ ratio suggests relative over-expression and dominance of ERβ mediated effects in the endometrium of OB-PCOS women. Animal studies revealed that ERα is essential for PR up-regulation (Critchley and Saunders, 2009). Altered expression of PRs was proposed to be associated with endometrial progesterone resistance and hyperplasia in PCOS patients (Li *et al.*, 2014). Thus, increased endometrial ERα/ERβ ratio following lifestyle intervention indicates improvement in estrogen signaling and thereby in endometrial function.

After lifestyle intervention, six women had restored ovulation and confirmed luteal phase while 12 remained anovulatory. Anovulatory women with PCOS had a higher expression of ERα mRNA on cycle day 21-23 as compared to controls. This finding is in agreement with a previous study where the ERα mRNA level was higher in midsecretory endometrium of PCOS women as compared to controls (Quezada *et al.*, 2006). Furthermore, anovulatory women had a higher ERα/ERβ immunostaining ratio in glandular epithelium on cycle day 21-

23. The same finding was observed in the study by Quezada et al., where higher ERα/ERβ ratio in midsecretory endometrium was suggested to show a predominance of ERα-mediated effects in this tissue (Quezada *et al.*, 2006). Moreover, both mRNA and protein levels of GPER were higher on cycle day 21-23 in anovulatory women with PCOS compared to controls. The higher GPER levels can be related to the absence of the regulatory effect of progesterone in this group of women (Kolkova *et al.*, 2010). An overexpression of GPER and higher ERα/ERβ ratio may further explain the higher proliferation activity, as revealed by Ki67 immunostaining in this group of women (Figure 26). Thus, our findings and previously published data support that anovulatory women with PCOS exhibit a higher proliferation activity and therefore the risk to develop endometrial abnormalities. Moreover, not only ERα but also GPER may play role in endometrial proliferative abnormalities observed in PCOS women.

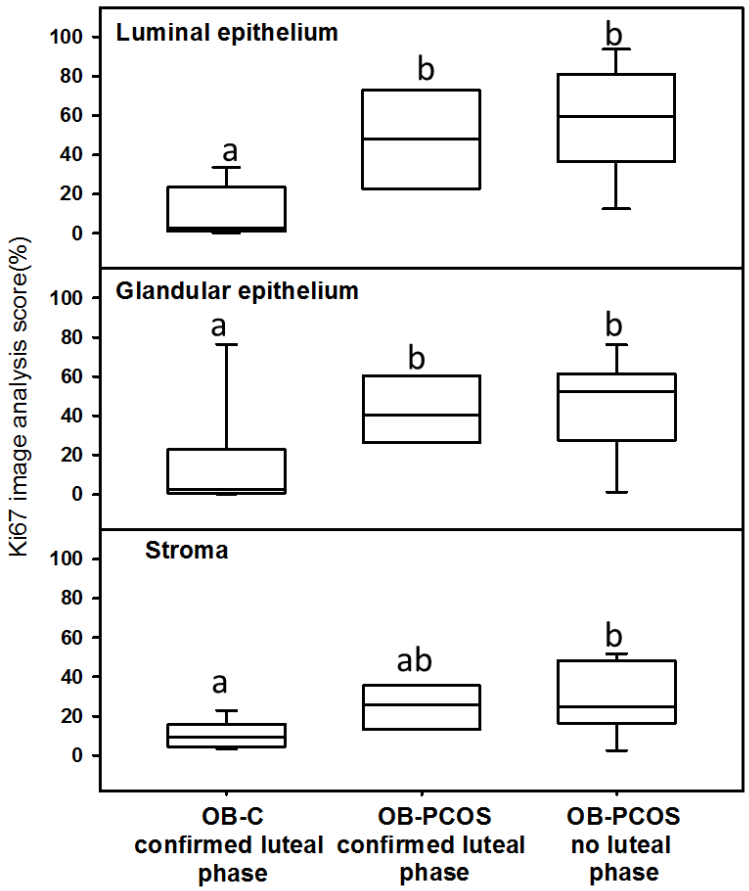

Figure 26. Immunostaining of proliferation marker Ki67 in the endometrium of OB-PCOS women with confirmed luteal phase, OB-PCOS women with no luteal phase and BMI-matched controls. Significant changes between groups are marked with letters ($p<0.05$).

Women with PCOS and restored ovulation were showing intermediate levels of ER mRNAs as compared to the other groups, which were neither different from anovulatory OB-PCOS women nor controls. However, GPER immunostaining in stroma was higher in seceretory endometrium as compared to the controls and correlated positively with the proliferation marker Ki67 (r= 0.52). Thus, higher GPER expression might be associated with endometrial abnormalities in PCOS women. Since progesterone is a known opposing factor of estrogen activity in midsecretory endometrium, our findings make us to speculate about possible alterations in progesterone signaling in the endometrium of PCOS women. Several studies have raised the suggestion that women with PCOS exhibit a state of progesterone resistance, which is characterized by a decreased responsiveness of the target tissue to progesterone (Li *et al.*, 2014). This is in agreement with our study where the rise in progesterone levels did not result in absolute suppression of proliferation activity.

We have shown that PCOS women exhibit decreased endometrial expression of ERα, ERα36 mRNA and ERα/ERβ ratio in the mid-follicular phase of the menstrual cycle that can aggravate in the presence of obesity. A low ERα/ERβ mRNA ratio in proliferative endometrium of PCOS women is associated with high testosterone levels, which may be the underlying mechanism for the impaired estrogen signaling in this tissue. Following life-style intervention the ERα/ERβ protein ratio was up-regulated in the group of OB-PCOS patients with improved menstrual function. We propose that up-regulation of the ERα/ERβ protein ratio in proliferative endometrium following lifestyle intervention improves estrogen signaling and thereby endometrial functioning in PCOS women. Furthermore, PCOS women with restored ovulation and confirmed luteal phase presented an ERα/ERβ mRNA and protein ratio in secretory endometrium comparable to that seen in the controls, while GPER expression was higher and correlated positively with the proliferative marker Ki67. It is likely that a high proliferation activity that is present in the secretory endometrium of PCOS women despite ovulation can be due to a non-genomic estrogen action via GPER.

5 A CRITICAL EVALUATION AND FUTURE PROSPECTS

Our results concerning the effects of long-term sex hormone treatment on uterine expression and distribution of receptors involved in steroid hormone and thyroid signaling are based on experimental animal data. Despite obvious similarities in reproductive biology of non-human primates and humans, the endometrial regulation remains a complex process which could differ between species. A variety of factors including age, metabolism, lifestyle as well as individual sensitivity can modify hormone responsiveness of the endometrium, and therefore our findings should be interpreted with caution.

A limitation with the monkey studies is the small number of subjects in each group and a limited amount of endometrial tissue. The same holds for the clinical studies. A larger sample size should minimize the risk of type II error. Furthermore, the follow-up after lifestyle intervention was limited to 3 months. A long-term follow-up period is needed to evaluate maintenance of both body weight and menstrual function.

The endometrial biopsies obtained in the clinical studies were sufficient for RT-PCR and immunohistochemistry (IHC) analyses. Gene expression was evaluated in all cell types together (endometrium was homogenized), while in IHC different compartments of the endometrium were evaluated separately. IHC is a semi-quantitative method that allows investigating expression and distribution of the protein in the tissue of interest, while western blot could be used to quantify protein levels. Western blot would have been a good complimentary method in addition to those used in our studies but was not employed because of the insufficient amount of endometrial tissue.

Our studies have raised several new questions regarding effects of sex steroid hormone disturbances, thyroid hormone dysfunction, insulin resistance and hyperinsulinemia on endometrial functioning. Further investigations are needed to clarify how these disorders can affect the endometrium. Increased knowledge about hormone signaling in the endometrium may in the long term contribute to better understanding and treatment of reproductive disorders.

6 CONCLUSIONS

I. Long-term sex hormone treatment influences protein expression of steroid hormone receptors in monkey uterus. TIB treatment affects the expression of sex hormone receptors differently from that observed by conventional hormone treatment. Enhanced expression of AR by TIB may be of importance for endometrial atrophy and the beneficial bleeding profile associated with this treatment.

II. All receptors involved in thyroid hormone function are expressed in the different compartments of the macaque uterus and are regulated by long-term sex hormone treatment. These findings raise the possibility of direct actions of TRH, TSH and thyroid hormones on endometrial function, which could be of clinical importance in women with thyroid dysfunction.

III. Lifestyle intervention up-regulates endometrial mRNA and protein expression of molecules involved in insulin signaling in relation to improved menstrual function in overweight/obese women with PCOS. These findings provide evidence of *in vivo* regulation of insulin signaling in proliferative endometrium of women with PCOS. We propose that up-regulation of endometrial IRS1 and GLUT1 improves the glucose homeostasis and thereby restores the functioning of the endometrium in these women.

IV. Estrogen receptor expression is altered in the endometrium of PCOS women. Lifestyle intervention changes genomic and non-genomic estrogen receptor levels towards normalization in proliferative and secretory endometrium of overweight/obese women with PCOS. Restored estrogen signaling may contribute to the improvement of endometrial functioning in these women. However, despite restored ovulation, the proliferative activity, as demonstrated by the proliferation marker Ki67, is higher in the secretory endometrium of the overweight/obese PCOS women. Elevated expression of GPER in the secretory endometrium may propose a mechanism behind high proliferation activity in PCOS women.

7 ACKNOWLEDGEMENTS

I would like to start by thanking the **Most High Lord Almighty** for the ability to complete this considerable work for He is the source of all wisdom and goodness. I am also grateful for my parents, my family and my colleagues at the Karolinska Institutet, who have welcomed me so warmly.

Without people - any science, however great it might be, is worth nothing. I have been blessed by all the women, who participated in clinical studies and to whom I would like to express my deep gratitude.

This work would have been impossible without **Angelica Lindén Hirschberg**, my main supervisor, to whom I am deeply indebted for her incredible character and absolutely phenomenal mind. She has always been a very kind, open and nourishing person, who helped me grow as a scientist and as a person. Thank you for always find the time to teach me scientific thinking and criticism, to encourage, support and correct. **Professor Angelica**'s motto may be summarized in one phrase: "My taste is simple. I like the best".

Lena Sahlin, my co-supervisor, entered my life as a meticulous teacher, who pays attention to the smallest detail and who always encouraged me to be critical and accurate in my conclusions. She was always mindful of my work and forwarded valuable materials with a note "you might find this useful". I will certainly remember those warm summer days that I spent at your summer house, comforted by kittens and your love of nature.

I am grateful to **Vasyl Podolskyy**, my co-supervisor, for being my invisible Guardian Angel. Even though I rarely had a blessed opportunity to communicate with you personally, your faith in me, encouragement and interest in my progress were certainly of great support for which I am grateful.

Volodymyr Kusch, my co-supervisor, who saw the potential in me from the early University years and who supported me in my quest for higher learning.

The unique primate material provided by **Mark Cline**, together with his keen interest in our research was instrumental in the success of this work. **Professor Cline**, I am deeply grateful for your permission and encouragement to use your invaluable material.

The dietician and co-author **Åsa Nybacka** helped participants in our studies to lose weight and gain shape. **Dorina Ujvari**, my other co-author, whom I travelled together with to the congress in Florence, shared her joy and energy that helped keep myself motivated. **Hua Zhang**, my co-author, for whom day and night are all the same when it comes to work, were always an extremely attentive reader. She would always find something that everybody else has missed.

Britt Masironi, my "lab mam" is truly blessed with a gift of making complex things simple. Thank you for teaching me everything in the lab – from the very basics to the most complex methods of analysis and lab work. You have that sublime gift of explaining a procedure in such a way, that one does not start thinking of being totally dumb – an otherwise inescapable phenomenon. You moved me as a very kind, soulful person.

Birgitta Byström, Yvonne Pierre and **Mo Pourian** are highly skilled technicians and lab experts with whom I had the pleasure to interact. I could not help but share their smile and joy whenever we communicated. I am also grateful to **Ivaka Palu** for her sense of purpose and energy.

I am deeply grateful to the research personnel at the Women's Health Research Unit: **Berit Legerstam**, **Siw Rödin Andersson** and **Lotta Blomberg**. **Berit's** inexhaustible enthusiasm, openness and kindness were always shining at the office no matter what the weather outside was like. She has more faith in my ability to master Svenska than myself and keeps talking to me in Swedish all the time for which I am deeply grateful. **Siw**'s joyful nature, inventiveness and knowledge of fancy recipes certainly brought joy into my work. **Lotta**'s, professionalism, sense of taste and design are truly delightful to witness.

Atmosphere is everything – without the air we can't survive for more than a few minutes at most. **Olle Söder**, Head of the Department of Women's and Children's Health knows everything about it. Your marvelous ability to create the atmosphere of celebrating life and science, your bright sense of humor and charisma made it a true joy to travel the rocky Ph.D. road.

Yellow and blue – colors that unite Ukraine and Sweden throughout the centuries are certainly dear to the heart of **Tommy Linné**, the Master Collaborator, the Principal Bridge Builder and the Martial Artist of Cooperation. His brilliant, positive and engaging personality, readiness to support and help in any way possible made him my true mentor, who also became a dear family friend.

Bo von Schoultz, former Head of the Department of Obstetrics and Gynecology, Karolinska University Hospital, has always found the precious moments to read our papers and frankly share his thoughts about them in such a way that encouraged our creativity rather than spreading a critical curtain of shadow over them.

No one should underestimate the role of a friendly chat by the office coffee maker and at the copy room in the overall scientific process. Thank you, **Anastasia Trouva** and **Gerasimos Tzortzatos** for your openness and smiley faces.

I was always fascinated to watch **Christine Carlsson-Skwirut** work at the lab - perfect order, not a single meaningless movement of her hands. I thank you for your kind words. I am also grateful to **Yvonne Löfgren Blomquist** for her iron fist of discipline and examples of proper lab behavior. I must admit though, that it felt rather good not to be around when the orders were being given out.

I thank **Astrid Häggland** for all her invaluable advices and assistance in properly filing the paperwork. I am grateful to **Catharina Karlsson** and **Susanne Hallberg,** for their expertise in technical and secretarial realms.

It would have taken a whole new volume to list every bright soul, whom I met at the Karolinska Institutet but I will try to mention the most of them **Blesson, Mi**, **Mona-Lisa**, **Emma**, **Ahmed**, **Jan**, **Luise**, **Rika**, **Valentina**, **Farasat**, **Leif**, **Isabel, Therese**, **Juliane**, **Ellie**, **Ondrej**, **Andrea, Bettina, Elham, Emelie, Halima, Myryam, Katja.**

I thank **Konstantin Svechnikov** for his friendly advices and the sense of humor. I am grateful to **Irina Svechnikova** for her bright smile. I thank **Andrei Chagin** for his encouragement and positivity.

I am grateful to all my five fellow students from Ukraine, with whom we shared one apartment during the first few months in Stockholm. I thank **Dmytro** for his friendly and cheerful character, his readiness to help. I thank **Gregory** for showing me the value of genuineness. I thank **Andrii** for showing me the value of Google. I thank **Olena** for her warm smile. I thank **Ievgeniia** for our alliance to get the male population of our apartment to respect the rule of cleanliness on our premises. I also thank **Volodymyr**, who recently came to join us for the profound talks about the meaning of life over multiple cups of coffee.

Alter ego is an indispensable fellow traveler in both life and work. Words cannot do justice to my overwhelming sense of gratitude to **Iuliiya Savchuk** who is my best friend and is as close to me as a family member. It is a true love-hate relationship on the edge of which lots of fun, emotions, travels and more fun are born. Thank you, my dear friend for everything! To our past and future travels!

I am profoundly grateful to my English teacher, **Tamara Yuryevna** without whose pedagogical talent I would not have had made it this far. A true teacher is a rare blessing. Her classes were always the best. Her creative approach to teaching the language opened the world to me.

To my beloved Yellow and Blue, Ukrainian – Swedish Family

To my **mam Olesya**, whose youthful look, dedication and the amazing inner strength remind me of the Iron Lady Margaret Thatcher. Mam, you are my true role model in life. To my **father Mykola**, always realistic and a bit skeptical. I hope I will never lose the sense of reality check you taught me. For your endless and unconditional love, confidence in my capacity to overcome all difficulties and obstacles even when I did not believe it myself. It is truly a great feeling of knowing that you are a part of something greater than yourself – a family with traditions, where you are always welcome and where one stands for all and all stand for one.

To my **grandmother Valentyna** and my **grandfather Petro**, who assembled without a doubt the biggest private library in my home city, probably comparable to the Alexandrian at least by the total number of pages. Thank you for all your love and support!

To my **uncle Platon,** an extremely talented journalist. I thank you for all your help and loyal support. It is a great feeling to know that I can count on you any time. For your bright sense of humor and all the laughs together. I was blessed to have you as my uncle!

To **Eva** and **Christoffer**, for the great dinners we had at your apartment in Göteborg and your wonderful company. I thank **Eva** for introducing me to a new joyful and sophisticated way of celebrating Christmas. To **Tomas** and **Liza**, for those magical moments we shared in the cozy comfort of your beautiful house in Fjällbacka. For picnics, candles, wine and the waterfront. To **Lo** and **Liv,** gorgeous children. Thank you **Lo** for fishing crabs together! I will never forget that experience. Thank you all for the great time together!

To **Anders,** if it wasn't for research, I would never have met you. It is not easy to be with someone who is writing a Ph.D. thesis. Thank you for all your support and understanding during the stormy days of thesis writing, for all your help and confidence in me. For being so gentle and incredibly caring. Your love means so much to me!

The studies were financially supported by the Swedish Institute (Visby Program), Swedish Research Council (projects 20324 (ALH) and 20137 (LS), Karolinska Institutet and the Stockholm Country Council (ALH/LS)

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