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MOLECULAR PROFILING OF HUMAN ENDOMETRIUM AND ENDOMETRIOSIS

by

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to my family

ABSTRACT

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Molecular profiling of human endometrium and endometriosis

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Endometriosis is a common hormone-dependent gynecological disease leading to severe menstrual and/or chronic pelvic pain with or without subfertility. The disease is defined by the presence of endometrium-like tissue outside the uterine cavity, primarily on the pelvic peritoneum, ovaries and infiltrating organs of the peritoneal cavity. The current tools for diagnosis and treatment of endometriosis need to be improved to ensure reliable diagnosis and effective treatment. In addition, endometriosis is associated with increased risk of ovarian cancer and, therefore, the differential diagnosis between the benign and malignant ovarian cysts is of importance.

The long-term objective of the present study was to support the discovery of novel tools for diagnosis and treatment of endometriosis. This was approached by exploiting genome-wide expression analysis of endometriosis specimens. A novel expression profiling -based classification of endometriosis indicated specific subgroups of lesions partially consistent with the clinical appearance, but partially according to unknown factors. The peritoneum of women with endometriosis appeared to be altered in comparison to that of healthy control subjects, suggesting a novel aspect on the pathogenesis of the disease. The evaluation of action and metabolism of sex hormones in endometrium and endometriosis tissue indicated a novel role of androgens in regulation of the tissues. In addition, an enzyme involved in androgen and neurosteroid metabolism, hydroxysteroid (17beta) dehydrogenase 6, was found to be highly up-regulated in endometriosis tissue as compared to healthy endometrium. The enzyme may have a role in the pathogenesis of endometriosis or in the endometriosis associated pain generation. Finally, a new diagnostic biomarker, HE4, was discovered distinguishing patients with ovarian endometriotic cysts from those with malignant ovarian cancer.

The information acquired in this study enables deeper understanding of endometriosis and facilitates the development of improved diagnostic tools and more specific treatments of the disease.

Keywords: endometriosis, endometrium, hormonal regulation, expression profiling, biomarker, HSD17B6, HE4

TIIVISTELMÄ

Kaisa Huhtinen
Endometrioositaudin ja terveen kohdun limakalvon molekyyliprofilointi

Biolääketieteen laitos, Fysiologia ja Kliininen laitos, Naistentaudit, Lääketieteellinen tiedekunta, Turun Yliopisto sekä Lääkekehityksen tutkijakoulu
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Endometrioosi eli kohdun sirottumatauti on yleinen hedelmällisessä iässä olevien naisten sairaus, joka aiheuttaa vaikeita kuukautisiin liittyviä tai kroonisia vatsanalueen kipuja sekä hedelmällisyysongelmia. Endometrioositaudissa kohdun limakalvon eli endometriumin kaltaista kudosta kasvaa poikkeavasti kohdun ulkopuolella, esimerkiksi vatsaontelon seinämällä, munasarjoissa tai vatsaontelossa sijaitsevien kudosten pinnalla. Taudin tunnistaminen vie usein vuosia oireiden ilmaantumisen jälkeen, pääosin sen vuoksi että tauti voidaan luotettavasti tunnistaa vain kirurgisessa toimenpiteessä. Endometrioositautia ei nykyisin kyetä parantamaan ja se uusiutuukin useissa tapauksissa. Taudin oireita voidaan helpottaa poistamalla pesäkkeitä kirurgisesti sekä hormonien toimintaan vaikuttavilla lääkkeillä.

Väitöskirjatyön tarkoituksena oli edistää uusien diagnostisten menetelmien ja hoidon kehittämistä. Pääasiallisena menetelmänä käytettiin nk. mikrosiruanalytiikkaa, jolla kyetään tutkimaan kaikkien geenien ilmenemistä samanaikaisesti. Näin jokaisesta tutkitusta kudosnäytteestä tunnistettiin ilmenemisprofiili, jonka perusteella erityyppiset endometrioosipesäkkeet ryhmiteltiin uudella tavalla. Tämä luokittelu havaitsi pesäkkeiden alaryhmiä, jotka olivat osin yhteneviä kliinisen luokituksen kanssa ja osin uusia, vielä tuntemattomiin tekijöihin perustuvia alaryhmiä. Lisäksi havaittiin, että endometrioosipotilaan vatsakalvon ilmenemisprofiili eroaa verrokkien vatsakalvon profiilista, mikä viittaa potilaan vatsakalvolla tapahtuneisiin muutoksiin. Väitöskirjatyössä tutkittiin myös endometrioosikudoksen ja terveen kohdun limakalvon hormonaalista säätelyä, minkä tuloksena havaittiin uusi endometrioositaudissa ilmentymiseltään häiriintynyt entsyymi, HSD17B6. Tällä entsyymillä saattaa olla merkitystä endometrioosin tai sen kipuoireiden kehittymisessä. Tervettä kohdun limakalvoa tutkimalla löydettiin miessukuhormonien eli androgeenien aiemmin tunnistamaton merkitys kudoksen erilaistumisessa Tutkimuksessa havaittiin myös, että verinäytteestä mitattavalla uudella munasarjasyövän merkkiaineella (HE4) kyetään erottelemaan potilaat, joilla on munasarjan endometrioosikysta, niistä joilla on munasarjan syöpäkasvain.

Tutkimustulokset syventävät ymmärrystä endometrioosista ja sen hormonaalisesta säätelystä sekä edistävät endometrioositaudin diagnostiikan ja lääkehoidon kehittämistä tulevaisuudessa.

Avainsanat: endometrioosi, kohdun limakalvo, hormonaalinen säätely, ilmenemisprofiili, merkkiaine, HSD17B6, HE4

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ABBREVIATIONS

4A	4-androstenedione
8-Br-cAMP	8- Bromoadenosine- 3', 5'- cyclic monophosphate
A-diol	androstanediol
AFS	American Fertility Society
AI	aromatase inhibitor
AKR1C3	aldo-keto reductase family 1, member C3
ANOVA	analysis of variance
AR	androgen receptor
ASRM	American Society for Reproductive Medicine
AUC	area under the curve
BMCC1	BCH motif-containing molecule at the carboxyl terminal region 1
BNIP-XL	BNIP2 motif containing molecule at the carboxyl terminal region 1
CA125	cancer antigen CA125
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CE	control endometrium; eutopic endometrium of healthy control
cRNA	complementary ribonucleic acid
CRP	C-reactive protein
ctrl	control, healthy study subject
COX-2	cyclo-oxygenase-2
CYP17A1	17α-hydroxylase (P450c17)
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1 (P450 Arom)
CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1
DAPI	4',6-diamidino-2-phenylindole
DEEP	deep infiltrated endometriosis
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DUSP3	dual specificity phosphatase 3
E1	estrone
E1-S	estrone sulfate
E2	estradiol
E2-S	estradiol sulfate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EmCa	endometrial cancer, patient with endometrial cancer
ESC	endometrial stromal cell
ESHRE	European Society of Human Reproduction and Embryology
ESR1	estrogen receptor 1; estrogen receptor alpha

ESR2	estrogen receptor 2; estrogen receptor beta
FC	fold change
FBS	fetal bovine serum
FSH	follicle stimulating hormone
G-418	antibiotic G-418
GABA	gamma-aminobutyric acid A
GABRP	gamma-aminobutyric acid A receptor, pi
GFP	green fluorescent protein
GnRH	gonadotropin releasing hormone
H&E	hematoxylin and eosin staining
HE4	human epididymal secretory protein E4
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESC	human endometrial stromal cell
HSD17B	hydroxysteroid (17beta) dehydrogenase
HSD3B2	hydroxysteroid (3beta) dehydrogenase type 2
IGF-I	insulin-like growth factor 1
IGFBP- 1	insulin-like growth factor binding protein 1
IHC	immunohistochemistry, immunohistochemical analysis
IL	interleukin
IL1R1	interleukin 1 receptor, type I
IL8RA	interleukin 8 receptor, alpha; chemokine (C-X-C motif) receptor 1
KCNK3	potassium channel, subfamily K, member 3
LH	luteinizing hormone
LNG-IUD	levonorgestrel-releasing intrauterine device
Luc	luciferase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1; chemokine (C-C motif) ligand 2
miRNA	micro RNA
MMP	matrix metalloproteinase
MPA	medroxyprogesterone acetate
mRNA	messenger ribonucleic acid
MUC-1	mucin 1, cell surface associated
MUC16	Mucin 16, cell surface associated; gene encoding CA125
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NK cell	natural killer cell
NSAID	non-steroidal anti-inflammatory drugs
OIP5	Opa interacting protein 5
OV	ovarian endometriosis, ovarian endometriotic cyst
OvCa	ovarian cancer, patient with ovarian cancer
OvEndo	ovarian endometriosis, patient with ovarian endometriosis
P4	progesterone
P450scc	side-chain cleavage enzyme
PAI-1	plasminogen activator inhibitor-1; serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
PCDH7	protocadherin 7
PCR	polymerase chain reaction
PE	patient endometrium; eutopic endometrium of patient with endometriosis

PeEndo	superficial peritoneal endometriosis
Plau-R	plasminogen activator, urokinase receptor
PGE-2	prostalandin E 2
PGR	gene encoding progesterone receptors
PGRMC1	progesterone receptor membrane component I
PIAS1	protein inhibitor of activated STAT, 1
PPARγ	peroxisome proliferator activated receptor γ
PR (A / B / C)	progesterone receptor (A, B or C)
PR-M	membrane associated progesterone receptor
PRL	prolactin
PRUNE2	prune homolog 2
qPCR, qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
RANTES	regulated upon activation, normally T-expressed, and presumably secreted; chemokine (C-C motif) ligand 5
RAR	retinoic acid receptor
RASD1	RAS, dexamethasone-induced 1
RNA	ribonucleic acid
ROC	receiver operator characteristic
ROS	reactive oxygen species
RPL19	ribosomal protein L19
RVE	rectovaginal endometriosis
RXR	retinoic X receptor
SAA	serum amyloid A
SC	stromal cell
SERM	selective estrogen receptor modulators
SHBG	sex hormone-binding globulin
siRNA	small interfering RNA, small interfering ribonucleic acid
SMRP	Soluble mesothelin-related peptide; ATP-binding cassette, sub-family C (CFTR/MRP), member 5
SPRM	selective progesterone receptor modulators
StAR	steroidogenic acute regulatory protein
STAT	signal transducer and activator of transcription
STS	steroid sulfatase
SULT1E1	estrogen sulphotransferase
SUMO-1	small ubiquitin-related modifier 1
T	testosterone
TGFβ/SMAD	transforming growth factor, beta / SMAD protein
TIMP	tissue inhibitor of metalloproteases
TNF-α	tumor necrosis factor α
Tris	tris(hydroxymethyl)aminomethane
TRITC	Tetramethyl Rhodamine Isothiocyanate
TWIST1	twist homolog 1 (Drosophila)
uNK	uterine natural killer cell
VEGF	vascular endothelial growth factor
WB	western blot analysis
WFDC2	WAP four-disulfide core domain 2; gene encoding HE4
WNT	wingless-type MMTV integration site family

LIST OF ORIGINAL PUBLICATIONS

The study is based on the following publications, which are referred to in the text by Roman numerals (I–IV). Unpublished data is also included.

I Hiissa J, Elo LL., **Huhtinen K**, Perheentupa A, Poutanen M, Aittokallio T (2009) Resampling reveals sample-level differential expression in clinical genome-wide studies. *OMICS* 13(5):381-96.

II Cloke B, **Huhtinen K**, Fusi L, Kajihara T, Yliheikkilä M, Ho K-K, Teklenburg G, Lavery S, Jones MC, Trew G, Kim JJ, Lam EW-F, Cartwright JE, Poutanen M, Brosens JJ (2008) The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium. *Endocrinology* 149:4462-74.

III **Huhtinen K**, Salminen A, Yli-Heikkilä M, Kujari H, Junnila J, Perheentupa A, Poutanen M (2010) Down-regulation of HSD17B2 and up-regulation of HSD17B6 in peritoneal, deep and ovarian endometriosis. *Manuscript.*

IV **Huhtinen K**, Suvitie P, Hiissa J, Junnila J, Huvila J, Kujari H, Setälä M, Härkki P, Jalkanen J, Fraser J, Mäkinen J, Auranen A, Poutanen M, Perheentupa A (2009) Serum HE4 concentration distinguish patients with malignant ovarian tumours from patients with ovarian endometriotic cysts. *Br J Cancer* 100:1315-9.

1. INTRODUCTION

Endometriosis is one of the most common benign gynecological diseases, while approximately 10% of women in reproductive age may be affected. The defining feature of endometriosis is the presence of endometrium-like tissue outside the uterine cavity, primarily on the pelvic peritoneum, ovaries and infiltrating organs within the peritoneal cavity. The main clinical features associated with the disease are severe menstrual or chronic pelvic pains and subfertility, and the frequency of endometriosis within women with pain, infertility, or both is 15-65% (Mahmood & Templeton, 1991; Olive & Schwartz, 1993). Thus, the disease has a significant impact on physical, mental, and social well-being of the patients (Kennedy *et al*, 2005; Siedentopf *et al*, 2008). Furthermore, endometriosis causes significant healthcare costs and loss of productivity (Simoens *et al*, 2007). The diagnosis of endometriosis is typically performed by laparoscopy while no reliable non-invasive methods exist. Moreover, ovarian endometriotic cysts increase the risk for ovarian malignancies (Ness, 2003; Nagle *et al.* 2008) and, in some cases, the differential diagnosis of endometriosis and malignant ovarian cysts may currently be unreliable without invasive methods.

Several factors have been suggested to be involved in the pathogenesis of the disease, including hormonal regulation, aberrant immune system as well as genetic and environmental factors. Endometriosis tissue proliferates in response to systemic estrogens, mainly similarly to endometrium. However, endometriotic cells also have their own estrogen production, which leads to continuous growth of the diseased tissue. Also other sex hormones *e.g.* progesterone and androgens affect estrogen-dependent endometrial and endometriotic cell proliferation. The current medical therapy is based on the inhibition of estrogen action resulting to restricted proliferation of the endometriotic tissue. However, the present medical therapy is often not sufficiently effective to treat endometriosis-related pain, and the treatment reducing pain does not improve infertility, but rather acts as reliable contraception (Guo, 2008). The most effective treatment for both endometriosis-associated pain and infertility is obtained by the excision of the lesions and normalization of the pelvic anatomy in a surgical operation. However, endometriosis cannot be cured and it often recurs.

Endometriosis lesions are classified to peritoneal, deep and ovarian diseases, which may have divergent etiology (Nisolle & Donnez, 1997). These subtypes also differ in the associated symptoms, recurrence and response to treatment. However, the current classification of the lesions is based on their appearance or location but not on their molecular profiles. Furthermore, no comparable genome-wide expression analysis of all the main lesion types has been available to evaluate the molecular differences between the types of endometriosis. Genome-wide gene expression microarray technology has already enabled the identification of cancer subtypes with specific gene expression patterns, which is exploited in several applications including identification of new diagnostic tools and personalized treatments for various cancers.

The long-term objective of the present study was to discover novel tools for diagnosis and treatment of endometriosis. This was approached by exploiting genome-wide expression analysis to determine transcriptomics-based classification of endometriosis, to evaluate novel insights on hormone actions on endometriosis, and to assess biomarkers for differentiation of ovarian endometriosis from ovarian cancer.

2. REVIEW OF THE LITERATURE

2.1 ENDOMETRIOSIS

Endometriosis is a common and chronic benign gynecological disorder in which endometrial tissue forms lesions outside the uterine cavity *(Fig. 1)* and results in pelvic pain and subfertility. Histopathologically, endometriosis is characterized by the presence of endometrial glands and stroma in ectopic locations. The lesions are typically located on the pelvic peritoneum, in the ovaries and in the rectovaginal septum, while infrequently observed also in the pericardium, pleura, and even the brain (Giudice & Kao, 2004). A severe disease typically results in extensive pelvic adhesions and disformation of pelvic anatomy, which often leads to pain and infertility (Giudice & Kao, 2004). The appearance of endometriosis is related to menstruation and estrogen action, and the reduction of estrogen effects *e.g.* following menopause typically diminishes the disease. The incidence of endometriosis is estimated to be 10% in women in reproductive age, while the frequency rises to 15–65% within women with pain with or without infertility (Mahmood & Templeton, 1991; Olive & Schwartz, 1993). The annual costs of endometriosis have been calculated to be ~4000 USD per patient as direct healthcare costs and loss of producibility (Simoens *et al*, 2007). In the United States, the total annual costs have been estimated as ~22 billion USD (Rogers *et al*, 2009; Simoens *et al*, 2007). Current knowledge of the pathogenesis of endometriosis, and the pathophysiology of the related infertility and pelvic pain, remain incomplete (Rogers *et al*, 2009).

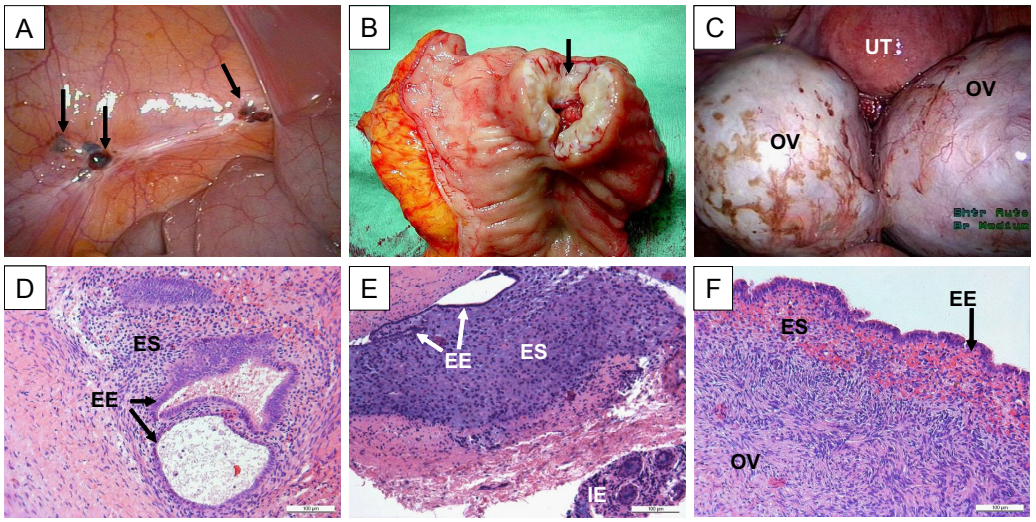

Figure 1: *Macroscopical (A-C) and histological (D-F) appearance of endometriosis. A and D – peritoneal lesions, B and E – deep lesion in the bowel, C and F – ovarian endometriotic cysts. The lesions are shown in arrows, UT = uterus, OV = ovary with endometriotic cyst, EE = endometriotic epithelium, ES = endometriotic stroma, IE = intestinal epithelium. The macroscopical pictures have been taken by Dr:s Pia Suvitie (A), Marjaleena Setälä (B), and Päivi Härkki (C).*

2.1.1 Symptoms

Endometriosis is associated with painful menstruation (dysmenorrhoea), painful sexual intercourse (deep dyspareunia), chronic pelvic pain, ovulation pain, cyclical or perimenstrual symptoms (*e.g.* bowel or bladder associated) with or without abnormal bleeding, infertility and chronic fatigue (Simoens *et al*, 2007). The disease has a significant impact on the physical, mental, and social well-being of the patients (Kennedy *et al*, 2005; Siedentopf *et al*, 2008). However, some affected women remain asymptomatic.

The endometriosis-associated **pain** may be a consequence of several mechanisms. The lesions proliferate and bleed similarly to endometrium during menstruation causing pressure to attached tissue and pain. The number of nerve fibers is increased in endometriosis tissue as compared to endometrium, suggesting increased pain signaling. Importantly, peritoneal inflammation leads to elevated production of cytokines and prostaglandins, which are involved in pain generation (Bokor *et al*, 2009; Guo, 2008). In addition, pelvic adhesions distort pelvic anatomy which may cause mechanical pain.

Similarly, there are multiple mechanisms of endometriosis-associated **subfertility** (for review, see Guo, 2008). The oocyte quality of women with endometriosis may be reduced due to changes in apoptosis, cell cycle, and oxidative stress in granulosa cells (Saito *et al*, 2002). In addition, the ovarian reserve may decrease due to repeated operations (Hachisuga & Kawarabayashi, 2002; Ragni *et al*, 2005; Somigliana *et al*, 2003). Increased levels of reactive oxygen radicals and cytokines may have adverse effects on sperm function and integrity (Gupta *et al*, 2008). Endometrial defects, including decreased decidualization capacity (Klemmt *et al*, 2006), aberrant tissue remodeling by matrix metalloproteinases, down-regulation of integring and homeobox genes, and aberrant immune cell trafficking may impair the endometrial receptivity and embryo implantation (Gupta *et al*, 2008; Kao *et al*, 2003) in women with endometriosis. Finally, distorted pelvic anatomy, mainly due to endometriosis-associated pelvic adhesions may impair oocyte release from the ovary or interfere with ovum pickup or transport (Schenken *et al*, 1984).

2.1.2 Pathophysiology

Endometriosis is a multifactorial disease as several molecular mechanisms have been identified to be involved in the pathophysiology of the disease (for review, see Bulun, 2009; Giudice & Kao, 2004): **Altered immunity** and factors involved in **adhesion, invasion, and angiogenesis,** as well as **proliferation,** and **impaired apoptosis** are essential in the formation of the lesions. Aberrant **estrogen metabolism** enhances the growth of endometriotic cells. **Chronic inflammation** has an important role in the regulation of multiple pathophysiological mechanisms *e.g.* angiogenesis, estrogen metabolism and **oxidative stress** (for review, see Giudice & Kao, 2004; Lebovic *et al*, 2001). In addition, genetic, epigenetic and environmental factors may influence the development of the disease (for review, see Foster & Agarwal, 2002). These mechanisms are further discussed in the following paragraphs.

2.1.2.1 Cellular origin

There are several theories for the etiology of endometriosis: retrograde menstruation together with altered cellular immunity, coelomic metaplasia, and possibly metastasis (Nap *et al*, 2004; Nisolle & Donnez, 1997). It has been suggested that the superficial lesions, deep rectovaginal endometriosis, and ovarian endometriotic cysts would be of different origin (Nisolle & Donnez, 1997). The development of peritoneal endometriosis can be explained by Sampson's theory of retrograde menstruation (Nisolle & Donnez, 1997; Sampson, 1927). However, the retrograde menstruation occurs in ~ 90% of women (Blumenkrantz *et al*, 1981; Halme *et al*, 1984), while the incidence of endometriosis is around 10% (Olive & Schwartz, 1993). Thus, it has been suggested that endometrial cells of women with endometriosis have the ability to survive, adhere, invade and proliferate in ectopic locations, which enables the endometrial fragments in menstrual blood to attach and grow in peritoneal wall (for review, see Donnez *et al*, 2002b). The deep endometriosis may be a consequence of infiltration of peritoneal endometriosis (Nap *et al*, 2004). However, the deep rectovaginal nodules may be of different origin: poor differentiation and hormonal independence suggest a metaplasia of Müllerian remnants, similarly to adenomyotic nodules (Donnez *et al*, 2002b; Nisolle & Donnez, 1997). Ovarian endometriotic cysts may originate from invagination of superficial endometriotic implants or from metaplasia of the invaginated celomic mesothelium (Nisolle & Donnez, 1997). Recently, the endometrial stem/progenitor cells have been suggested to be an origin of endometriosis (for review, see Sasson & Taylor, 2008). Understanding the cellular origin of endometriosis would be important to better comprehend and restrain the recurrence of the disease.

2.1.2.2 Adhesion, invasion and angiogenesis

The molecular mediators for adhesion of endometrial cells to peritoneum are not well known. Various integrins are present in menstrual endometrium and the blockage of the integrin beta-1 subunit partly disrupts the adhesion (Koks *et al*, 2000). This implies a role of integrins in cell adhesion but other mechanisms are likely to be involved. Integrins are cell-surface glycoproteins that act as receptors for extracellular matrix (ECM) proteins. In normal endometrium, they are important in the interaction between glandular and stromal elements, and essential for implantation (Lessey *et al*, 1992). The invasion of endometriotic cells to the attached tissue requires local degradation of ECM by matrix metalloproteinases (MMPs). In normal endometrium, increased synthesis and activation of MMPs in late secretory phase is essential for appropriate tissue breakdown and menstruation (Salamonsen & Woolley, 1996). In peritoneal and ovarian endometriosis, MMPs are present independent of the cycle phase (Kokorine *et al*, 1997). In fact, the invasion index of endometriotic cells corresponds to that of metastatic bladder cell lines (Gaetje *et al*, 1995). The survival of endometriotic lesions is dependent on angiogenesis. Increased levels of angiogenic factors such as vascular endothelial growth factor (VEGF) are present in peritoneal fluid of endometriosis patients, where they may originate from peritoneal macrophages, retrogradely menstruated endometrial cells or endometriotic lesions themselves (Oosterlynck *et al*, 1993). Thus, the peritoneal environment supports the vascularization of newly formed lesions.

2.1.2.3 Proliferation and apoptosis

The proliferation of endometrial and endometriotic cells is induced by estrogens. In contrast, progesterone stimulates cellular differentiation and suppresses cellular proliferation. In endometriosis, the increased estrogen effect and abnormal progesterone action lead to enhanced cell proliferation. This subject is discussed in more detail in chapter 2.2.

Simultaneously, impaired apoptosis in endometrial and endometriotic cells of women with endometriosis may contribute to the pathogenesis of the disease (for review, see Agic *et al*, 2009; Harada *et al,* 2004). Apoptosis, the programmed cell death, minimizes the leakage of cellular contents such as proteases from dying cells, thereby reducing the likelihood of an inflammatory response (Wyllie *et al*, 1980). In the healthy endometrium, apoptosis facilitates the maintenance of cellular homeostasis during the menstrual cycle (Kokawa *et al*, 1996). In women with endometriosis, the percentage of apoptotic cells in sloughed endometrium and in glandular epithelium is reduced implying the increased number of surviving cells entering the peritoneal cavity with retrograde menstruation (Agic *et al*, 2009; Dmowski *et al*, 2001; Gebel *et al*, 1998). Increased expression of anti-apoptotic factors and decrease of pro-apoptotic factors observed in endometriosis support the anti-apoptotic phenotype (Agic *et al*, 2009).

2.1.2.4 Inflammation and immune response

Endometriosis is typically associated with an inflammatory process that takes place in the peritoneal cavity of the patient. Immune cell trafficking and their cytokine release are important components of the cyclic development of the normal endometrium in each menstrual cycle. However, an increased number of activated macrophages and lymphocytes have been detected in the peritoneal fluid of these patients (Lebovic *et al*, 2001). The production of cytokines by endometriotic lesions and associated immune cells modulate the growth and inflammation in endometriosis: Increased levels of proinflammatory cytokines, MMPs, as well as chemokines and their receptors are involved in different steps of endometriotic cell survival: adhesion, invasion, vascularization and growth of the lesions. Induction of prostaglandin E2 (PGE-2) synthesis by cyclo-oxygenase 2 (COX-2) may also be essential for the pathogenesis of endometriosis as well as pain generation The proinflammatory cytokines and chemokines suggested to be involved in pathogenesis of endometriosis include interleukins (IL) 1β and 6, tumor necrosis factor alpha (TNF-α), regulated upon activation, normally T-expressed, and presumably secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), IL-8, and IL-8 receptor α (IL8RA) (for review, see Lebovic et al, 2001). Moreover, the activity of natural killer (NK) cells, which are involved in recognition and destruction of foreign cells in the body, is decreased in the endometrium of endometriosis patients (Oosterlynck *et al*, 1991). That may increase the survival of endometriotic cells in the peritoneal cavity. Endometriosis has also been suggested to be an autoimmune disease as autoantibodies recognizing endometrial antigens are produced by the patients (Kennedy *et al*, 1990; Mathur *et al*, 1982).

Therefore, it has been suggested that anti-inflammatory drugs, like peroxisome proliferator activated receptor (PPAR) γ agonists, would be useful in the treatment of endometriosis (Demirturk *et al*, 2006; Lebovic *et al*, 2007; Wu & Guo, 2009). In addition, the presence of endometrial autoantibodies and increased concentration of inflammatory molecules in the peritoneal fluid and peripheral blood of women with endometriosis have been suggested to be potential biomarkers for endometriosis.

2.1.2.5 Oxidative stress

Oxidative stress is caused by imbalance between production of reactive oxygen species (ROS), which are produced by normal oxygen metabolism, and the antioxidant system controlling their synthesis and inactivation. Oxidative stress is increased in women with pelvic endometriosis (for review, see Jackson *et al*, 2005; Van Langendonckt *et al*, 2002) mainly due to elevated production of ROS by macrophages (Murphy *et al*, 1998). Also endometriotic cells display increased ROS production and decreased ROS detoxification leading to higher endogenous oxidative stress (Ngô *et al*, 2009). ROS may contribute to increased endometrial and endometriotic cell growth (Ngô *et al*, 2009; Foyouzi *et al*, 2004). Oxidative stress is also involved in the formation of pelvic adhesions (for review, see Alpay *et al*, 2006), due to enhanced production and decreased turnover of extracellular matrix by inhibition of MMP action and increase of their inhibitors (TIMPs). Thus, oxidative stress may be one of several factors involved in endometriosis and the related symptoms.

2.1.3 Classification and staging

The extent of endometriosis varies from a few, small lesions on otherwise normal pelvic organs to large, ovarian endometriotic cysts (endometriomas), and/or extensive fibrosis and adhesions causing marked distortion of pelvic anatomy (Kennedy *et al*, 2005). Disease severity is commonly classified in stages 1-4: minimal, mild, moderate and severe, according to the revised classification system of the American Society for Reproductive Medicine (ASRM; 1997). This staging correlates with the degree of subfertility, but not properly with the severity of pelvic pain (D'Hooghe *et al*, 2003; Fauconnier & Chapron, 2005; Kennedy *et al*, 2005).

Endometriosis lesions are typically classified into three main types: 1) peritoneal (*i.e.* superficial) lesions located on the peritoneal surface, 2) deep lesions infiltrating at least five mm under the peritoneum, and 3) ovarian endometriotic cysts called endometriomas or chocolate cysts. **The peritoneal lesions** typically appear as red, black or white, representing distinctive steps in the evolutionary process (Donnez *et al*, 2002b). Red lesions are considered to be the first phase of this process, as they are the most active and highly vascularized. Due to bleeding of these lesions and accumulation of old blood, the lesions turn black. The white lesions are considered as inactive latent stages (Nisolle & Donnez, 1997). In addition, the more rarely observed clear endometriotic vesicles on peritoneum may be the very first step of attachment of endometriotic cells (Donnez *et al*, 2002b). **The deep lesions** are subclassified according to their location in the rectovaginal

pouch, uterosacral ligaments, bowel, or bladder. Their appearance is associated with pain while superficial lesions are typically found in patients with infertility (Cornillie *et al*, 1990). **Ovarian endometriosis** is typically less symptomatic than deep and peritoneal disease, but it responds poorly to conventional treatments and often recurs.

2.1.4 Diagnosis of endometriosis

Endometriosis is typically diagnosed by visualization of lesions in **surgery** *i.e.* in laparoscopy or laparotomy. Because of its invasive nature, surgery often causes a delay in diagnosis and treatment of endometriosis, especially in symptomatic teenagers and young women (Brosens *et al*, 2003). The time to diagnosis can be very long (mean 11.7 years in the USA and 8.0 years in the UK) mainly because of variability in symptoms and signs, and confusion with other disorders (Hadfield *et al*, 1996). However, the early diagnosis of endometriosis is of importance in reducing the occurrence of the disease and infertility problems, and will thus make the possibility of a successful of pregnancy more likely (Yang et al, 2004). In laparoscopy, the superficial endometriosis and ovarian endometriomas can be identified due to the presence of old or recent bleeding (Brosens *et al*, 2003). The noninvasive imaging technologies *i.e.* high resolution transvaginal ultrasound and magnetic resonance imaging can be used to diagnose large ovarian endometriotic cysts (Moore *et al*, 2003; Stratton *et al*, 2003) and also deep endometriotic nodules (Chamié *et al*, 2009; Goncalves *et al*, 2009; Guerriero *et al*, 2007). However, they do not allow accurate staging of endometriosis as superficial lesions, small ovarian endometriomas and endometriosis-related adhesions cannot be detected (Brosens *et al*, 2003).

In addition to the diagnosis of endometriosis in the patients with subfertility and/or pelvic pain, the differential diagnostics of benign and malignant ovarian cysts is of importance (Rogers *et al*, 2009). The coexistence of endometriosis and **ovarian cancer** has been reported to range between 0.7% and 5.0% of all cases with ovarian endometriosis (Erzen & Kovacic, 1998; Nishida *et al*, 2000; Ogawa *et al*, 2000; Stern *et al*, 2001). Moreover, endometriosis is shown to increase the risk of ovarian cancer (Ness, 2003), especially endometrioid and clear cell carcinomas (Nagle *et al*, 2008). These cancer types have been suggested to arise, at least partly, from endometriosis (Nishida *et al*, 2000; Sato *et al*, 2000). As neoplastic ovarian cysts can resemble endometriomas in ultrasound, they need to be carefully considered in the differential diagnostics.

Currently, no reliable markers for the diagnosis and prognosis of endometriosis are available. To improve the diagnostics, a variety of **potential molecular markers** have been identified (for review, see Bedaiwy & Falcone, 2004; Brosens *et al*, 2003; Othman *et al*, 2008; Yang *et al*, 2004). Both the noninvasive tests including serum or plasma and menstrual fluid samples, and semi-invasive methods including peritoneal fluid or endometrium biopsy, have been evaluated. However, multiple distinct pathways could be involved in the pathogenesis of endometriosis, and many of its essential features, such as inflammation and neoangiogenesis, are shared with many other diseases. Therefore, it is unlikely that a single biochemical marker will yield sufficient sensitivity (proportion

of true positives) and specificity (proportion of true negatives) to be used in clinical practice (Brosens *et al*, 2003). The relevant biomarker(s) should be useful to diagnose patients of all disease stages (especially minimal to mild endometriosis) with high sensitivity (D'Hooghe *et al*, 2006) and independently of the phase of menstrual cycle. Comparison of the biomarkers in individual studies is difficult as the group of patients differ in number, stage of endometriosis and reporting the variability of symptoms. Despite few promising results, further studies are needed to evaluate the relevance of the suggested diagnostic biomarkers for endometriosis. New potential diagnostic tools may be identified by applying genome-wide expression analyses and evaluation of proteomic profiles in addition to applying conventional biochemical assays.

2.1.4.1 Serum biomarkers

The most extensively studied and used serum biomarker for endometriosis is the cancer antigen 125 (**CA125**) even though it has limited diagnostic utility. With a threshold value of 35 IU/ml, the specificity is 90% and the sensitivity 47% for moderate to severe endometriosis (for review, see Mol *et al*, 1998). However, for minimal to severe disease a specificity was 89% while sensitivity was as low as 28% (Mol *et al*, 1998). Therefore, CA125 alone cannot be used in the detection of patients with mild endometriosis. However, the high specificity of CA125 indicates its usefulness in disease monitoring and follow-up (Bedaiwy & Falcone, 2004). Combining CA125 with other potential biomarkers and/or clinical data may increase its usefulness as a diagnostic marker (Gagné *et al*, 2003).

Several other **potential serum biomarkers** for the diagnosis of endometriosis include *e.g.* cytokines (IL-1β, IL-6, IL-8, MCP-1, TNF-α), growth factors (VEGF, insulin-like growth factor 1 (IGF-I), and epidermal growth factor receptor (EGF-R)), soluble adhesion molecules (sICAM, sE-cadherin), and cancer biomarker CA19-9 (for review, see Agic *et al*, 2006; Bedaiwy & Falcone, 2004; Yang *et al*, 2004). Interleukin 6 (IL-6) is one of the most promising individual biomarkers. It has a sensitivity of 71-90% and a specificity of 66-67% to diagnose minimal to severe endometriosis, with a threshold value of 1.9-2 pg/ml (Bedaiwy *et al*, 2002; Othman *et al*, 2008). Recent studies suggest that elevated levels of serum urokortin and follistatin, may discriminate ovarian endometrioma from other benign ovarian cysts with specificities of 90% and 92%, and sensitivities of 88% and 92%, (Florio *et al*, 2009; Florio *et al*, 2007). The use of autoantibodies as a screening tool is limited by their low diagnostic sensitivity (Bedaiwy & Falcone, 2004). Some of the suggested biomarker candidates *e.g.* C-reactive protein (CRP) and serum amyloid A (SAA) are elevated in inflammatory processes meaning that other inflammatory processes must be excluded before using them in the diagnosis of endometriosis (Agic *et al*, 2006).

2.1.4.2 Peritoneal and endometrial fluid biomarkers

Peritoneal fluid, sampled by ultrasonographically guided transvaginal aspiration (Bedaiwy & Falcone, 2004), may serve as a basis for a semi-invasive diagnostic test for endometriosis. Levels of several cytokines are elevated in the peritoneal fluid of endometriosis patients mainly because of their production by activated macrophages (Bedaiwy *et al*, 2002).

According to a prospective controlled trial, the most promising individual peritoneal fluid biomarker is tumor necrosis factor α (TNF-α) (Bedaiwy *et al*, 2002). A threshold value of 15 pg/ml has resulted in 100% sensitivity and 89% specificity. The peritoneal fluid level of CA125 is higher than in serum, but there is no difference between women with and without endometriosis (Kruitwagen *et al*, 1991). The possible limitation for the use of peritoneal fluid is the low amount of peritoneal fluid in some patients. Proteomic profiling of peritoneal and endometrial fluids may also be useful for diagnosing endometriosis (Ametzazurra *et al*, 2009; Casado-Vela *et al*, 2009; Ferrero *et al*, 2009).

2.1.4.3 Endometrial biomarkers

Several tissue biomarkers have also been suggested for diagnosis of endometriosis from endometrial biopsy according to the whole genome expression analysis. However, their usefulness as diagnostic markers remains to be evaluated. The expression of aromatase (CYP19A1), one of the most studied proteins, may correlate with the presence of endometriosis (Kitawaki *et al*, 1999). However, the marker lacks the specificity for endometriosis as it is also associated with other hormone-dependent proliferative disorders of the uterus (Dheenadayalu *et al*, 2002). The altered expression of cell adhesion molecules like integrins, and matrix metalloproteinases (MMP1, 2, 3, 9) or their inhibitors (TIMP1, 2, 3) is present in endometrium of patients with endometriosis as compared to healthy controls (Yang *et al*, 2004). Recently, the detection of nerve fibers in endometrial biopsy has been suggested to predict the presence of endometriosis (Al-Jefout *et al*, 2009) based on the result that the small nerve fibers are present in the functional layer of endometriosis patients but not that of healthy controls (Bokor *et al*, 2009).

2.1.5 Treatment

There is no known cure for endometriosis. The ESHRE guidelines (Kennedy *et al.* 2005) for patient managements are largely based on the reduction of systemic estrogen effect. Combined hormonal contraceptive methods are typically used, either cyclically or continuously, as a first-line treatment for symptomatic patients. In addition, pain symptoms suggestive of the disease can be treated without a definitive diagnosis using a therapeutic trial of a hormonal drug to reduce menstrual flow. Also non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat endometriosis-associated pain based on their ability to reduce prostanoid synthesis (Kauppila *et al*, 1979; Ylikorkala & Viinikka, 1983). However, the scientific proof of their efficacy in treating endometriosis-associated pain is inconclusive (Allen *et al*, 2005). In addition, current medical treatments for endometriosis-associated pain include progestins (*e.g.* medroxyprogesterone (MPA)), androgenic progestins (*e.g.* levonorgestrel-releasing intrauterine device (LNG-IUD)), and gonadotropin releasing hormone (GnRH) analogs (for review, see Fedele *et al*, 2008a; Guo, 2008; Kennedy *et al*, 2005; Lessey, 2000; Olive & Pritts, 2001; Vercellini *et al*, 2009a). The medical therapy alone is relatively inefficacious (Donnez *et al*, 2002a) while the symptomatic disease often recurs after cessation of the therapy. In addition, a long term use of GnRH agonists causes side effects because of systemic estrogen deficiency (Waller

& Shaw, 1993). Moreover, some degree of proliferation of endometrial and endometriotic cells is detected even after prolonged estrogen suppression using GnRH agonist (Nisolle *et al*, 1997). Finally, the recovery of estrogen levels after the discontinuation of the therapy causes a relapse of the lesions (Kitawaki *et al*, 2002).

Surgical excision of endometriosis lesions aims at removing all disease tissue and restoring normal anatomy. In mild stage disease, it is an effective treatment for endometriosis-associated pain (D'Hooghe *et al*, 2003; Jones & Sutton, 2003; Sutton *et al*, 1997). Also fertility may be improved by surgical excision of lesions. Unfortunately, disease with pain symptoms recurs in 21.5% of women within 2 years and in 40-50% within 5 years (Guo, 2009). Further surgery is needed in many cases (Guo, 2009; Olive & Pritts, 2001; Vercellini *et al,* 2009b). Repeated operations are associated with the decreased ovarian reserve (Hachisuga & Kawarabayashi, 2002; Ragni *et al*, 2005; Somigliana *et al*, 2003), increased morbidity and health care costs. Thus, in addition to the use as a first line treatment, non-surgical medical therapy is of importance to decrease number of repeat operations.

Putative therapeutic options for the future treatment of endometriosis include recently discovered hormonal and non-hormonal drugs (for review, see Guo, 2008). These therapies are typically developed to target factors synthesized by the endometriosis tissue and to the immune response associated with endometriosis (for review, see Fedele *et al*, 2008b; Guo, 2008). The hormonal treatments include aromatase inhibitors, GnRH antagonist, non-steroidal progesterone receptor agonists, selective progesterone receptor modulators (SPRMs), estrogen receptror 2 (ESR2) agonists and selective estrogen receptor modulators (SERMs). The non-hormonal therapies include anti-angiogenic (antagonists of VEGF), immunostimulatory (*e.g.* IL-2 and interferon alpha (IFN-α)), and anti-inflammatory agents (COX-2 inhibitors, PPAR-γ agonists, retinoid X receptor (RXR) ligands, statins, and TNF-α inhibitors). In addition, inhibitors of mitogen-activated protein kinase (MAPK), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB), histone deacetylase, and MMPs have been suggested to be used to treat endometriosis (Guo, 2008). The anti-angiogenic and immunostimulatory agents are aimed to prevent recurrences rather than to treat the disease, but their expected side effects may prevent their long-term use (Fedele *et al*, 2008b). The anti-inflammatory agents are expected to prevent endometriosis rather than diminish already established lesions. However, many medical therapies effective in pain reduction are typically not useful for infertility treatment (Guo, 2008).

2.2 HORMONAL REGULATION OF ENDOMETRIAL AND ENDOMETRIOTIC CELLS

Endometriosis responds to hormones mainly similarly to cycling eutopic endometrium. However, aberrant local estrogen and progesterone actions in endometriosis tissue are known factors involved in the pathophysiology of the disease.

2.2.1 Synthesis and action of endocrine hormones in cycling endometrium

Endometrium, and more specifically its functional layer, goes through remarkable changes during **the menstrual cycle** *(Fig 2)*. Synchronously developing glandular and surface epithelium and stromal cells, together with appropriate development of vascular endothelium and smooth muscle cells, connective tissue, extracellular matrix, and transiently resident cells including macrophages and monocytes are essential for successful endometrial function and implantation (Giudice & Ferenczy, 1996). These events are regulated mainly by ovarian hormones estradiol (E2) and progesterone (P4) but also by locally produced growth factors, acting primarily or secondarily as mediators of steroid hormone action. The production of E2 and P4, and their release from the ovaries to blood circulation, is coordinated by the hypothalamus-pituitary-ovarian axis with a major role played by gonadotropin-releasing hormone (GnRH) and ovarian steroid feed-back loops. GnRH, released by the hypothalamus, stimulates the pituitary gland to release the

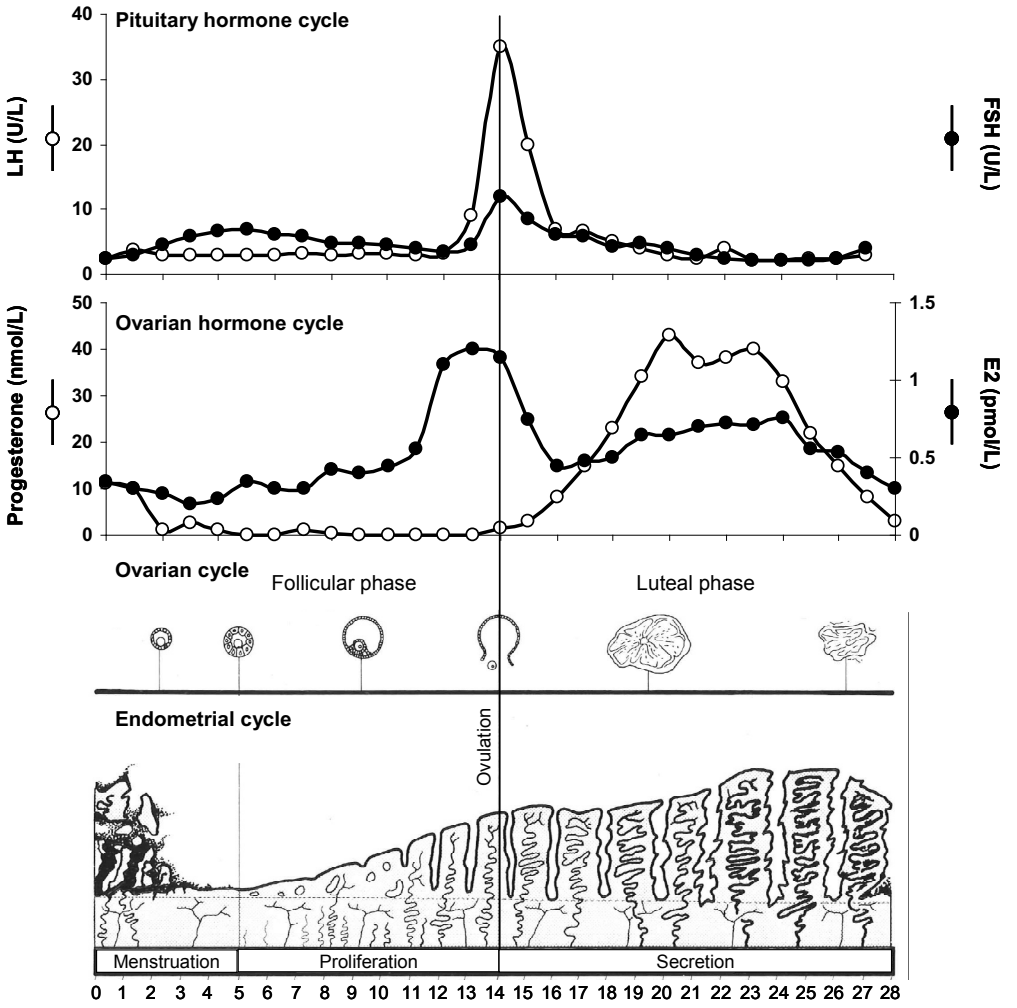

Figure 2*: Changes in the ovarian follicle, endometrial thichness, and serum hormone concentrations during a normal menstrual cycle. (Modified from Burkitt et al, 1993 and Marshall, 2006.)*

gonadotropins luteinizing hormone (LH) and follicle stimulating hormone, which further regulate follicular development, ovulation, development of corpus luteum, and the release of E2 and P4 (Giudice & Ferenczy, 1996). These circulating steroid hormones easily penetrate the cells in the target tissue *e.g.* endometrium, due to their lipofilic nature.

In the target tissue, the major effects of estrogens and progesterone are mediated by their nuclear receptors. These are members of the nuclear receptor superfamily and bind their ligands with high specificity and affinity (Evans, 1988). Activated by their ligand, the nuclear receptors interact with a specific DNA sequence *i.e.* response element, in the promoter regions of the target genes (Mangelsdorf *et al*, 1990; Mangelsdorf *et al*, 1995), and recruits a number of other proteins called transcription coregulators. Depending on the recruited coactivators and corepressors, the expression of the target gene is either up- or down-regulated (Glass & Rosenfeld, 2000; Jepsen *et al*, 2000).

2.2.2 Estrogen actions in endometrium

Estrogens, mainly the highly active E2, induce the proliferation of the endometrial epithelial, stromal and endothelial cells during the proliferative (*i.e.* follicular) phase of the menstrual cycle. In premenopausal women, the main source of circulating estrogens is the **ovary** where it is synthesized by the growing follicle during the mid and late proliferative phase of the menstrual cycle. Estrogens are also released into circulation by **peripheral tissues** *i.e.* adipose tissue and skin (Bulun & Simpson, 1994; Harada, 1992). This source is important especially in the early follicular phase and after menopause, when follicular steroidogenesis does not occur. Importantly, estradiol is also synthesized in estrogen **target tissues** from circulating precursors via two pathways: 1) aromatase pathway where circulating precursors dehydroepiandrosterone (DHEA), its sulfate (DHEA-S), and 4-androstenedione (4A; released by adrenals) are converted to estrone by action of hydroxysteroid (3beta) dehydrogenase (HSD3B) and P450 aromatase, or 2) sulfatase pathway in which circulating, peripherally synthesized estrone sulfate (E1-S) is converted to estrone (E1) by steroid sulfatase (STS; Labrie, 1991; Simpson, 2003). Finally, E1 is converted to the more potent E2 by reductive hydroxysteroid (17β) dehydrogenases (HSD17Bs).

Estrogen action is mediated primarily by **estrogen receptors (ESR) 1 and 2** (previously called as α and β, respectively). They are both expressed in the human endometrium, although ESR1 predominates over ESR2 and their expression differs during the menstrual cycle. Estrogen-mediated proliferation in endometrium is promoted mainly through the activation of ESR1. Paradoxically, the expression of ESR1 is decreased in endometriosis (Brandenberger *et al*, 1999; Matsuzaki *et al*, 2001), due to down-regulation by E2 (Trukhacheva *et al*, 2009). In contrast, ESR2 expression is increased in endometriosis (Fujimoto *et al*, 1999; Trukhacheva *et al*, 2009), possibly because of hypomethylation of ESR2 promoter (Xue *et al*, 2007) leading to the imbalanced ESR1/ESR2 ratio. Thus, there is some controversy between increased E2-dependent proliferation and decreased ESR1 expression in endometriosis. Moreover, high levels of ESR2 suppress ESR1 expression

and response to estradiol in endometrial and endometriotic stromal cells and, thus, possess antiuterotrophic effects (Moutsatsou & Sekeris, 2003; Trukhacheva *et al*, 2009). ESR2 also regulates cell cycle progression and might contribute to the proliferation of endometriotic stromal cells (Trukhacheva *et al*, 2009). It has been suggested that part of the proliferative estrogen actions in endometrium are mediated also by non-genomic or non-ER mediated manner *e.g.* via membrane ERs, MAPK or AKT signaling pathways (Kayisli *et al*, 2004; Vivacqua *et al*, 2006). Thus, E2 may exert its proliferative effect via decreased ESR1, non-genomic actions, or even via ESR2 in endometriosis (Rizner, 2009; Trukhacheva *et al*, 2009). ESR2 also mediates repression of epithelial expression of the progesterone receptor (PR) while ESR1 induces PR expression (Moutsatsou & Sekeris, 2003). The increased ESR2/ESR1 ratio may, thus, contribute to progesterone resistance (Chapter 2.2.6; Trukhacheva *et al*, 2009). Estrogens also regulate the effects of androgens and retinols, which also have a role in the regulation of endometrial cell proliferation (Deng *et al*, 2003; Mertens *et al*, 1996).

Estrogens also show **anti-inflammatory actions** by repressing the expression of inflammatory genes *e.g.* IL-6, IL-8, or TNF-α through the NF-κB pathway (Cvoro *et al*, 2008; Ray *et al*, 1997). These events may be mediated by both ESR1 and ESR2, while ESR2 is more potent in mediating the anti-inflammatory E2 actions (Cvoro *et al*, 2008). In fact, selective ESR2 agonists repress proinflammatory genes and, thus, have beneficial effects in preclinical models involving inflammation without causing growth-promoting effects on the uterus (Cvoro *et al*, 2008; Xiu-li *et al*, 2009).

2.2.3 Aberrant local estrogen metabolism in endometriosis

In endometriosis, the local estrogen synthesis *(Fig. 3)* is increased by elevated aromatase and the presence of reductive HSD17B activity. Moreover, the local inactivation of E2 is decreased by loss of HSD17B2 action, which further increases local E2 concentration and estrogen-dependent proliferation of endometriotic cells. That is supported by the detection of elevated E2 level in menstrual blood of endometriosis patients as compared with that of healthy women (Takahashi *et al*, 1989).

2.2.3.1 Aromatase

Aromatase (P450 aromatase, CYP19A1) catalyzes the conversion of androstenedione and testosterone to estrone and estradiol, respectively. Aromatase is expressed in endometriosis analyzed by measuring the mRNA, protein, and enzymatic activity (for review, see Colette *et al*, 2009). **Increased aromatase expression** has been detected in ovarian (Bukulmez *et al*, 2008b; Noble *et al*, 1996; Smuc *et al*, 2007; Heilier *et al*, 2006), peritoneal (Bukulmez *et al*, 2008b; Heilier *et al*, 2006), and deep (Dassen *et al*, 2007; Heilier *et al*, 2006; Matsuzaki *et al*, 2006b) endometriosis without cyclical changes (Bukulmez *et al*, 2008b). In addition, aromatase mRNA and protein have been detected in the endometrium of endometriosis patients, while in the endometrium of healthy controls the transcript level is hardly detectable (Aghajanova *et al*, 2009; Bukulmez *et al*, 2008b; Kitawaki *et al*, 1997; Noble

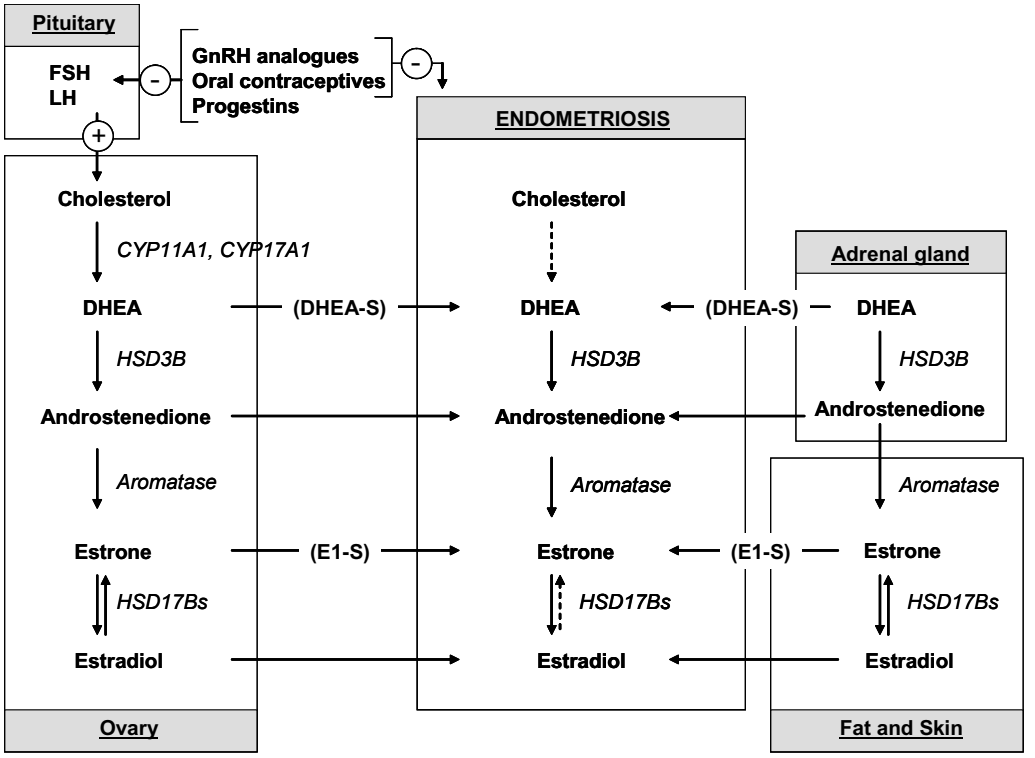

Figure 3: *Sources of estradiol in endometriosis tissue. (Modified from Bulun, 2009)*

et al, 1996). However, there is some controversy in endometriotic aromatase expression. Increased aromatase mRNA expression has been detected in 61% of endometriosis patients (Velasco *et al*, 2006), while totally absent or only marginal aromatase activity (Delvoux *et al*, 2009; Izawa *et al*, 2008) and protein expression (Colette *et al*, 2009) have recently been reported. Among different types of endometriosis the highest level of aromatase expression has been observed in ovarian endometriosis (Heilier *et al*, 2006), which was suggested to support the theory of distinct entities of different types of endometriosis. However, another study (Bukulmez *et al*, 2008b) demonstrated highest aromatase mRNA levels in peritoneal implants where the expression correlates with the inflammatory stage of endometriosis. It is notable that aromatase is highly expressed in ovarian follicles and present also in adipose tissue and intact peritoneum (Bulun *et al*, 1993; Kyama *et al*, 2008), which may cause the false positive expression result, especially in endometriosis specimens (Colette *et al*, 2009). The localization of aromatase protein in endometriosis tissue is also controversial as some reports demonstrate glandular localization (Kitawaki *et al*, 1997; Bukulmez *et al*, 2008b; Fechner *et al*, 2007; Hudelist *et al*, 2007; Ishihara *et al*, 2003; Matsuzaki *et al*, 2006b) while others show aromatase protein in stromal cells (*e.g.* Acién *et al*, 2007; Velasco *et al*, 2006; Zeitoun & Bulun, 1999).

It has been suggested that **a positive feedback loop** *(Fig. 4)* enhances the aromatase expression in endometriosis (Attar & Bulun, 2006a): Peritoneal fluid cytokines, TNFα and IL-6, stimulate aromatase activity in cultured endometriotic stromal cells (Velasco *et*

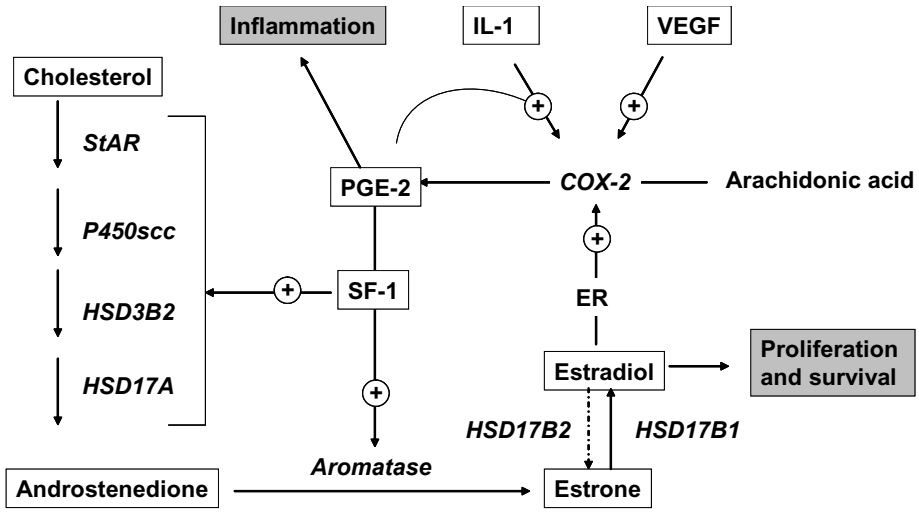

Figure 4: *Survival and inflammation of endometriotic tissue. (Modified from Bulun, 2009)*

al, 2006). Aromatase increases the local level of estradiol, which directly induces COX-2 enzyme. COX-2 increases the level of PGE-2, which is the most potent known stimulator of steroidogenic acute regulatory protein (StAR) and aromatase in endometriotic stromal cells. The effect of PGE-2 is mediated by a transcription factor SF-1, which is highly present in endometriosis but not in healthy endometrium (Attar *et al*, 2009). StAR is involved in the first major step of estrogen synthesis. The action of aromatase and StAR leads to continuous estrogen and prostaglandin formation in endometriosis. On the other hand, prostaglandins and cytokines mediate pain, inflammation and infertility (for review, see Attar & Bulun, 2006a; Bulun *et al*, 2009). Thus, the aberrant regulation of aromatase seems to be closely connected in both estrogen-dependent proliferation and inflammation in endometriosis tissue.

Aromatase inhibitors (AI) have been successfully used to treat endometriosis in pilot studies (for review, see Fedele *et al*, 2008b; Guo, 2008). However, in premenopausal women, AI treatment alone may induce ovarian folliculogenesis and increase the risk of ovarian cysts. Therefore, when treating premenopausal women, AIs need to be combined with a progestin, a combination oral contraceptive, or a GnRH analogue (Attar & Bulun, 2006b; Ailawadi *et al*, 2004). A randomized clinical trial showed that endometriosis patients receiving AI anastrozole with GnRH agonist had a lower endometriosis recurrence risk (43% vs. 90%) as compared to those treated with GnRH agonist only (Soysal *et al*, 2004). However, some studies also documented a quick return of the symptoms after the completion of combined AI and progestin (Desogestrel) treatment (Remorgida *et al*, 2007a; Remorgida *et al*, 2007b).

2.2.3.2 Hydroxysteroid (17beta) dehydrogenases (HSD17Bs)

Hydroxysteroid (17beta) dehydrogenases (HSD17Bs) catalyze the interconversion between the active and inactive forms of sex steroids including estrogens, androgens

Table 1: *Human hydroxysteroid (17beta) dehydrogenases, their preferred substrates and catalytic activities*

HSD type	Gene name	Other names	Activity	Substrate specificity
1	HSD17B1	EDH17B1,	17beta-HSD	estrogens, androgens
2	HSD17B2	E2DH	17beta-HSD, 20alpha-HSD	estrogens, androgens, progestins
3	HSD17B3		17beta-HSD	androgens, (estrogens)
4	HSD17B4	MFP-2, MFE-2, DBP	3-ketoacyl-DH, 17beta-HSD	fatty acids, bile acids, (estrogens, androgens)
5	AKR1C3	HSD17B5	17beta-HSD, 3alpha-HSD	androgens, progestins, prostaglandin, (estrogens)
6	HSD17B6	HSE, RODH	3-alpha/beta epimerase, retinal reductase/DH, 17beta-HSD	androgens, neurosteroids, retinoids, (estrogens)
7	HSD17B7		3-keto reductase, 17beta-HSD	sterols, estrogens, (androgens, progestins)
8	HSD17B8		17beta-HSD, (fatty acid CoA-dehydrogenase)	estrogens, androgens, (fatty acids)
10	HSD17B10	ERAB, SCHAD, SDR5C1, HADH2	17beta-HSDm 20beta-OH DH, 21beta-OH DH, 3alpha-HSD, 7alpha/beta-OH DH	fatty acids, bile acids, estrogens, androgens, progestins, corticosteroids
11	HSD17B11	DHRS8, PAN1B, retSDR2	17beta-HSD	estrogens, androgens
12	HSD17B12	KAR	Ketoacyl-CoA reductase, 17beta-HSD	fatty acids, estrogens, (androgens)
13	HSD17B13	SCDR9, DHRS8	not known	not known
14	HSD17B14	DHRS10, retSDR3	17beta-HSD	estrogens, androgens

HSD= hydroxysteroid dehydrogenase, DH = dehydrogenase, OH =hydroxy, CoA = coenzyme A
Modified from Moeller & Adamski, 2006; Moeller & Adamski, 2009

and progesterone. In addition, some of the HSD17Bs metabolize retinoids, fatty acids and neurosteroids (reviewed by Moeller & Adamski, 2006; Moeller & Adamski, 2009). Currently, 14 different HSD17B enzymes with individual cell-specific expression profiles, substrate specificities, and unique regulatory mechanisms have been identified *(Table 1)*. In mammals, increasing evidence suggests variable substrate specificities and differential physiological roles for these enzymes. It is likely that some of the HSD17B enzymes act in multiple metabolic pathways. The HSD17B-enzymes may possess an important **pre-receptor regulation** of steroid action by catalyzing the inactivation and activation of ligands for various nuclear receptors in a range of target tissues (Poutanen *et al*, 1993). While many of the HSD17Bs have a wide variety of substrates, one substrate and catalytic activity is typically shared by several HSD17B-enzymes.

The catalytic activity towards estrogens and androgens has been detected at some level in almost every HSD17B-enzyme (reviewed by Moeller & Adamski, 2006; Moeller & Adamski, 2009). The data suggest that HSD17B1, 2, 4, 7, 12 and 14 mainly utilize estrogens as substrates, and HSD17B3, 5 and 6 primarily convert androgens. However, the only HSD17Bs exclusively utilizing sex steroids as substrates are HSD17B1 and HSD17B3. HSD17B2 is mainly known as an enzyme catalyzing the conversion of

estradiol to estrone but it also acts as a 20α-hydroxysteroid dehydrogenase activating 20α-hydroxyprogesterone into progesterone (Lu *et al*, 2002; Puranen *et al*, 1999). It may also be involved in retinoic metabolism (Rantakari *et al*, 2008; Zhongyi *et al*, 2007). HSD17B4, 8, 10 and 12 possess activity for fatty acids (Moeller & Adamski, 2006; Moeller & Adamski, 2009), and HSD17B7 is involved in cholesterol biosynthesis (Marijanovic *et al*, 2003; Shehu *et al*, 2008). In addition to estrogens and androgens, HSD17B5 possesses activity on progesterone and prostaglandins (Matsuura *et al*, 1998; Penning *et al*, 2000), and HSD17B6 on neurosteroids and retinoids (Biswas & Russell, 1997; Huang & Luu-The, 2000).

2.2.3.3 Estrogen inactivating HSD17Bs

One of the major estradiol inactivating enzymes, HSD17B2 catalyzes the conversion of estradiol to its less active form estrone. In the healthy endometrium, its expression is highly increased in secretory phase due to increased P4 action, thus, decreasing the local E2 level. Its aberrant expression, *i.e.* lack of increase in secretory phase, is confirmed both at mRNA and protein level in deep endometriosis (Dassen *et al*, 2007; Matsuzaki *et al*, 2006b), ovarian endometriotic cysts (Banu *et al*, 2008; Cheng *et al*, 2007; Matsuzaki *et al*, 2006a), and in inadequately described endometriosis (Absenger *et al*, 2004; Zeitoun *et al*, 1998). This results to continuously high E2 levels in endometriosis lesions. No differences have been detected in proliferative phase samples (Carneiro *et al*, 2007; Smuc *et al*, 2007). Accordingly, decreased inactivation of E2 to E1 has recently been demonstrated in endometriosis lesions (Delvoux *et al*, 2009) as compared with the normal endometrium. As HSD17B2 is most likely increased by P4 in secretory phase endometrium, its aberrant expression in endometriosis may be caused by impaired P4 action (Attia *et al*, 2000; Zeitoun *et al*, 1998). However, the deficient HSD17B2 expression may be a result of stromal defect: Progesterone may stimulate epithelial HSD17B2 mRNA expression via stromal PR-B, which induces the secretion of paracrine factors inducing HSD17B2 promoter activity in epithelial cells (Bulun, 2009). Recently, the HSD17B2 gene has been suggested to be methylated in 31% of breast cancers (Bhavani *et al*, 2009). As hyper-methylation is responsible for gene silencing, this epigenetic regulation may be partly responsible for the decreased HSD17B2 expression. However, the HSD17B2 methylation in endometriosis has not been reported. Concerning to other E2 inactivating HSD17Bs, no differences have been detected in the expression of estradiol inactivating HSD17B4 or 8 in ovarian endometriotic cysts (Smuc *et al*, 2009) while a significant down-regulation of HSD17B4 has been reported in deep infiltrating endometriosis (Dassen *et al*, 2007). Currently, no data is available for HSD17B10 or HSD17B14 in endometriosis.

2.2.3.4 Estrogen activating HSD17Bs

Human HSD17B1 is the main enzyme converting the weak estrogen, E1, to highly active E2 (Rizner, 2009). There is also evidence suggesting that it also activates 4A to T, as overexpression of human HSD17B1 in mouse results in female masculinization (Saloniemi *et al*, 2007; Saloniemi *et al*, 2009). As HSD17B1 has a central role in E2

formation, it has been widely studied as a drug target for several E2-dependent diseases, *e.g.* breast cancer and endometriosis (Husen *et al*, 2006; Kruchten *et al*, 2009; Messinger *et al*, 2009).

The conversion of E1 to E2 is increased in endometriosis (Delvoux *et al*, 2009). Several studies have demonstrated either presence (Zeitoun *et al*, 1998) or increased expression (Borghese *et al*, 2008; Dassen *et al*, 2007; Smuc *et al*, 2007) of HSD17B1 in ovarian (Borghese *et al*, 2008; Smuc *et al*, 2007) or deep infiltrating (Dassen *et al*, 2007) endometriosis at mRNA level. It must be noted that, similarly to aromatase, HSD17B1 is highly expressed in ovarian granulosa cells (Ghersevich *et al*, 1994), which may be adjacent to endometrioma cyst wall, and therefore, be present also in the endometrioma specimens (Colette *et al*, 2009). The increased expression of HSD17B1 in ovarian endometrioma may, thus, result from a contamination of ovarian granulosa cells. However, in the induced endometriosis model in marmoset monkey HSD17B1 and aromatase proteins were up-regulated in the established endometriotic foci, whereas only weak immunohistochemical staining was detected in early endometriotic foci (Einspanier *et al*, 2006). Thus, the data on HSD17B1 expression in endometriosis is inconclusive.

The expression of other enzymes possessing HSD17B-activity has been presented in only few reports. The mRNA expressions of estrogen activating AKR1C3, HSD17B7 and HSD17B12 have been shown to be up-regulated in ovarian endometriosis as compared to healthy endometrium (Smuc *et al*, 2009; Smuc *et al*, 2007). However, no data is available for these enzymes in peritoneal or deep endometriosis.

2.2.3.5 Other estrogen metabolizing enzymes

Interestingly, endometriosis lesions have been shown to express all the enzymes required to synthesize estrogens *de novo* from cholesterol without the need for androgenic precursors (Attar & Bulun, 2006a). The rate limiting step for steroidogenesis is the intake of cholesterol from cytosol into the mitochondrion by StAR (Stocco, 2001). Estradiol is then synthesized via several steps catalyzed by side-chain cleavage enzyme (P450scc), 17α-hydroxylase (P450c17, CYP17A), hydroxysteroid (3beta) dehydrogenase type 2 (HSD3B2), HSD17B1 and aromatase (Attar *et al*, 2009; Tsai *et al*, 2001).

In addition, endometriosis is able to synthesize estrogens from E1-S and E2-S, which are circulating in high concentrations. That is due to the activity of steroid sulfatase (**STS**; (Pasqualini *et al*, 1989) detected in ovarian and peritoneal endometriosis (Carlström *et al*, 1988; Purohit *et al*, 2008). The STS activity in peritoneal endometriotic implants may be even higher than that of aromatase, and was shown to correlate with the severity of the disease (Purohit *et al*, 2008). Thus, the sulfatase pathway of E2 synthesis may to be important in pathogenesis of endometriosis, and STS inhibitors have been suggested for the treatment of endometriosis (Purohit *et al*, 2008). One of the major estrogen inactivating enzymes, in addition to HSD17B2, is estrogen sulphotransferase (**SULT1E1**), which catalyzes the sulphate conjugation of estrone (Pasqualini, 2009). Its expression is increased in secretory phase endometrium most probably via P4 regulation (Rubin *et al*,

1999). In contrast to HSD17B2, SULT1E1 is expressed in ovarian, peritoneal and deep infiltrating endometriosis similarly to normal endometrium (Dassen *et al*, 2007; Hudelist *et al*, 2007; Smuc *et al*, 2007).

2.2.4 Progesterone and decidual differentiation in endometrium

After ovulation *i.e.* during the secretory or luteal phase of the menstrual cycle, the estrogen-primed endometrium undergoes secretory differentiation. That is a result of increased P4 release by the corpus luteum (Fig.2; Giudice & Ferenczy, 1996). Furthermore, P4 induces **decidualization**, the endometrial remodeling following fertilization. In a broad sense decidualization includes the secretory transformation of the uterine glands, influx of specialized uterine natural killer (uNK) cells, and vascular remodeling. A more restricted definition denotes decidualization as a progesterone-induced differentiation of fibroblast-like endometrial stromal cells (ESCs), located in the proliferative estrogen-primed endometrium, into round epithelial-like decidual cells with enlarged nuclei and increased cytoplasm (Gellersen *et al*, 2007; Maruyama & Yoshimura, 2008). The major secretory products of decidual cells include prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1), which have also been used as phenotypic markers of these cells (Gellersen & Brosens, 2003). Decidual cells also produce cytokines, growth factors, neuropeptides, and other signaling molecules, which may enhance and spread the decidual process by autocrine or paracrine action. This differentiation process is dependent entirely on the convergence of the cyclic adenosine monophosphate (cAMP) and P4 signaling pathways that drives integrated changes at both the transcriptome and the proteome level. Cyclic AMP is produced in the endometrial stromal cells by stimulation of a variety of local and endocrine factors, such as PGE-2, and pituitary gonadotrophins (Gellersen & Brosens, 2003).

Increased P4 level inhibits the proliferative activity of estrogen-primed endometrium, induces secretory activity in the glandular compartment, and triggers influx of specialized uNK cells in response to local production of chemokines (Gellersen *et al*, 2007). These cells are a source of growth and angiogenic factors, and therefore, they have a critical role in the remodeling of the endometrial spiral arteries before and during pregnancy (Gellersen *et al*, 2007). During implantation and gestation, P4 appears to decrease the maternal immune response to allow the pregnancy. Progesterone also represses MMPs thereby preventing menstrual breakdown (Henriet *et al*, 2002).

The main P4 actions are mediated by **progesterone receptors (PR) A and B**, which regulate different target genes (Wen *et al*, 1994). While PR-B is suggested to mediate primary P4 actions, PR-A has been shown to act as a dominant repressor of PR-B (Vegeto *et al*, 1993). Thus, the relative concentrations of the two PR isoforms regulate the tissue response to P4 (Lessey, 2003; Mote *et al*, 2001). In endometrium, both isoforms are expressed in the glandular epithelium with the highest concentration in the late proliferative phase. In stromal cells, PR-A expression is predominant over PR-B and more constant throughout the cycle (Mote *et al*, 1999). In addition to traditional genomic

action of nuclear PRs, **non-genomic mechanisms** have been suggested especially for rapid P4 actions (for review, see Gellersen *et al,* 2009). These optional mechanisms include non-nuclear variants of nuclear PRs (cytosolic inhibitory PR-C and probably membrane associated PR-M), G protein-coupled membrane progestin receptors (mPRα, β, and γ), progesterone receptor membrane component I (PGRMC1), and allosteric modulation or direct binding of P4 or its metabolites (like allopregnanolone) to other receptors *e.g.* gamma-aminobutyric acid (GABA)-A or oxytosin receptor.

2.2.5 Progesterone resistance in endometriosis

The molecular response to P4 or synthetic progestins in endometriosis differs from that of eutopic endometrium (Bulun *et al*, 2006; Burney *et al*, 2007). The decreased progesterone action may be primarily due to a significant reduction of PRs, especially PR-B, in endometriosis (Attia *et al*, 2000; Bulun *et al*, 2006). In fact, the promoter region of PR-B, but not PR-A, is silenced in endometriosis by hypermethylation (Wu *et al,* 2006a). The loss of PR-B leads to aberrant regulation of P4-dependent genes, such as HSD17B2 (Attia *et al*, 2000; Zeitoun *et al*, 1998), which is expected to cause an increased local estrogen concentration. In addition to aberrant PR action, the increased expression of P4 inactivating enzymes AKR1C1 and AKR1C3 in endometriosis have been reported (Smuc *et al*, 2009). The increased conversion of P4 into the biologically less active 20α-hydroxyprogesterone may further impair the P4 actions, and effect also via non-genomic mechanisms. As P4 action is critical for the inhibition of estrogen-dependent proliferation, and for decidual differentiation (Brosens & Gellersen, 2006), the P4 resistance may play a role both in the pathogenesis of endometriosis as well as in endometriosis-related subfertility. In fact, stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity (Klemmt *et al*, 2006)

2.2.6 Direct androgen actions

There is increasing evidence suggesting that, in addition to estrogens and progestins, also androgens may have direct effects on the normal endometrial function and not only by the conversion to estrogens by CYP19A1. Androgens antagonize the proliferative effects of E2 and induce atrophy in the human endometrium, cultured endometrial cells and in rat uterus (Miller *et al*, 1986; Nantermet *et al*, 2005; Rose *et al*, 1988; Tuckerman *et al*, 2000). In addition, androgens have been suggested to play a role in decidualization and/or implantation. Both lack and excess of circulating androgens are associated with an increased risk of early fetal loss and impaired placental function (Bjercke *et al*, 2002; Castracane & Asch, 1995; de Vries *et al*, 1998; Diamant *et al*, 1982). Testosterone also inhibits the production of MMP-1 through androgen receptor (AR) in cultured human endometrial stromal cells (Ishikawa *et al*, 2007). MMP-1 is one of the MMPs involved in regulation of menstruation and embryo implantation in human endometrium (Hampton & Salamonsen, 1994). Thus, androgens may mediate antiproliferative effects and regulate implantation by inhibition of MMP-1 synthesis. In a mouse model, a low dose of T (as

its propionate ester) leads to a delay in implantation, but a high dose disturbs the uterine prostaglandin system, which further may disturb peri-implantation development or may be involved in early pregnancy loss (Diao *et al*, 2008).

Serum **androgen levels** fluctuate throughout the menstrual cycle, with levels peaking around ovulation (Dawood & Saxena, 1976; Epstein *et al*, 1975; Massafra *et al*, 1999). If fertilization occurs, circulating androgen levels rise in the late secretory phase and continue to rise in early pregnancy (Castracane *et al*, 1998; Hines *et al,* 2002). However, endometrial androgen levels and conversion of A4 to T are higher in secretory than proliferative endometrium (Bonney *et al*, 1984; Hausknecht *et al*, 1982; Vermeulen-Meiners *et al*, 1988). In addition to T and E2, A4 is metabolized in endometrial stromal cells to dihydrotestosterone (DHT) with highest affinity to AR, and to inactivce metabolite androstanediol (A-diol) (Bukulmez *et al*, 2008a).

Androgen receptor is immunolocalized to the nuclei of stromal, epithelial and endothelial cells in the endometrium (Burton *et al*, 2003; Slayden *et al*, 2001), as well as stromal and epithelial cells in decidua (Burton *et al*, 2003; Critchley & Saunders, 2009; Milne *et al*, 2005). However, the protein expression is predominant in endometrial stromal cells and possibly under regulation of androgens, E2 and P4 (Mertens *et al*, 1996; Slayden *et al*, 2001). In rhesus macaque, the expression of AR is induced by E2 and decreased by P4 (Slayden *et al*, 2001). Similarly, during the natural cycle in women, the stromal AR staining is predominant in the proliferative as compared to the late secretory phase (Slayden *et al*, 2001). Furthermore, the expression of AR and its coactivators is increased in the endometrium of women with polycystic ovarian syndrome (Giudice, 2006), indicating its regulation by androgens. In glandular epithelium, AR expression increases due to the decrease of P4 levels in late secretory phase, which is consistent with the upregulation of AR by PR antagonists (Narvekar *et al*, 2004). It has been suggested that the upregulation of AR expression in glandular epithelium is a key component in the mechanism through which progestins induce antiproliferative effects in the endometrium in the presence of estrogens (Brenner *et al*, 2003; Narvekar *et al*, 2004).

It has been suggested that AR has a role in uterine diseases associated with increased cell proliferation (McGrath *et al*, 2006; Terakawa *et al*, 1988). However, little is known about androgen action in endometriosis. In fact, androgens have been effectively used to treat endometriosis-associated pain but the side effects have limited their clinical use in women. It is also known that AR and 5α-reductases 1 and 2, converting T to DHT, are expressed in pelvic endometriosis similarly to eutopic endometrium of the patients and controls (Carneiro *et al*, 2008).

These findings suggest that, in addition to estrogens and progestins, also androgens have a direct role in regulating morphological changes in human endometrium and not only by their conversion to estrogens by aromatase. It is likely that a complex interplay between ER-, PR-, and AR-mediated signaling regulates the proliferation and differentiation of endometrial and endometriotic cells.

2.3 GENOME-WIDE EXPRESSION ANALYSIS

2.3.1 Utilization possibilities in cancer diagnostics

Genome-wide gene expression microarray technology has enabled the identification of gene expression patterns with several applications including new diagnostic tools and personalized medicine for various cancers. As the simplest, the method can be used in identification of differentially expressed genes between diseased and healthy tissue. The gene expression signatures have been used as a basis for tumor classification (Bild *et al*, 2006), and they also improve the understanding of the histological heterogeneity of the tumors. The gene expression data is used to identify putative biomarkers for biochemical assays, but the gene signature *per se* can also be used as a biomarker for certain cancers. For example, gene expression arrays have been used to identify high-risk patients based on the differential expression profiles of the aggressive disease types (*e.g.* Bonome *et al*, 2008). Thus, the method enables the establishment of new diagnostic tools for disease prognosis (Sun *et al*, 2007) and prediction of survival (Bonome *et al*, 2008; Shedden *et al*, 2008). As recurrent disease may appear drug resistant and an increasing number of cancer drugs are designed to target specific cell-signaling pathways, the gene expression signature of the tumor may help to predict the response of individual tumor to the specific treatment (*e.g.* De Smet *et al,* 2006; Golub *et al*, 1999; Staunton *et al*, 2001). Moreover, the necessity of adjuvant therapy can be predicted by the molecular signature (Bild *et al*, 2006; Downward, 2006; van 't Veer *et al*, 2002; van't Veer & Bernards, 2008) to maximize its efficacy, and to avoid unnecessary treatments. The individually tailored treatment is expected to improve patients' quality of life, and reduce overall cancer mortality and health care costs (van't Veer & Bernards, 2008).

Several reports have demonstrated that integration of gene expression profiling with associated clinical, pathological and other information improves the prediction of cancer recurrency (Bonome *et al*, 2008; Shedden *et al*, 2008; Sotiriou & Piccart, 2007; Stephenson *et al*, 2005). It is also shown that larger gene sets predict the disease recurrence with better accuracy, and the prediction may not be possible with just a few exceptional genes (Shedden *et al*, 2008).

2.3.2 Methodological aspects

Different approaches are used to identify a relationship between the gene expression patterns and the behavior of the tumor cells. These can be categorized as the data-driven approach, the knowledge-driven approach and the model-driven approach (van't Veer & Bernards, 2008). The most straightforward is the data-driven approach, where the whole-genome expression data is used to search correlations between patterns of gene expression and selected tumor traits. The knowledge-driven approach utilizes only a limited number of known genes which have been suggested to be relevant for the hypothesis, and therefore the findings are limited to current knowledge. The model-driven approach is typically used to evaluate transcriptional changes caused by specific

stimuli, such as activation of a selected signaling pathway or administration of a treatment of interest.

The classification of samples to subgroups by genome-wide expression analysis is typically performed using **unsupervised hierarchical clustering** (van't Veer & Bernards, 2008). The identified subgroups of samples, thus, have similar gene-expression patterns. On the contrary, in supervised classification the samples are first divided into predefined groups according to the clinical data, and then the genes predictive of those groups are searched. Thereafter, the predictive panel of genes is typically tested subsequently on independent set of samples.

2.3.3 Technical criteria

The outliers in expression-based classification of tumors may reflect biological heterogeneity of tumor types. However, the misclassification of tumors could be due to inaccurate clinical information, tissue sampling problems or bad classifiers (Shedden 2008). Accordingly, factors which significantly affect the results of expression analysis include sample collection methods, processing protocols, limited subject cohorts, small sample sizes, and the use of different microarray technologies (Shedden 2008, Dumur 2008). Variability in gene expression might also be caused by RNA degradation or tissue hypoxia during excision of the sample (Dash *et al*, 2002; Huang *et al*, 2001). Reducing technical variability by using similar protocols for inclusion, collection and annotation of heterogenous sample types, collection of clinical data, and the use of same reagents, platforms and data analysis (Dobbin *et al*, 2005) are of importance specifically in multicenter studies. This would ensure that the main uncontrolled variables represent the biology of the samples and associated clinical data.

2.3.4 Genome-wide expression analysis of endometriosis

In endometriosis research, the genome wide gene expression analysis has mainly been used to reveal differentially expressed genes and affected signaling cascades (for review, see Groothuis *et al,* 2007). The **pathophysiology** of the disease has been studied by evaluation of changes in expression pattern between control and patient endometrium (Kao *et al*, 2003; Klemmt *et al*, 2007; Matsuzaki *et al*, 2005; Sha *et al*, 2007), between endometrium and ovarian endometriomas (Arimoto *et al*, 2003; Zafrakas *et al*, 2008), deep (Matsuzaki *et al*, 2005; Matsuzaki *et al*, 2004) and peritoneal (Eyster *et al*, 2007) endometriosis*,* or in endometriosis-associated ovarian cancer (Banz *et al*, 2009; Kobayashi *et al*, 2009). In case of deep and ovarian endometriosis, the tissue surrounding endometriotic epithelial and stromal cells may affect the resulting gene signature. Therefore, **laser capture microdissection** has been used to isolate only the cells of interest for microarray analysis (Matsuzaki *et al*, 2005; Matsuzaki *et al*, 2004; Van Langendonckt *et al*, 2007; Wu *et al*, 2006b). However, the technical restrictions have limited the use of this method. In addition to mRNA expression, also the expression and regulation of **microRNAs (miRNAs)** and miRNA-regulated pathways in endometriosis

have recently been evaluated by microarray experiments (Burney *et al*, 2009; Ohlsson Teague *et al*, 2009; Pan *et al*, 2007; Toloubeydokhti *et al*, 2008). Several studies have evaluated the disease mechanisms in the **animal models** of endometriosis (Hull *et al*, 2008; Konno *et al*, 2007; Nap *et al*, 2008; Umezawa *et al*, 2009) and their comparability to the human disease (Flores *et al*, 2007; Pelch *et al*, 2009). An increasing number of reports concerning the differential expression of a single gene or a group of genes in endometriosis is initially based on microarray analysis. In addition, diagnostic **markers** (Eyster *et al*, 2002; Flores *et al*, 2006; Sherwin *et al*, 2008) and molecular signature for hormonal treatments (Berrodin *et al*, 2009; Nap *et al*, 2008) have been searched by genome-wide expression analysis. Finally, the endometriosis-related pathways have been identified by a cross-study gene enrichment analysis exploiting the published whole-genome expression analysis of endometriosis (Zhao *et al,* 2009).

Expression profiling has not been performed comparably in peritoneal, deep and ovarian endometriosis, even though the disease types vary in their clinical outcome, macroscopical appearance, and possibly in the etiology of the lesion types as well. The microarrays have mainly been exploited in the evaluation of aberrantly expressed genes and pathways in endometriosis and in the endometrium of patients with endometriosis. The differentially expressed genes have been suggested to be involved in pathogenesis, as biomarkers or as drug targets for the disease. However, attempts to generate a diagnostic array for endometriosis have not been successful (Sherwin *et al.* 2008) while the data observed suggests that endometrial transcriptome at late secretory phase is not likely to form the basis of a minimally invasive diagnostic test for endometriosis. However, such a diagnostic tool would be of importance in the prediction of infertility and the recurrence of the disease.

3. AIMS OF THE PRESENT STUDY

Endometriosis is a multifactorial disease of which growth is dependent on estrogen action. The disease cannot be definitely cured with the currently available therapies while it often recurs. The present medical therapies are often not sufficiently effective to treat endometriosis-associated pain, and typically do not improve fertility. Similarly, there are no reliable non-invasive methods for the diagnosis of endometriosis. Different types of endometriosis, *i.e.* peritoneal, deep and ovarian lesions, may differ in origin, associated symptoms, and response to treatment. The current classification of endometriosis is based on the appearance and / or location of the lesions. However, the expression profiling-based classification has been shown to be useful for prognosis of various cancers and for the development of individually tailored cancer treatments. The present study is part of our long-term objective to discover novel tools for classification, diagnosis and treatment of endometriosis. In the present study, this aim was approached by exploiting genome-wide expression analysis as a tool to evaluate classification, hormone action and biomarkers of endometriosis.

The specific aims of the study were:

1) To study how different types of endometriosis are classified based on their transcriptomic profiles, and to correlate the classification with the corresponding clinical data.

2) To study the sex steroid action in endometrium and endometriosis with special reference to androgen receptor regulated gene networks and sex steroid metabolizing enzymes.

3) To evaluate the usefulness of a new serum biomarker, HE4, in differentiating ovarian endometriosis from ovarian cancer.

4. MATERIALS AND METHODS

Detailed description of materials and methods used in this study are found in the original publications I-IV.

4.1 STUDY SUBJECTS (I, III, IV)

The patients with endometriosis, endometrial cancer or ovarian cancer were enrolled into the study in Turku University Central Hospital, Helsinki University Central Hospital, Päijät-Häme Central Hospital, and North Carelian Central Hospital in Finland between October 2005 and November 2007. A written informed consent was required from all study subjects prior to sampling. The study protocol was approved by the Joint Ethics Committee of Turku University and Turku University Central Hospital, Turku, Finland (II-IV) or The Local Research and Ethics Committee at Hammersmith Hospitals NHS Trust (I).

4.1.1 Healthy controls (I, III, IV)

Control subjects (n=66 for biomarker analysis and 54 for microarray analysis) were verified to be free from endometriosis or ovarian cancer by laparoscopy during the tubal sterilization, and the possibility of endometrial cancer was excluded by endometrial biopsy. The mean age of control women was 38.5 years. The phase of the menstrual cycle of each patient was determined by endometrial histology at the day of sampling.

4.1.2 Endometriosis patients (I, III, IV)

A total of 137 patients with endometriosis was diagnosed per operatively in laparoscopy or laparotomy and confirmed by histopathological evaluation. The patients with endometriosis were classified to stage I-IV according to the Revised American Fertility Society (AFS) classification of endometriosis (1985) / revised American Society for Reproductive Medicine (ASRM) criteria (1997; Schenken & Guzick, 1997): 18 patients (13.2%) were stage I, 18 (13.2%) stage II, 34 (25.0%) stage III, and 65 (47.8%) stage IV. For the biomarker analysis, the serum samples of patients with ovarian endometrioma (OvEndo, n=69, ASRM stage 3-4) were evaluated as a separate group in the analysis. The mean age of the patients with endometriosis was 31.8, respectively. The phase of the menstrual cycle of each patient was determined by endometrial histology at the day of sampling.

4.1.3 Patients with endometrial or ovarian cancer (IV)

The serum samples of women diagnosed for ovarian cancer (OvCa, n=14), or endometrial cancer (EmCa, n=16) were included in the study. The diseases were diagnosed per operatively in laparoscopy or laparotomy and confirmed by histopathological evaluation. The patients with ovarian and endometrial cancer were staged according to the FIGO guidelines (Benedet *et al,* 2000). The 14 ovarian carcinomas included seven serous, three mucinous, two clear cell, one endometrioid and one small cell carcinomas. Four of the

ovarian cancers were local stage I cancers and the remaining 10 were advanced stage II-IV. All endometrial carcinomas were endometrioid adenocarcinomas. In 14 patients the cancer was limited to the uterus (stage I-II), while in 2 cases metastatic pelvic lymph nodes were present (stage III). Control subjects (n=66) were verified to be free from endometriosis or ovarian cancer by laparoscopy during the tubal sterilization, and the possibility of endometrial cancer was excluded by endometrial biopsy. The mean age of patients with ovarian cancer and endometrial cancer, were 63.8 and 60.5 years, respectively.

4.2 RNA PURIFICATION (I-IV)

The total RNA was isolated from tissue specimens with Trizol-reagent (Invitrogen, Carlsbad, CA, USA), further purified with RNeasy columns (Qiagen, Valencia, CA, USA) and DNase treated (RNase-free DNase Set, Qiagen, Valencia, CA, USA or DNase I, Invitrogen, Carlsbad, CA, USA). The RNA concentrations where measured using Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer and RNA quality was controlled by Experion analysis (Bio-Rad Laboratories, Hercules, CA, USA). From cultured HESCs, the total RNA was isolated using Stat-60 (Tel- Test, Friendswood, TX). Genomic DNA was removed by DNase treatment and the quality of the RNA was evaluated using a Bioanalyser 2100 (Agilent Technologies Inc., Santa Clara, CA).

4.3 MICROARRAY ANALYSIS (I-IV)

The microarray analysis was performed for a total of 335 tissue samples *(Table 2)*.

Table 2: *Samples in collection and microarray analysis*

Sample type	Collection	In microarray analysis (N)		
	(N)	All	Proliferative	Secretory
Eutopic endometrium	200	105	32	35
Control	62	41	16	14
Patient	138	64	16	21
Unaffected peritoneum	188	52	8	12
Control	53	24	3	6
Patient	135	28	5	6
Peritoneal endometriosis	152	72	10	18
Red	50	26	2	6
Black	63	25	6	5
White	39	21	2	7
Ovarian endometrioma	87	28	10	9
Deep endometriosis	131	78	10	16
RVE	30	22	2	4
Sacro	41	23	2	6
Intestine	52	30	5	7
Bladder	8	3	2	0

All steps of the microarray analysis were carried out at the Finnish DNA-Microarray Centre utilizing the Sentrix® Human Illumina 6 V1 (I) or V2 (II-IV) Expression BeadChips (Illumina, San Diego, CA, USA), which contain over 47 000 known genes, gene candidates

and splice variants. Three hundred ng RNA of each sample was used as a template to produce double-stranded cDNA and, further, biotinylated cRNA using the Illumina RNA TotalPrep Amplification Kit (Ambion Inc., Austin, TX, USA). The labeled cRNA was purified and hybridized to the BeadChip at 55°C, for 16 hours following the Illumina Whole-Genome Gene Expression Protocol for BeadStation. Hybridization was detected with Cyanine3-streptavidine (GE Healthcare, Little Chalfont, UK) and the arrays were scanned with the Illumina BeadArray Reader. Normalization and analyses of the microarray data were performed using the statistical software R package limma (http://www.R-project.org).

4.4 CLASSIFICATION OF ENDOMETRIOSIS SPECIMENS BY GENE EXPRESSION PROFILES

To generate the endometriosis classification based on the genome-wide expression profiles, the microarray data of 335 specimens of endometriosis, endometrium and unaffected peritoneum *(Table 2)* were analyzed using three different clustering methods: 1) the conventional hierarchical clustering with Euclidean distance and complete linkage (Hovatta *et al*, 2005) performed by M.Sc. Jouni Junnila (University of Turku), 2) a novel ReScore procedure (I) performed by M.Sc. Jukka Hiissa (University of Turku), and 3) Bayesian clustering (Marttinen *et al*, 2009) performed by Prof. Jukka Corander (Åbo Akademi University) with some modification of the prior distributions to reflect the characteristics of microarray data. As an exception, the ovarian endometriomas were excluded from the ReScore method.

In general, clustering means the grouping of the samples into subsets or "clusters", so that the samples within each cluster are more similar to one another than those assigned to different clusters. Essential to all of the cluster analyses is the concept of the degree of similarity (or dissimilarity) between the individual objects (samples) being clustered. The raw intensity data from Illumina Human 6 V2 array analysis were extracted using the Illumina BeadStudio Gene Expression Module, then quantile-normalised, and finally log-transformed for the analysis. Hierarchical and ReScore clusterings were visualized by a two-dimensional diagram known as dendrogram, which illustrates the fusions or divisions made at analysis.

4.5 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (II, III)

The microarray results were re-evaluated by quantitative reverse transcriptase PCR (qRT-PCR). The analysis was performed using QuantiTect SYBR Green RT-PCR Kit (QIAGEN) (II-IV) or Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) followed by SYBR Green Master Mix (Applied Biosystems, Foster City, CA)(I) according to the manufacturer's instructions. All measurements were performed in triplicate. The data was normalized by ribosomal protein L19 (RPL19). The primers used for qRT-PCR analysis are presented in *Table 3*.

Table 3: *Primers used in qRT-PCR analysis*

Gene	Sense	Antisense
AKR1C3	5'-GCCAGGTGAGGAACTTTCAC-3'	5'-CAATTTACTCCGGTTGAAATACG-3'
AR	5'-CGACTTCACCGCACCTGAT-3'	5'-CCCATTTCGCTTTTGACACA-3'
CYP19A1	5'-TGGCTACCCAGTGAAAAAGG-3'	5'-CCATGGCGATGTACTTTCCT-3'
DUSP3	5'-AGGAGTTCAACCTCAGCGCTTA-3'	5'-AGCACCCGGCCATTCTTT-3'
ESR1	5'-TGGAGATCTTCGACATGCTG-3'	5'-GCCATCAGGTGGATCAAAGT-3'
HSD17B1	5'-GAAGTGTTCGGCGACGTT-3'	5'-AGACCCAGGGGACAAAGAAG-3'
HSD17B1 2.3	5'-CAACGCCTTTACTTTCACAGC-3'	5'-ACAACAAACTGTCCTGGTTGC-3'
HSD17B2	5'-AACTGATGGGGAGCTTCTTCTTAT-3'	5'-CCTCCTCCCATGCTGCTGACA-3'
HSD17B4	5'-TCTCTCTCTTTCTTGTTGGC-3'	5'-TCAAAACCTGCTAGACTAGC-3'
HSD17B6	5'-CTCCAGCATTCTGGGAAGAG-3'	5'-AATATGCTTGGGGGCTTCTT-3'
HSD17B7	5'-GCTGACCCAGGGTGATAAGA-3'	5'-CTTGCACTGCGAGATGATGT-3'
HSD17B12	5'-CCTGTCCCACTCTTGACCAT-3'	5'-AAAGTTGGCTTCCGGATTTT-3'
IGFBP-1	5'-CGAAAACTCTCCATGTCACCA-3'	5'-TGTCTCCTGTGCCTTGGCTAAAC-3'
IL1R1	5'-AATGTCACCGGCCAGTTGAG-3'	5'-TTCCCCTAGCAGTGGGTCATC-3'
KCNK3	5'-CGCACTGGAGGTTCAAGCTAA-3'	5'-GTGTCTGGAAGGCTGAAGTCCTA-3'
MMP10	5'-ATGTACCCACTCTACAACTCATTCACA-3'	5'-AGACTGAATGCCATTCACATCATC-3'
OIP5	5'-GGCTGCCTTGAGAGGTCACTT-3'	5'-GCATTTACTATGGCTTTTGTTTTTAAGA-3'
PR	5'-TTAATACAATTC CTTTGGAAGGGC-3'	5'-CCTTGATGAGCTCTCTAATGTAGCTTG-3'
PCDH7	5'-AGAAACACCAAGCAGTAAGAGTTCATC-3'	5'-GGCCTGGTCCTTTCATAGTTGT-3'
PRL	5'-AAGCTGTCGAGATTGAGGAGCAAAC-3'	5'-TCAGCATGAACCTGGCTGACTA-3'
RASD1	5'-GACACGTCCGGCAACCA-3'	5'-TTGTCCAGACTGAACACCAGGAT-3'
RPL19 (I)	5'-GCAGCCGGCGCAAA-3'	5'-GCGGAAGGGTACAGCCAAT-3'
RPL19 (II)	5'-AGGCACATGGGCATAGGTAA-3'	5'-CCATGAGAATCCGCTTGTTT-3'
TWIST1	5'-GGCCAGGTACTACATCGACTTCCT-3'	5'-TCCATCCTCCAGACCGAGAA-3'
WNT4	5'-GGAACAAGCAGATACCAGGTCAA-3'	5'-TATCGAACCTCTAGCTGTCCATGTAA-3'

4.6 PRIMARY CULTURED ENDOMETRIAL CELLS (II)

Human endometrial stromal cells (HESC) from normal proliferative endometrial tissues were isolated from women with normal menstrual cycles by endometrial biopsy at the time of diagnostic laparoscopy. The primary culture was established as described in (I). Cultures were decidualized with 0.5 mM 8-Br-cAMP (Sigma, St Louis, MO) and MPA (medroxyprogesterone acetate; Sigma), P4 (Sigma), DHT (dihydrotestosterone; Sigma) or bicalutamide (Casodex; AstraZeneca, London, UK), all at 1 μM except DHT, which was used at 0.1 μM concentration unless stated otherwise.

Primary HESCs were transfected with DNA vectors or siRNA by the calcium phosphate coprecipitation method using the Profection mammalian transfection kit (Promega, Madison, WI). The expression plasmids for AR, PR-B, PIAS1, PIAS1(C351S, W372A), and EGFPSUMO1 and the reporter constructs dPRL3000/Luc and PRE2/-32dPRL/Luc were used in the concentration of 100 ng/well and 400 ng/well, respectively (Brosens *et al*, 1999; Jones *et al*, 2006). The control vector pCH110 (50 ng/well), was used to compare transfection efficiency. For gene silencing studies, HESCs were transiently transfected with 100 nM of the following siRNA reagents (Dharmacon, Lafayette, CO): si*CONTROL* Non-targeting (NT) siRNA Pool, AR si*GENOME* SMARTpool siRNA, PR si*GENOME* SMARTpool siRNA, and PIAS1 si*GENOME* SMARTpool siRNA.

Immunofluorescence analysis was performed as described in (I). Cell motility was assessed by time-lapse microscopy using an inverted microscope with a motorized stage. Images were captured every 15 minutes over a 48 hour period, and the distance each

cell moved was analyzed in triplicate experiments. Proliferation was ascertained using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega).

4.7 WESTERN BLOT ANALYSIS (II, III)

Tissue specimens and whole cell protein extract of HESCs used for whole cell protein extraction as described in II and I, respectively. Nuclear extracts were obtained using the modified method of Rittenhouse and Marcus (Rittenhouse & Marcus, 1984). Hundred (II) or 30 (I) micrograms of total protein was separated on a 10-12% SDS-polyacrylamide gel, electro transferred (15V for 30 min) onto nitrocellulose membrane (Amersham, Little Chalfont, UK) and the specific protein was detected using primary antibodies shown in *Table 4*. Secondary antibodies were used to bind primary antibody at 1:1000-5000 dilution and protein complexes were visualized with a chemoluminescent detection kit (Amersham).

Table 4: *Antibodies used for Western blot and immunohistochemical analysis*

Antibody	Source	Species raised in	Clonality	Dilution (WB)	Dilution (IHC)
HSD17B1	Solvay Pharmaceuticals	rabbit	polyclonal	1:1000	1:1000
HSD17B2	PTGlab	rabbit	polyclonal	1:1000	5.73 µg/ml
HSD17B4	Prof. K. Hiltunen	rabbit	polyclonal	1:1000	1:1000
HSD17B6	PTGlab	rabbit	polyclonal	1:200	4.67 µg/ml
HSD17B12	AbNova	mouse	polyclonal	1:500	1:200
AR	Biogenix		monoclonal	1:1000	
PR	Novocastra Laboratories	mouse	monoclonal	1:1000	
AKT	Cell Signaling	rabbit	polyclonal	1:1000	
Phosphorylated (Ser_{473}) AKT	Cell Signaling	rabbit	polyclonal	1:1000	
ERK1/2	Cell Signaling	rabbit	polyclonal	1:1000	
Phosphorylated ($Thr_{202/204}$) ERK1/2	Cell Signaling	rabbit	polyclonal	1:1000	
-catenin	Santa Cruz Biotechnology	mouse	monoclonal	1:100 000	
phosphorylated ($Ser_{807/811}$) pRB	Cell Signaling	rabbit	polyclonal	1:1000	
phosphorylated MLC (Ser_{19})	Cell Signaling	rabbit	polyclonal	1:1000	
IL1R1	Abcam	rabbit	polyclonal	1:1000	
STAT3	Upstate Biotechnology	rabbit	polyclonal	1:1000	
STAT5b	Upstate Biotechnology	rabbit	polyclonal	1:1000	

4.8 PRL AND IGFBP- 1 ASSAYS (II)

PRL in the HESC culture media was measured by microparticle enzyme immunoassay (AxSYM system, Abbott Laboratories, North Chicago, IL). IGFBP-1 levels in culture media were determined using an amplified “two-step” sandwich-type immunoassay (R&D Systems, Minneapolis, MN).

4.9 HISTOLOGICAL ANALYSIS AND IMMUNOHISTOCHEMISTRY (III)

For histological analysis, tissue specimens were fixed in 10% formalin, dehydrated and embedded in paraffin. Five µm sections were stained with hematoxylin and eosin (H&E) or used in immunohistochemical analysis. Histological evaluation of a single

endometrial biopsy was used to determine the hormonal status (Mazur & Kurman, 2005) of each patient with endometriosis and healthy controls. The samples were classified as menstrual, proliferative, secretory, atrophic, or inactive. The presence of endometrial glands and stroma in endometriosis specimens were determined to verify the disease.

Immunohistochemical staining was performed using the antibodies described in (III) and in *Table 4*. The staining was scored visually as strong (3), moderate (2), weak (1) or no staining (0) in glandular epithelial cells.

4.10 SERUM HORMONE AND BIOMARKER ANALYSIS (III, IV)

Serum samples were collected just prior to surgery and stored at -20ºC or -80ºC. The concentrations of E2, P4, sex hormone-binding globulin (SHBG), cortisol, LH, and FSH were analyzed by Delfia fluoroimmunoassays (Perkin Elmer, Waltham, MA, USA) following the manufacturer's instructions, and a radioimmunoassay was used for T measurement (Huhtaniemi *et al*, 1985). Human epididymal secretory protein E4 (HE4) and CA125 concentrations were analysed in serum samples by ELISA analysis (Fujirebio Diagnostics Inc, Malvern, PA) according to the manufacturer's instructions.

4.11 STATISTICAL ANALYSIS (I-IV)

The normalized microarray data of siRNA treated HESCs was analyzed by pair-wise comparisons in order to create a list of differentially expressed genes *(II, Tables 1-4)*. Differentially expressed genes were defined by a lower boundary of a 99% confidence interval of fold change greater than 1.2 as validated by Student's t-test ($P < 0.01$). To interpret the biological significance of differentially expressed genes, a gene ontology analysis was conducted using Ingenuity Pathways Analysis (IPA, Ingenuity Systems).

To compare mRNA expressions of HSD17B1-14 (III), either a one-way or a two-way ANOVA with Bonferroni adjusted t-test was used as described in III. Multiple Linear Regression Analysis and Pearson Product Moment Correlations were examined between serum hormone concentrations and gene expression in microarray analysis. The genes whose expression associated with the expression of HSD17B2 and HSD17B6 were evaluated using Pearson Product Moment Correlations. Statistical analyses were performed using the statistical software R package limma (http://www.R-project.org) or Sigma Stat 3.11 (Systat Software Inc., Chicago, IL, USA).

The statistical analyses of serum HE4 and CA125 concentrations (IV) alone and in combination were performed using Tukey's multiple comparisons of means with 95% family-wise confidence level. The classification capability of the HE4 and CA125 markers, alone and together, was assessed and the sensitivity at 95% specificity and accuracy was calculated. The receiver operator characteristic (ROC) curves were constructed and the area under the curve (AUC) was used to summarize the overall performance of the regression model.

5. RESULTS

5.1 CLASSIFICATION OF ENDOMETRIOSIS SPECIMENS BY GENE EXPRESSION PROFILES

The 335 tissue specimens (*Table 2*) were clustered based on their whole genome expression using three methods: the conventional hierarchical clustering with Euclidean distance and complete linkage, a novel ReScore procedure, and Bayesian clustering. The purpose of this study was to analyze the biological relevance of the data obtained by the various clustering methods and, therefore, the mathematical aspects are not presented. The results of hierarchical and Bayesian clustering are not included in original publications. The clustering of endometriosis samples by ReScore method is a part of the original paper I, while the other sample sets are outside the scope of this study.

5.1.1 Hierarchical clustering

The conventional hierarchical clustering resulted in the dendogram shown in *Figure 5*. The distribution of sample types in the dendogram, including different types of endometriosis as well as eutopic endometrium and unaffected peritoneum of endometriosis patients and healthy controls, are shown below the dendogram.

The samples were grouped to three main clusters. The leftmost cluster included unaffected peritoneum samples of patients and control subjects, as well as deep and peritoneal endometriosis specimens of different subtypes. Therefore, the cluster was defined as 'peritoneal-like endometriosis'. Interestingly, the unaffected peritoneum samples of patients and controls were partially separated, suggesting that these sample types might have differential gene expression profiles. The middle cluster included part of the peritoneal and deep endometriosis lesions. The peritoneal lesions were grouped together with the few macroscopically normal patient peritoneum samples in the cluster. The ovarian endometriotic cysts were strictly grouped together, suggesting differential expression profiles in comparison to other types of samples. Similarly, most of the deep endometriotic lesions were clustered together. The third cluster (on the right) contained the eutopic endometrial samples with some endometriosis specimens. The endometrium specimens of patients and controls were not separated from another in the genome-wide expression profiles. However, combining the clustering data with the clinical and endometrial histology data resulted in defining of subclusters according to the phase of menstrual cycle or hormonal medication.

5.1.2 ReScore method (I)

The novel ReScore procedure was developed for identification of hidden subgroups in the endometriosis microarray dataset and to identify the correlation of subgroups with molecularly, histologically or pathologically defined sample subsets. The resulting dendogram *(Fig. 6)* shows that given control and disease groups differentiated into two

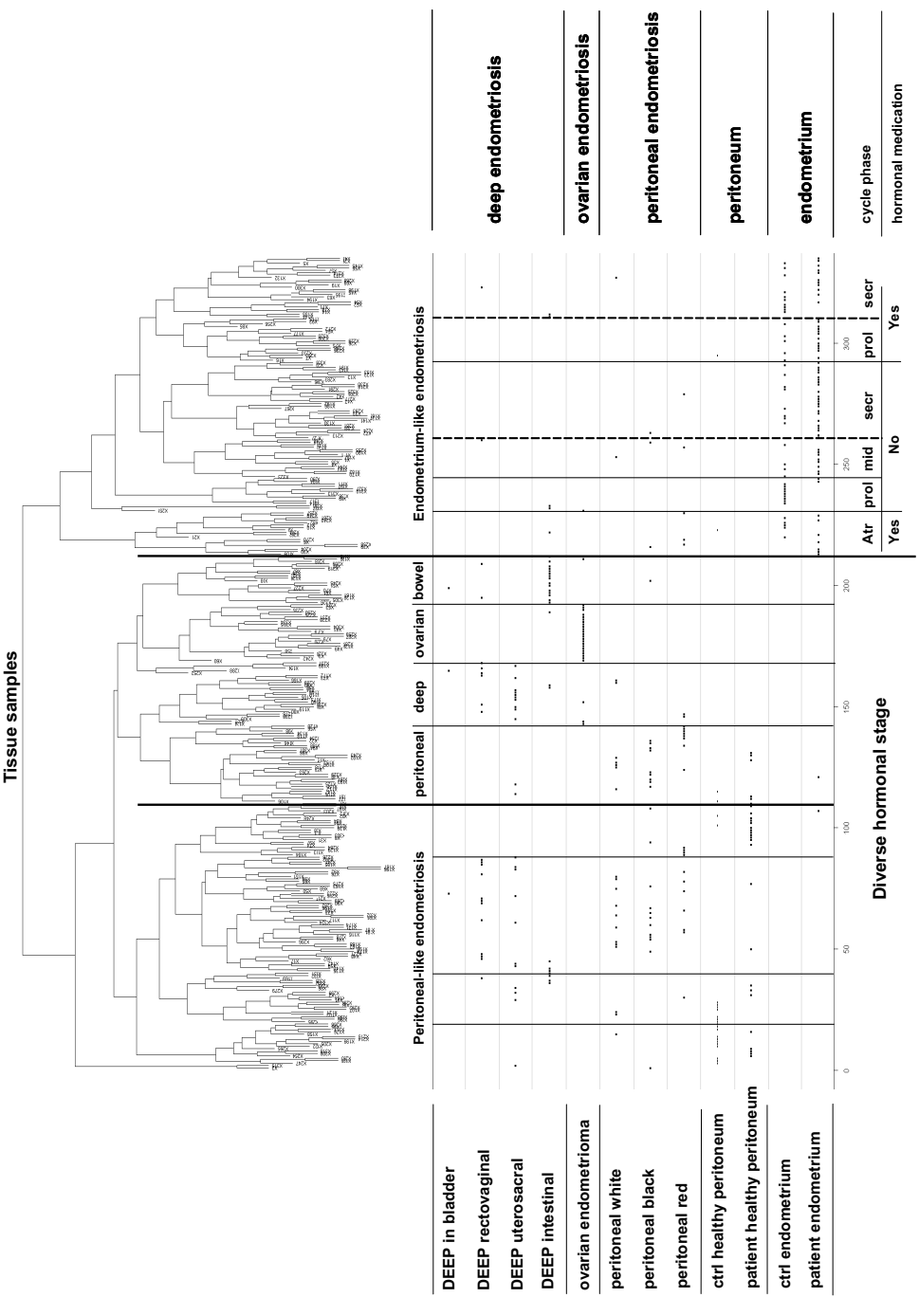

Figure 5: *Hierarchical clustering of the samples according to genome-wide gene expression analysis. (Jouni Junnila, 2007)*

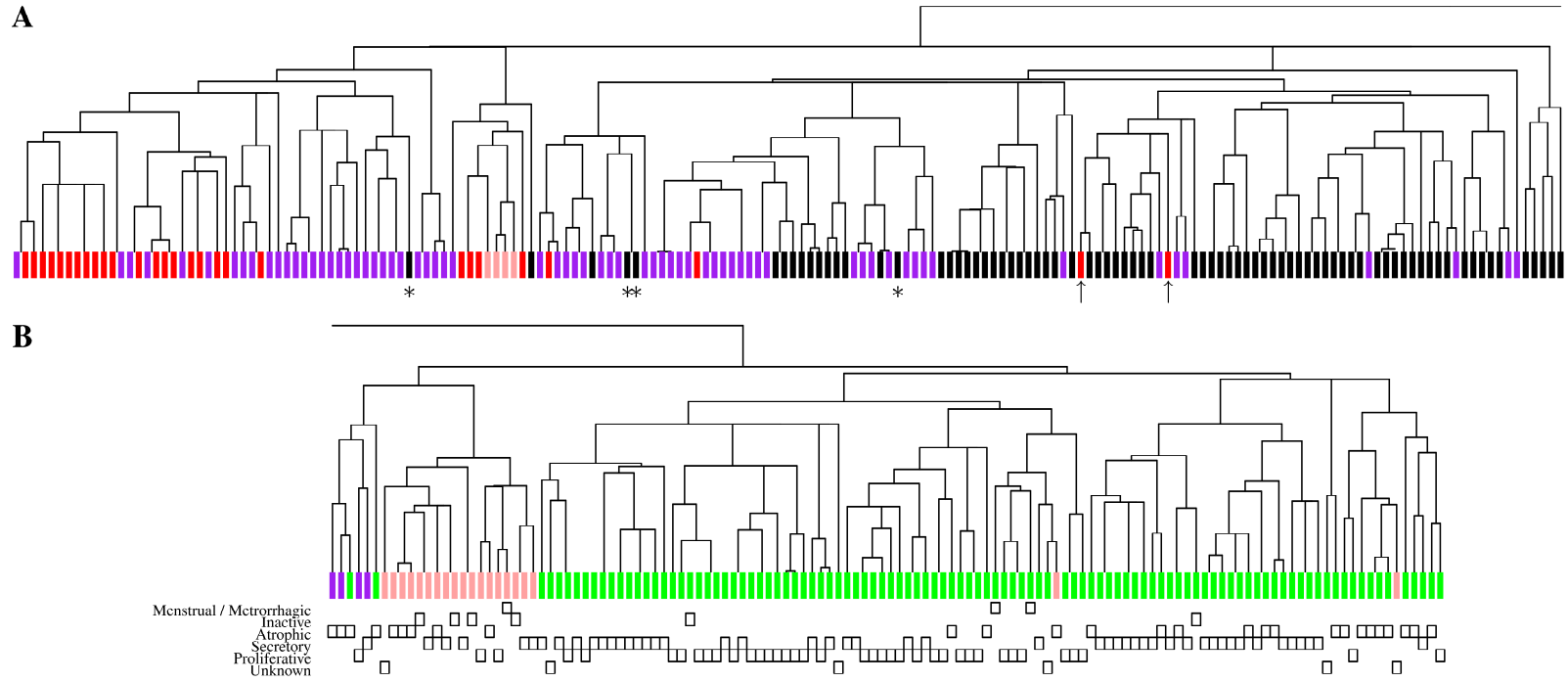

Figure 6: *Grouping of the endometriosis samples. The clustering result was split into two parts displayed on top of each other. (A) The patient samples were identified as a separate cluster, where colours indicate the origin of the sample material (red, patient peritoneum samples; magenta, peritoneal endometriotic lesions; black, deep endometriotic lesions). The asterisks below the samples indicate the four mislabelled cases discovered. The arrows indicate two interesting findings discussed in the text. (B) The grouping of the control samples was nearly perfect (pink, control peritoneum samples; green, endometrium). The boxes below the samples indicate the phase of the menstrual cycle. According to Hiissa et al. OMICS J Integrat Biol 2009*

main clusters, as expected. Similarly to that observed in the hierarchical clustering, the ovarian endometriotic cysts highly separated as one group. To increase the sensitivity to identify the hidden subgroups, ovarian endometriosis was excluded from the final analysis. As a result, the ReScore procedure was more successful to identify the endometriosis subtypes in the heterogeneous endometriosis data set than the conventional hierarchical clustering. The method well distinguished the subgroups of patient peritoneum, peritoneal endometriosis and deep endometriosis with high accuracy.

The ReScore method also revealed several important practical and clinical findings. For example, four of the deep endometriosis samples were found to be mislabelled on the basis of their grouping and a re-check of the surgical report confirmed that these samples were in fact superficial peritoneal lesions. Similarly to that observed by hierarchical clustering, the grouping of the endometrium samples associated with the endometrial histology corresponding to the different phases of the menstrual cycle and hormonal medication. Interestingly, two of the patient peritoneum samples clustered together with the deep endometriotic lesions. These samples appeared to be from patients with stage 3-4 disease with divergent disease condition. It remains to be determined whether these peritoneal samples included macroscopically invisible endometriosis, or if the peritoneums of these patients were remarkably changed, and whether that change would be associated with rapid progression of the disease.

5.1.3 Bayesian clustering

The Bayesian clustering of the endometriosis dataset (n = 335) resulted in 36 clusters of tissue samples. Also the subsets of genes being cluster specific, or informative for the clustering, were identified. Using a theoretically suggested threshold for a critical evidence for a gene being informative (log Bayes Factor >10), appr. 20,000 genes among the 48701 observed genes were considered informative. However, due to the small sizes of some clusters, other methods had to be used in conjuction with the Bayes Factors to pinpoint the genes that were considered to have a characteristic behavior in any given cluster.

Figure 7 presents an example of the visualization of the Bayesian clustering including the genes involved in steroid action (*i.e.* nuclear receptors, their co-regulators, metabolic enzymes). The data is binarized according to the normalized mean of the whole data for the particular gene: the expression of each gene is either above (white color) or below (black colour) the normalized mean in each sample. Thus, the figure can be used to identify differential expression between the clusters rather than actual biological up- or down-regulation. Basically, the Bayesian sample clusters *(Table 5)* showed a similar pattern to the other two methods: The endometrium samples grouped according to their hormonal status: phase of the menstrual cycle, evaluated by endometrial histology, and the possible hormonal medication (clusters # 1, 9, 10, 21, 29, 32 and 35). Ovarian and intestinal endometriosis mainly clustered as separate groups (clusters #23 and #27, respectively). Finally, peritoneal endometriosis clustered either with the unaffected peritoneums (clusters #12, #14, #30, #31) or with the deep lesions especially from uterosacral ligaments and rectovaginal septum (clusters #17 and #28).

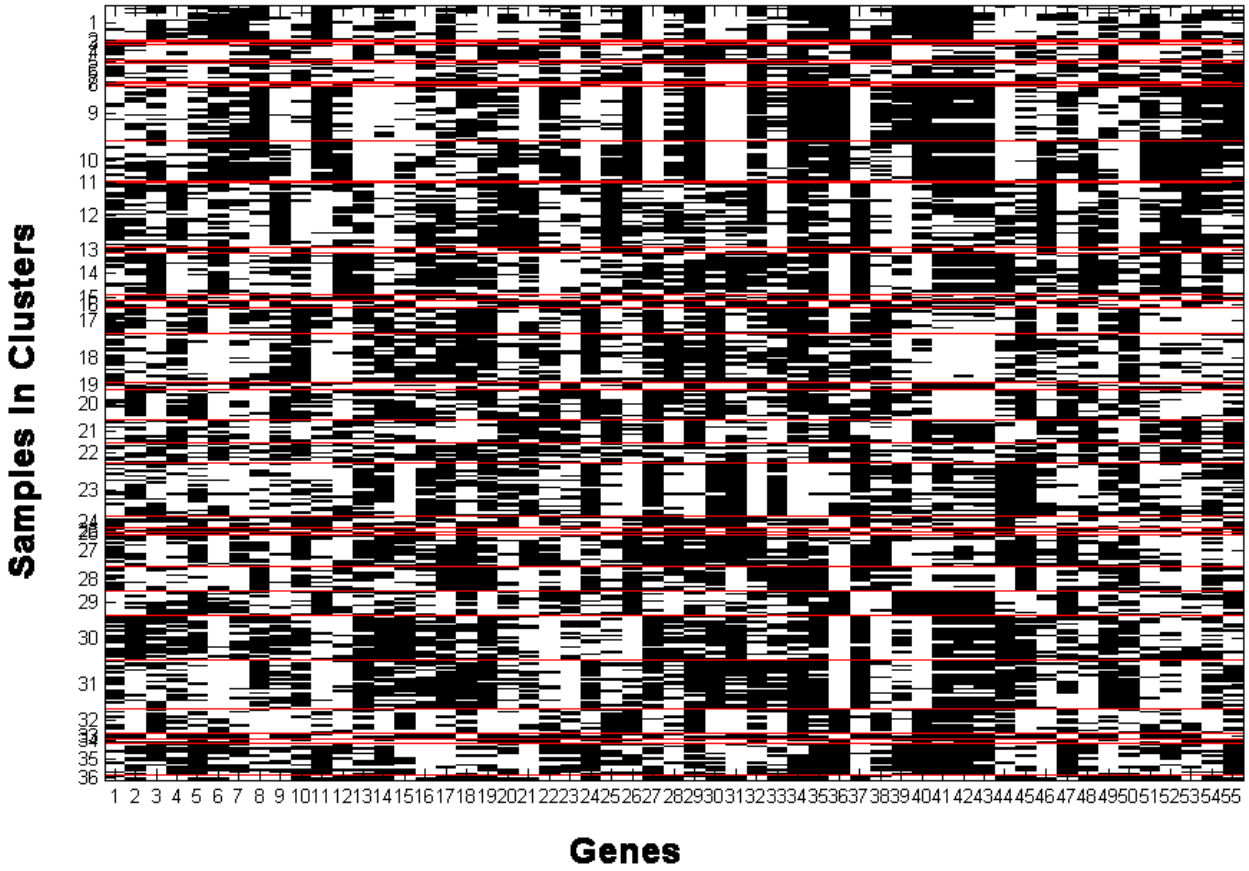

Figure 7: *Visualization of tissue sample clusters identified by Bayesian clustering. Each sample is a horizontal segment (row) and each gene is a vertical segment (column) in the image. White color indicates an expression value that is above the normalized mean of the whole data for the particular gene. Black color indicates an expression value that is below the normalized mean of the whole data for the particular gene. (Prof. Jukka Corander, 2008)*

Table 5: *Bayesian clusters with a minimum of 10 samples*

Cluster	Samples	N	% of the samples in the cluster	Hormonal status	N	%	Sample characteristics in the cluster
#10	total	17		Prolif	17	100.0 %	**Endometrium of healthy controls, Proliferative**
	CE	11	64.7 %	Secr	0	0.0 %	
	PE	4	23.5 %	Atrophic / Inactive	0	0.0 %	
	Other (endo)	2	11.8 %	Hormonal medication	1	5.9 %	
#32	total	11		Prolif	9	81.8 %	**Endometrium of endometriosis patients, proliferative, low PGR**
	PE	9	81.8 %	Secr	0	0.0 %	
	CE	1	9.1 %	Atrophic / Inactive	1	9.1 %	
	DEEP	1	9.1 %	Hormonal medication	1	9.1 %	
#9	total	24		Prolif	14	58.3 %	**Endometrium, Mid phase**
	PE	15	62.5 %	Secr	6	25.0 %	
	CE	7	29.2 %	Atrophic / Inactive	4	16.7 %	
	PeEndo	2	8.3 %	Hormonal medication	3	12.5 %	
#1	total	15		Prolif	3	20.0 %	**Endometrium, Secretory**
	PE	10	66.7 %	Secr	12	80.0 %	
	CE	4	26.7 %	Atrophic / Inactive			
	PeEndo	1	6.7 %	Hormonal medication	3	20.0 %	
#29	total	11		Prolif	0	0.0 %	**Endometrium, secretory, lower ESR**
	PE	6	54.5 %	Secr	8	72.7 %	
	CE	5	45.5 %	Atrophic / Inactive	3	27.3 %	
				Hormonal medication	3	27.3 %	
#35	total	14		Prolif	1	7.1 %	**Endometrium, secretory / atrophic**
	PE	5	35.7 %	Secr	6	42.9 %	
	CE	5	35.7 %	Atrophic / Inactive	5	35.7 %	
	PeEndo	2	14.3 %	Hormonal medication	4	28.6 %	
	CP	2	14.3 %				
#21	total	10		Prolif	2	20.0 %	**Endometrium, atrophic with medication**
	PE	5	50.0 %	Secr	0	0.0 %	
	CE	2	20.0 %	Atrophic / Inactive	8	80.0 %	
	PeEndo	3	30.0 %	Hormonal medication	7	70.0 %	
#30	total	19		Prolif	1	5.3 %	**Peritoneum (mainly control peritoneums)**
	CP	10	52.6 %	Secr	6	31.6 %	
	PP	4	21.1 %	Atrophic / Inactive	11	57.9 %	
	DEEP	3	15.8 %	Hormonal medication	10	52.6 %	
	PeEndo	2	10.5 %				
#14	total	18		Prolif	3	16.7 %	**Peritoneum (mainly patient peritoneums)**
	PP	8	44.4 %	Secr	7	38.9 %	
	CP	4	22.2 %	Atrophic / Inactive	7	38.9 %	
	PeEndo	4	22.2 %	Hormonal medication	4	22.2 %	
	DEEP	3	16.7 %				
#31	total	21		Prolif	5	23.8 %	**Peritoneum + endo, mixed**
	PP	8	38.1 %	Secr	3	14.3 %	
	CP	6	28.6 %	Atrophic / Inactive	10	47.6 %	
	PeEndo	6	28.6 %	Hormonal medication	11	52.4 %	
	DEEP	1	4.8 %				
#12	total	28		Prolif	9	32.1 %	**Peritoneal endometriosis, mixed hormonal status**
	PeEndo	18	64.3 %	Secr	8	28.6 %	
	PP	5	17.9 %	Atrophic / Inactive	6	21.4 %	
	CP	2	7.1 %	Hormonal medication	14	50.0 %	
	other	3	10.7 %				
#28	total	10		Prolif	2	20.0 %	**Peritoneal endometriosis, ESR and PGR high**
	PeEndo	8	80.0 %	Secr	5	50.0 %	
	DEEP	2	20.0 %	Atrophic / Inactive	3	30.0 %	
				Hormonal medication	3	30.0 %	
#17	total	11		Prolif	1	9.1 %	**DEEP endometriosis: RVE (N=6)+Sacro (N=3), hormonal medication**
	DEEP	9	81.8 %	Secr	4	36.4 %	
	PeEndo	2	18.2 %	Atrophic / Inactive	4	36.4 %	
				Hormonal medication	7	63.6 %	
#18	total	21		Prolif	3	14.3 %	**DEEP endo, mixed hormonal status**
	DEEP	16	76.2 %	Secr	7	33.3 %	
	OvEndo	2	9.5 %	Atrophic / Inactive	8	38.1 %	
	Other	2	9.5 %	Hormonal medication	12	57.1 %	
#20	total	13		Prolif	0	0.0 %	**DEEP endo + medication (corresponding endometrium samples are atrophic)**
	DEEP	11	84.6 %	Secr	3	23.1 %	
	PeEndo	1	7.7 %	Atrophic / Inactive	10	76.9 %	
	PE	1	7.7 %	Hormonal medication	10	76.9 %	
#27	total	14		Prolif	2	14.3 %	**DEEP endometriosis: intestinal 12/14 (85.7%)**
	DEEP	14	100.0 %	Secr	4	28.6 %	
	Intestinal	12	85.7 %	Atrophic / Inactive	6	42.9 %	
				Hormonal medication	7	50.0 %	
#23	total	23		Prolif	9	39.1 %	**Ovarian endometriosis**
	OvEndo	22	95.7 %	Secr	9	39.1 %	
	DEEP	1	4.3 %	Atrophic / Inactive	4	17.4 %	
				Hormonal medication	5	21.7 %	

5.1.4 Comparison of the clustering results

Part of the sample groups clustered similarly independent of the clustering method. For example, 83-96% of ovarian endometriosis were separated as a separate group *(Table 6)* in the three analyses. Similarly, 43-57% of deep intestinal endometriosis were located in the same cluster. The best separation for unaffected peritoneum samples of patients and control subjects was identified by ReScore, which was expected by the preclassification as 'control' and 'disease' samples. In all of the three analyses, endometrium samples clustered by the hormonal status and not according to the classification to patients and controls. With each of the methods, the proliferative and secretory samples were grouped in at least two rather specific clusters. In addition, Bayesian clustering separated the mid-phase endometrial samples including cycle days 9-16.

Table 6: *Comparison of the clustering of the tissue samples by conventional hierarchical clustering, ReScore method, and Bayesian clustering*

Sample characteristics	Hierarchical			ReScore			Bayesian		
	Cluster	Samples in the cluster (N, % of the sample type)		Cluster	Samples in the cluster (N, % of the sample type)		Cluster	Samples in the cluster (N, % of the sample type)	
Endometrium by histology									
Endometrium, Proliferative (N=35)	4.2.1.	10	28.6 %	4.3.	12	34.3 %	#10	14	40.0 %
	4.3.2.	10	28.6 %	4.1.	11	31.4 %	#9	13	37.1 %
	4.3.1.	6	17.1 %	4.4.	7	20.0 %			
	4.3.4.	6	17.1 %						
Endometrium, Secretory (N=37)	4.3.3.	14	37.8 %	4.5.	17	45.9 %	#1	10	27.0 %
	4.3.6.	8	21.6 %	4.2.	10	27.0 %	#32	9	24.3 %
	4.3.1.	6	16.2 %				#9	6	16.2 %
Endometrium, Atrophic (N=17)	3.1.1-3.1.2.	7	41.1 %	4.7.	7	41.1 %	#21	5	29.4 %
	4.3.5.	4	23.5 %	4.5.	5	29.4 %	#35	3	17.6 %
Endometrium by cycle day									
Endometrium, day 2-8 (N=19)	4.3.4.	11	57.9 %	4.4.	11	57.9 %	#10	7	36.8 %
	4.2.1.	6	31.6 %	4.3.	4	21.1 %			
Endometrium, day 9-16 (N=28)	4.3.2.	11	39.3 %	4.1.	13	46.4 %	#9	16	57.1 %
	4.3.1.	8	28.6 %	4.3.	6	21.4 %	#1	5	17.9 %
				4.2.	5	17.9 %			
Endometrium, day 17-31 (N=36)	4.3.3.	13	36.1 %	4.5.	22	61.1 %	#32	10	27.8 %
	4.3.5-4.3.6	16	44.4 %	4.2.	5	13.9 %	#29	8	22.2 %
Endometrium, day >32, medication (N=10)	3.1.1-3.1.2	5	50.0 %	4.7.	5	50.0 %	mixed		
Endometriosis by lesion type									
Ovarian endometriosis (N=23)	2.3.2.	20	87.0 %	2.3.	22	95.7 %	#23	19	82.6 %
DEEP intestinal endometriosis (N=21)	2.3.3.	12	57.1 %	2.1.	9	42.9 %	#27	9	42.9 %
				2.2.	10	47.6 %			
DEEP endometriosis, RVE +Sacro (N=43)	2.1.3.	17	39.5 %	2.2.	23	53.5 %	#18	13	30.2 %
	2.2.2.	12	27.9 %	2.1.	17	39.5 %	#17	8	18.6 %
							#20	6	14.0 %
Peritoneal endometriosis (N=54)	2.2.1.	20	37.0 %	2.1.	25	46.3 %	#12	15	27.8 %
	2.1.3.	19	35.2 %	1.2.	12	22.2 %	#28	7	13.0 %
	2.1.4.	6	11.1 %	1.1.	11	20.4 %	#31	6	11.1 %
Patient peritoneum N=27)	2.1.4.	13	48.1 %	1.1.	21	77.8 %	#31	9	33.3 %
	2.1.1.	5	18.5 %	1.5.	4	14.8 %	#14	8	29.6 %
	2.2.1.	5	18.5 %				#12	5	18.5 %
Control peritoneum (N=24)	2.1.1.	11	45.8 %	5.1.	18	75.0 %	#30	10	41.7 %
	2.1.2.	7	29.2 %	1.5.	4	16.7 %	#31	6	25.0 %

5.2 HORMONAL REGULATION OF ENDOMETRIUM AND ENDOMETRIOSIS

5.2.1 Androgen responses in decidualizing endometrial stromal cells (II)

Dehydrotestosterone (DHT), a non-aromatisable androgen, markedly enhanced PRL but not IGFBP-1 secretion in cultures treated with 8-Br-cAMP plus P4. This androgen response in decidualizing cultures increased in magnitude over time in a dose-dependent manner *(II, Fig. 1B)*. The transcript levels mirrored those at the protein level *(II, Fig. 1C)*. In addition, the pattern of PRL expression in response to cAMP, P4, and DHT corresponded to activation of the decidua-specific PRL promoter region (II, Fig. 1D). The antiandrogen bicalutamide entirely negated the ability of DHT to enhance PRL secretion *(II, Fig. 1E)* suggesting that the effect is mediated by AR. The depletion of AR by targeting siRNA *(II, Fig. 3A)* abolished the ability of DHT to enhance PRL secretion in cells differentiated with 8-Br-cAMP and P4 *(II, Fig. 1F)*. Together, the results unequivocally demonstrate that androgen actions in decidualizing HESCs are dependent upon AR activation. However, the decidualization of HESCs by treatment of primary cultures with 8-Br-cAMP alone resulted in a rapid and sustained decrease in cellular AR levels. DHT strongly increased AR levels in undifferentiated cells *(II, Fig. 2A)*, but only partially antagonized the down-regulation of the receptor in decidualizing cells. Untreated and decidualized cultures were transfected with expression vectors encoding AR and enhanced green fluorescent protein (EGFP)-tagged SUMO-1 and pulsed 24 h later with DHT. Immunoblotting of cell lysates with an anti-AR antibody demonstrated the presence of two slower migrating forms of AR in undifferentiated cells, first apparent after 10 min DHT stimulation. Compared with undifferentiated HESCs, DHT-dependent sumoylation of AR was attenuated in decidualized cells *(II, supplemental Fig. 1, Fig. 2)*. Furthermore, PIAS1 knockdown in undifferentiated HESCs was sufficient to induce PRL expression in response to DHT without the need of additional decidualizing stimuli *(II, Fig. 2E)*.

5.2.2 AR-mediated effects in decidualizing endometrium (II)

The AR target genes in decidualizing HESCs were exploited using siRNAs targeting AR. The knockdown of the receptor caused efficient attenuation of PRL mRNA expression. The whole genome expression analysis of the siRNA treated HESCs resulted in 92 transcripts deregulated by AR depletion when compared to nontargeting siRNA-treated cells *(II: Tables 1 and 2, Fig 3)*. Of them, 39 (42.4%) were induced and 53 (57.6%) repressed. Only 29 genes *(II: Table 5, Fig 3)* were under control of both AR and PR receptors in decidualizing HESCs, although 10 were regulated in an opposing manner. The results were confirmed by qRT-PCR analysis for the genes under the control of AR (IL1R1, DUSP3, and OIP5), or both AR and PR (KCNK3, PCDH7, and WNT4). The gene lists were cross-referenced and annotated with the Endometrium Database Resource (http://endometrium.bcm.tmc.edu/edr/) to indicate genes already reported to be regulated during endometrial differentiation.

AR-regulated genes were mainly involved in cytoskeletal organization and inhibition of cell motility and proliferation. Forty percent (21 of 53) of genes repressed by AR knockdown appeared to be involved in regulation of cell morphology, cytoskeletal organization, and cell motility. Furthermore, fluorescence microscopy demonstrated that a dramatic increase in F-actin polymerization and stress fiber formation takes place during decidualization *(II: Fig 6)*. Also a dramatic decrease in basal cell motility was associated with decidualization. The genes up-regulated by AR depletion in decidualizing HESCs, and thus normally repressed in an AR-dependent manner, are involved in the regulation of cell cycle, including DNA replication licensing, and chromatid separation *(II: Tables 2 and 5)*. Functionally, AR knockdown enhanced the proliferation of HESCs while proliferation was reduced by PR knockdown *(II: Fig. 6)*.

5.2.3 AR-responsive genes in endometriosis

The expression of AR and three selected AR-responsive genes (IL1R1, DUSP3, and OIP5) identified in cultured endometrial stromal cells were analysed also in the endometriosis microarray data. The expression levels of these genes were regulated as expected according to the decidualization data. Small but significant differences were detected in the secretory phase samples as compared to those in proliferative phase with fold changes (FCs) of 2.5, 1.27, and 0.57 for IL1R1 ($p<0.001$), DUSP3 ($p=0.027$), and OIP5 ($p=0.002$). The expression of AR in proliferative and secretory control endometrium was not significantly different ($p=0.063$). In peritoneal, ovarian, and deep endometriosis samples, the cyclical regulation was lost ($p>0.05$) between proliferative and secretory phase. In addition, the expression levels of these genes in proliferative phase endometriosis samples were significantly higher (for IL1R1 and DUSP3) or lower (for OIP5) as compared to healthy endometrium *(Table 7)*.

Table 7: *Expression of AR responsive genes as log2 values in the proliferative and secretory endometrium and endometriosis tissue samples.*

		CE	PE	PeEndo	OV	DEEP	**P-value for**
ENZYME	N prolif	**16**	19	10	10	10	**One-Way**
	N secr	**14**	21	18	9	16	**ANOVA**
AR	prolif	**9.95**	10.13	10.30	10.22	9.73	**0.341**
	secr	**9.43**	9.20	9.97	9.76	9.98	**0.005**
IL1R1	prolif	**8.22**	9.05 *	9.31 *	9.62 *	8.97	**<0.001**
	secr	**9.51**	9.72	9.41	9.84	9.22	**0.184**
DUSP3	prolif	**11.17**	11.36	11.99 *	11.65 *	12.34 *	**<0.001**
	secr	**11.52**	11.61	11.86	11.68	12.55 *	**<0.001**
OIP5	prolif	**7.78**	7.78	6.67 *	6.60 *	6.67 *	**<0.001**
	secr	**6.97**	6.98	6.67	6.76	6.50 *	**0.031**

* P<0.05 in Multiple Comparisons versus CE within the cycle phase (Student's t-test with Bonferroni correction)

5.2.4 PR-mediated gene networks in decidualizing endometrium (II)

The PR target genes in decidualizing HESCs were exploited using siRNAs silencing the translation of PR *(II: Fig. 3)*. The knockdown of the receptor was controlled by efficient attenuation of PRL mRNA expression. The whole genome expression analysis of the PR siRNA treated HESCs disturbed the expression of 860 transcripts *(II: Tables 3 and 4)*, of which 478 (55.6%) were up-regulated and 382 (44.4%) down-regulated. The result was validated by qRT-PCR analysis for the expression of MMP10, TWIST1, RASD1, KCNK3, PCDH7, and WNT4. Several genes down-regulated in a PR-dependent manner encode for matrix metalloproteinases, death receptors of the tumor necrosis factor receptor superfamily, apoptosis mediators, and oxidative stress defenses and DNA repair. A significant number of PR-dependent genes encode for ligands, membrane bound receptors, and intermediates in WNT/β-catenin, TGFβ/SMAD, and STAT signal transduction pathways *(II: supplemental Fig. 4)*.

5.2.5 Expression of estrogen metabolizing HSD17B enzymes in endometrium and endometriosis (III)

The gene expression levels of HSD17B1-14 in endometrium and in various types of endometriotic lesions were studied as part of the whole genome microarray analysis. The most remarkable difference between the control or patient endometrium and different types of endometriosis specimens was observed for HSD17B2 and HSD17B6 *(III: Table 1 and Supplementary Table 2)*.

5.2.5.1 Decreased HSD17B2 expression

The microarray study revealed that HSD17B2 expression was strongly increased in control endometrium (CE) and patient endometrium (PE) during the secretory phase, as there was a markedly higher expression in the secretory endometrium as compared with the proliferative endometrium both within the patients (fold change, FC 6.5, $p<0.05$,) and controls (FC 3.8, $p<0.05$). However, increased expression during secretory phase was not observed in the endometriosis lesions. Thus, the level of HSD17B2 mRNA in peritoneal, ovarian and deep endometriosis was only 10% of that in the PE ($p<0.05$). Furthermore, in the proliferative phase, we did not detect a difference in the HSD17B2 expression between the CE or PE and endometriosis specimens. In endometriosis, the HSD17B2 mRNA level was, thus, similar throughout the menstrual cycle. Similar results were obtained by qRT-PCR measurements *(III, Fig. 1)*. To detect the genes with a similar expression pattern with HSD17B2 and HSD17B6, we evaluated the correlating genes within all 283 samples in the microarray study. The data addressed that Cytochrome P450, family 26, subfamily A, polypeptide 1 (CYP26A1) has a highly similar expression pattern with HSD17B2 (regression 0.808, correlation coefficient $R=0.743$, $p=7.57*10^{-51}$; *III Fig. 2*). Immunohistochemistry revealed that both HSD17B2 and HSD17B6 were localized into the cytoplasm of glandular and luminal epithelium of the endometrium and endometriosis specimens *(III: Fig 2)*. Based on the microarray analysis, the other estradiol oxidizing HSD17Bs 4, 8 and 10 were weakly down-regulated in the eutopic

and ectopic endometrium *(III: Supplementary Fig. 1)*. However, HSD17B14 expression was increased in peritoneal deep and ovarian endometriosis lesions by 1.6-2.0 fold ($p<0.05$) independent from cycle phase. Also the expression of HSD17B11 and 13 were low within the sample groups, and no statistical differences were observed.

5.2.5.2 Estrogen activating HSD17Bs

Only minor differences in mRNA expression for the estrone reducing HSD17B1, 5, 7 and 12 were observed between the eutopic and ectopic endometrium specimens. The mRNA and protein expression of HSD17B1 were not significantly changed between the sample groups *(III: Table 1)*. The HSD17B5 (**AKR1C3**) mRNA expression was significantly increased in the proliferative phase peritoneal (FC 1.8; $p<0.05$) and deep (FC 1.8; $p<0.05$) endometriosis lesions when compared to proliferative eutopic endometrium of women with endometriosis. In secretory phase, no significant differences were observed. Similarly, the mRNA expression of **HSD17B7** was increased in proliferative phase endometriosis (FC 3.7, 3.0, and 3.8 for peritoneal, ovarian and deep endometriosis; $p<0.001$) but also in patient endometrium (FC 2.5; $p<0.001$) as compared to control endometrium. Thus, no differences were observed when compared to patient endometrium. No differences were detected between the endometrial and endometriotic expression of **HSD17B12**.

5.2.5.3 Increased HSD17B6 expression

The expression of HSD17B6 was highly increased in deep and peritoneal endometriotic lesions as compared with eutopic endometrium of patients with endometriosis or healthy controls. This result was obtained both by microarray and qRT-PCR studies *(III: Fig. 1)*. Interestingly, HSD17B6 expression did not vary during the menstrual cycle in any of the sample groups. As compared with the endometrium samples, the expression was at its highest in the deep endometriosis (FC 6.8-7.0) but a markedly increased expression was also observed in the peritoneal endometriosis (FC 2.9-3.4), and ovarian endometriotic cysts (FC 2.1-2.6). The gene with the highest correlation (regression 0.697, R=0,740, $p=3.13*10^{-50}$) to HSD17B6 was Prune homolog 2 (Drosophila) (PRUNE2) *(III: Fig. 2)*. Interestingly, an inverse regression (-0.668, $p=1.968*10^{-17}$) was detected with GABA-A receptor, pi (GABRP; *III Fig. 2*) with a correlation coefficient of -0.476.

5.2.6 Correlation of endometrial and endometriotic HSD17B expression with serum hormone levels (III)

The correlations between mRNA expression revealed in the microarray study and serum hormone levels were analyzed in the specimens obtained from patients without hormonal medication *(III: Table 3 and Supplementary table 4)*. Interestingly, the expression of HSD17B2, HSD17B6 and HSD17B11 in control endometrium samples positively correlated with serum P4 concentration. Weaker or no correlation was observed between P4 concentration and the expression of these enzymes in the patient endometrium or endometriosis specimens. However, while the expression of HSD17B6 positively correlates with serum P4 in control endometrium no cyclical changes were detected in mRNA level

in any sample groups *(III: Fig 1)*. As expected, the mRNA expression of PGR negatively correlated with serum P4 both in patient (correlation coefficient -0.686; $p= 3.9x10^{-6}$) and control endometrium (correlation coefficient -0.428; p=0.018), which well validated the array studies. The expression of HSD17B2 also possesses moderate inverse correlation with serum FSH level in control and patient endometrium but not in endometriosis specimens. No correlation between HSD17B6 expression and other serum hormones were observed.

Interestingly, HSD17B14 expression in control or patient endometrium possessed significant inverse correlation with serum estradiol level, similarly to that of ESR1, while no correlation was observed in the endometriosis specimens *(III: Table 2)*. The expression of these genes in endometrium also weakly correlated with serum FSH levels. Noteworthily, the serum estradiol levels did not correlate with the expression of estrogen metabolizing enzymes *e.g.* HSD17B1, 2, 4, 5, 7, and 12, CYP19A1, STS or SULT1E1.

5.3 SERUM HORMONE AND BIOMARKER CONCENTRATIONS IN PATIENTS WITH ENDOMETRIOSIS AND HEALTHY CONTROLS

5.3.1 Serum hormone concentrations

The concentrations of serum LH, FSH, E2, P4, cortisol, and total or free (T/SHBG) T were not significantly different in patients with endometriosis and healthy controls *(Table 8)*. Serum LH and FSH levels significantly decreased while P4, E2 and SHBG concentrations increased in secretory phase when compared to proliferative phase. No combined effect of the disease status and menstrual cycle on serum hormone levels was observed.

Table 8: *Serum hormone concentrations by disease status and cycle phase*

	FSH (U/L)	LH (U/L)	P4 (nmol/L)	E2 (nmol/L)	Cortisol (nmol/L)	T (nmol/L)	T / SHBG	SHBG (nmol/L)
Least square means for disease :								
ctrl	6.817	8.398	9.786	0.353	401.3	2324.3	0.414	7429.1
endometriosis	5.953	10.222	8.756	0.379	350.7	2254.1	0.694	7497.9
P-value	*0.195*	*0.447*	*0.675*	*0.652*	*0.113*	*0.806*	*0.372*	*0.934*
Least square means for cycle :								
prol	7.736	12.066	3.048	0.307	377.9	2424.9	0.765	6585.4
secr	5.034	6.555	15.494	0.425	374.1	2153.4	0.343	8341.6
P-value	***1.01E-04***	***0.024***	***2.19E-06***	***0.044***	*0.905*	*0.344*	*0.180*	***0.036***
Least square means for disease x cycle :								
ctrl x prol	8.587	10.295	2.172	0.265	383.4	2558.1	0.483	6945.3
ctrl x secr	5.047	6.502	17.400	0.441	419.3	2090.5	0.345	7912.9
endometriosis x prol	6.884	13.837	3.924	0.349	372.4	2291.8	1.047	6225.5
endometriosis x secr	5.022	6.608	13.589	0.409	328.9	2216.4	0.341	8770.3
P-value	*0.208*	*0.474*	*0.258*	*0.315*	*0.212*	*0.494*	*0.365*	*0.342*

Also the correlations between serum hormone concentrations, disease state (healthy, endometriosis), and cycle phase (proliferative/secretory) were evaluated *(Table 9)*. Serum FSH and P4 concentrations correlated with cycle phase. The concentrations of LH and P4 correlated with serum FSH level.

Table 9: *Correlations between serum hormone concentrations, disease state, and cycle phase*

		cycle	FSH	LH	P4	E2	Cortisol	T	T / SHBG	SHBG
disease	Correlation Coefficient	0.114	-0.164	0.044	0.003	0.062	-0.191	-0.031	0.070	0.047
	P Value	0.296	0.132	0.685	0.975	0.570	0.078	0.779	0.524	0.666
	Number of Samples	86	86	86	86	86	86	86	86	86
cycle	Correlation Coefficient		**-0.405**	-0.256	**0.476**	0.209	-0.060	-0.095	-0.158	0.253
	P Value		**1.10E-04**	0.017	**3.70E-06**	0.054	0.585	0.384	0.147	0.019
	Number of Samples		86	86	86	86	86	86	86	86
FSH (U/L)	Correlation Coefficient			**0.421**	**-0.428**	-0.268	0.023	-0.088	0.143	-0.067
	P Value			**5.55E-05**	**3.93E-05**	0.013	0.831	0.422	0.190	0.538
	Number of Samples			86	86	86	86	86	86	86
LH (U/L)	Correlation Coefficient				-0.201	0.334	0.038	-0.065	0.032	-0.141
	P Value				0.064	0.002	0.726	0.553	0.773	0.196
	Number of Samples				86	86	86	86	86	86
Progesterone (nmol/L)	Correlation Coefficient					0.170	0.128	-0.028	-0.082	0.151
	P Value					0.117	0.240	0.799	0.453	0.165
	Number of Samples					86	86	86	86	86
Estradiol (nmol/L)	Correlation Coefficient						0.021	0.100	0.222	0.142
	P Value						0.851	0.361	0.040	0.193
	Number of Samples						86	86	86	86
Cortisol (nmol/L)	Correlation Coefficient							0.069	-0.018	0.056
	P Value							0.528	0.867	0.608
	Number of Samples							86	86	86
Testosterone (nmol/L)	Correlation Coefficient								0.165	-0.050
	P Value								0.130	0.646
	Number of Samples								86	86
Testo / SHBG	Correlation Coefficient									-0.021
	P Value									0.848
	Number of Samples									86

5.3.2 Serum HE4 and CA125 concentrations in differential diagnosis of ovarian endometriotic cysts from ovarian cancer (IV)

The concentration of CA125 *(IV: Table 2)* was highly increased in the sera of patients with ovarian cancer (mean 1117.1 U/ml, $p<0.001$) in comparison to healthy controls (8.9 U/ml). Significantly ($p<0.001$) elevated CA125 levels were observed also in patients with ovarian endometrioma (44.3 U/ml) and advanced non-ovarian endometriosis (ASRM stage 4, 40.8 U/ml). These concentrations were also higher than the generally used limit for elevated CA125 value (35 U/ml). The CA125 level increased with increasing ASRM stage of endometriosis. In the sera of patients with endometrial cancer, the level of CA125 (22.0 U/ml) was also significantly ($p=0.029$) higher than in healthy controls even though clearly lower than the threshold value.

The serum HE4 concentration *(IV: Table 2)* was increased both in patients with ovarian cancer (1125.4 pM) and those with endometrial cancer (99.2 pM, $p<0.001$) as compared to healthy controls (40.5 pM). The levels of HE4 in different types of ovarian cancer were the highest in serous (2031.1 pM, n=7) carcinomas, while it was clearly elevated also in clear cell (397.6 pM, n=2) and mucinous (202.6 pM, n=3) carcinomas. However, the concentration was below the threshold value for elevated HE4 (70 pM according to (Moore *et al*, 2008b) and similar in healthy controls and patients with endometriosis (mean 45.5 pM) irrespective of the disease stage or the presence of ovarian endometrioma.

In order to differentiate the ovarian cancer patients from healthy controls, the combination of CA125 and HE4 relative to CA125 or HE4 alone resulted to the highest accuracy

(96.3%) and sensitivity (92.9%; *IV Table 3)*. Furthermore, the combination had the highest accuracy (94.0%) and sensitivity (78.6%) also for differential diagnosis of patients with ovarian cancer from those with ovarian endometriosis. The combination also differentiates ovarian endometriosis from healthy controls almost as accurately as CA125 alone, even though HE4 alone is a poor marker for endometriosis. Finally, the combination of HE4 and CA125 had the highest accuracy (81.9%) also in the three-wise comparison between the ovarian cancer, ovarian endometriosis and healthy controls.

In line with the differential serum concentrations observed for HE4 in the different patient groups and controls, also the mRNA expression *(IV, Table 4)* of the gene encoding HE4 (WFDC2) was significantly ($p<0.05$) increased in ovarian cancer. The median of $\log_2$ intensity value in ovarian cancer specimens (9.25) was 5.7 fold higher than in the ovarian endometrioma (6.73). However, the 1.9 fold expression in endometrial cancer (8.61) did not reach significance when compared with healthy endometrium (7.67). In contrast, the mRNA expression of CA125 encoding gene (MUC16) was similar in the tissue groups, while the fold change between all comparisons was between 0.7 and 1.3.

6. DISCUSSION

6.1 CLASSIFICATION OF ENDOMETRIOSIS SPECIMENS BY GENE EXPRESSION PROFILES

The 335 tissue specimens were clustered based on their whole genome expression using conventional hierarchical clustering, a novel ReScore procedure, and Bayesian clustering. These methods have technical differences with their *pros and cons*, which also affects the results and are, therefore, shortly discussed. The conventional hierarchical clustering results in a clustering tree, which is only one of the possible options. Therefore, it can be used as a basis for further studies, but it may also contain artifacts and result in misinterpretation. Regardless of that, this method is conventionally used. The ReScore and Bayesian clustering were carried out using repeated analysis runs, which improves the relevance of the result. The successful combining of the clinical data with resulted sample clustering supports the use of the ReScore method to identify novel disease or sample subgroups. The evaluation of the differentially expressed genes between the clusters can be used in identification of biomarkers that differentiate the samples within these subgroups. Based on the pre-determined grouping of the samples to 'disease' and 'control' samples, it cannot be used as a diagnostic array *per se.* Finally, in Bayesian clustering, the binarization of the gene expression data (to be above or below the normalized mean of all of the samples) results in a loss of detailed expression levels. However, it enables a powerful method for detecting similarly behaving sample groups, with the genes that are informative for the grouping. Combining the Bayesian clustering results with the clinical data showed that the clustering obtained based on the expression profiles was compatible with the biological and clinical characteristics.

The results given by the three methods presented very similar sample grouping indicating the reliability of the main findings. Ovarian endometriomas were clearly separated as their own group, which suggests markedly different expression profiles in these samples as compared with the other types of endometriosis. Similarly, ~50% of bowel endometriotic lesions were separated as a discrete cluster. The rectovaginal endometriosis and deep lesions in uterosacral ligaments were typically located in the same clusters. These findings are contradictory to the theory of differential etiology, which suggests that rectovaginal endometriotic nodules originate from metaplasia of Müllerian rests, while the deep uterosacral and bowel lesions are the result of infiltration of peritoneal lesions. However, these results are only indicative, and can be used to direct further studies. The detailed analysis of the differentially expressed genes between the clusters will clarify the source and relevance of the differential grouping of the samples.

The peritoneal lesions were clustered partly together with deep lesions from the rectovaginal wall or uterosacral ligament, and partly with unaffected peritoneum samples. That may be due to the size of the peritoneal lesion: Those clustering together with deep lesions may be large lesions and probably in early intermediate phase of infiltration

(Cornillie *et al*, 1990). Small lesions would have a greater proportion of peritoneal cells, which may have an impact on differential expression profiles. Differential clustering of the peritoneal lesions may also indicate their differential activity but red, black and white lesions were not separated by hierarchical or Bayesian clustering. However, one subcluster (1.2) in ReScore analysis consisted of red lesions by 75% (12 out of 16 samples in the cluster), thus, including 46% (12/26) of all red lesions, which may indicate the more sensitive identification of these active lesions by this method.

The majority of unaffected peritoneum samples of endometriosis patients and control subjects were separated by ReScore, which was expected as they were given as 'control' and 'disease' groups, respectively. However, the differential expression pattern was supported by hierarchical clustering results, even though not equally clearly by Bayesian clustering. Despite the fact that the dendogram of hierarchical clustering is only one of the possible options, the similar trend given by these three methods supports the hypothesis of the differential gene expression between peritoneums of the two groups. Biologically, this difference may be due to macroscopically invisible endometriotic changes in patient peritoneum. In fact, there is evidence suggesting that macroscopically normal pelvic peritoneum is biologically different between women with and without endometriosis (Kyama *et al*, 2008). Interestingly, two of the patient peritoneum samples clustered together with the deep endometriotic lesions by the ReScore method. These samples appeared to be from patients with stage 3-4 disease with divergent disease condition. It remains to be determined if these peritoneal samples included macroscopically invisible endometriosis, or if the peritoneums of these patients are remarkably changed, and if that will further the development of a more severe disease state. Thus, it would be of importance to evaluate if patient peritoneum is altered and whether that may be actively involved in the pathogenesis of endometriosis. Similarly, this putative difference may be used as a source for identification of prognostic markers.

In all of the three analyses, endometrium samples clustered by the hormonal status rather than by the groups of patients and controls. By each of the methods the proliferative and secretory samples were grouped in at least two rather specific clusters. In addition, Bayesian clustering separated the mid-phase samples including cycle days 9-16. Interestingly, a proportion of control and patient endometria in the proliferative phase of the menstrual cycle appeared in different clusters (#10 and #32).

The ability of microarray-based gene expression signatures to define cancer subtypes, to predict recurrence of the disease, and to predict response to specific therapies has been demonstrated in multiple studies (Ramaswamy & Golub, 2002). Predictions of pathway deregulation in cancer cell lines are also shown to predict the sensitivity to therapeutic agents that target components of the pathway. Similarly, the successful classification of endometriosis would enlighten the etiology and behavior of the different lesion types, and their relation to the varying symptoms. The identification of the differentially expressed genes within the sample subtypes can be used to identify the putative biomarkers for the prediction of endometriosis-associated infertility and the recurrence of the disease. In

the future, the gene expression signature together with clinical data may be used *per se* as a biomarker for prognosis of the disease and the related symptoms, and the prediction of the most effective treatment.

6.2 HORMONAL REGULATION OF ENDOMETRIUM AND ENDOMETRIOSIS

6.2.1 AR mediated androgen responses in decidualizing endometrial stromal cells

Secretion of PRL and IGFBP-1 in response to cAMP and P4 signaling is the characteristic of decidual transformation of HESCs (Brosens *et al*, 1999). The role of androgens in this differentiation process has not been evaluated. The present results demonstrate that androgens modify this differentiation process by an AR-dependent manner. DHT markedly enhanced PRL, but not IGFBP-1, secretion in decidualizing HESC in culture via decidual-specific PRL-promoter activation (Gellersen *et al*, 1994). The induction was negated by an AR antagonist bicalutamide. It was postulated that enhanced AR expression could explain the gradual increase in androgen sensitivity upon HESC differentiation. However, combined 8-Br-cAMP plus P4 treatment resulted in a rapid and sustained decrease in cellular AR levels. DHT strongly increased AR levels in undifferentiated cells *(II: Fig. 2A)*, as described in other cell systems (Yeap *et al*, 1999), but only partially antagonized the down-regulation of the receptor in decidualizing cells. Thus, as reported for P4 (Brosens *et al*, 1999), increased sensitivity to androgens in HESCs is paradoxically associated with decreasing receptor levels. In the case of P4, increased responsiveness has been linked to global changes in cellular small ubiquitin-like modifier (SUMO)-1 modification upon HESC differentiation (Jones *et al*, 2006). More specifically, decidualization is characterized by a gradual decline in the expression of PIAS1, resulting in decreased ligand-dependent sumoylation of PR and increased transcriptional activity. The present results demonstrate that down-regulation of PIAS1 upon decidualization sensitizes HESCs not only to P4 (Jones *et al*, 2006) but also to androgen signaling.

6.2.2 Decidual AR and PR target genes

Medroxyprogesterone acetate (MPA), a 17-OH P4 derivative with known androgenic actions (Ghatge *et al*, 2005), is widely used in combination with 8-Br-cAMP to differentiate HESCs *in vitro* (Brosens *et al*, 1999; Gellersen *et al*, 1994). The present study confirmed that MPA, like DHT but not P4, enhances cellular AR levels in HESCs, induces its nuclear accumulation, and transactivates the receptor in a reporter assay *(II: supplemental Fig. 2)*. The progestogenic and androgenic properties of MPA were exploited to search for specific AR- and PR-dependent genes in decidualizing HESCs by siRNA-mediated silencing of the gene expression. The knockdown of either receptor was equally efficient in attenuating PRL mRNA expression in differentiating HESCs *(II: Fig. 3B)*. The whole genome expression analysis showed that AR depletion regulated a

relatively small number of transcripts (92 mRNAs) while PR knockdown perturbed the expression of 860 genes. Only 29 genes were identified to be under control of both nuclear receptors in decidualizing cells, although 10 were regulated in an opposing manner *(II: Table 5)*. The data confirmed the major role of PR in regulating decidual gene expression and define, for the first time, a smaller but distinct set of genes under AR control.

Microarray analyses have been extensively used to examine endometrial responses to P4 in humans and various animal models (Cheon *et al*, 2002; Jeong *et al*, 2005; Kao *et al*, 2002). The present gene profiling complements these studies and confirms that in decidualizing HESCs PR controls the expression of a network of at least 860 genes within 28 different functional molecular and cellular categories. Thus, although insufficient to trigger HESC differentiation, P4 is essential for maintaining the decidual phenotype both *in vivo* and *in vitro*. Compelling evidence has emerged indicating that sustained expression of the decidual phenotype is also dependent on autocrine or paracrine signals, resulting in the activation of various secondary signaling pathways (Dimitriadis *et al*, 2006; Gellersen & Brosens, 2003; Mohamed *et al*, 2005). A significant number of PR-dependent genes encode for ligands, membrane bound receptors, and intermediates in various signal transduction pathways. The data imply that a substantial proportion of PR-dependent decidual genes are regulated indirectly, via autocrine or paracrine activation of the WNT/β-catenin, TGFβ/SMAD, and STAT pathways. Thus PR is essential for the activation of secondary signaling pathways upon decidualization.

6.2.3 AR regulates cytoskeletal organization and cell cycle inhibition

Ingenuity pathway analysis complemented by manual mining of available literature implicated 40% (21 of 53) of genes down-regulated upon AR silencing in the regulation of cell morphology, cytoskeletal organization, and cell motility (II: Tables 1 and 5). The present results show that decidualization is characterized by a dramatic increase in F-actin polymerization and stress fiber formation. However, the proportion of cells that express elongated stress fibers was reduced by approximately 50% upon AR knockdown. Decidualization was also associated with a dramatic decrease in basal cell motility. That may be regulated at least partially via AR, which attenuates the phosphorylation of the the regulatory light chain of myosin 2(MLC2), and further, declines the actin-myosin interactions that are essential for cell motility (Fumoto *et al*, 2003).

In addition to cell motility, the actin cytoskeleton is involved in many other biological functions, including endo and exocytosis, cytokinesis, and signal transduction (Disanza *et al*, 2005; Lanzetti, 2007), underscoring the importance of AR in regulating decidual cell function. Importantly, induction of the IL1R1 in decidualizing cells is under AR control *(II: Fig. 4)*. Embryonic signals, and in particular IL-1β, have been shown to promote cytoskeletal reorganization in decidual cells (Ihnatovych *et al*, 2007). Together, these observations suggest that AR plays a major role in coordinating decidual-trophoblast interactions during early pregnancy. This speculation is further supported by the observation that inactivation of decidual RhoA, a Rho GTPase family member essential

for cytoskeletal organization, blocks outgrowth but not attachment of blastocysts in a co-culture model (Shiokawa *et al*, 2000). *In silico* analysis further revealed that several genes up-regulated in decidualizing cells upon AR depletion, and thus, normally repressed in an AR-dependent manner, are involved in various aspects of cell cycle regulation including DNA replication licensing and chromatid separation. This expression profile points toward a role for AR in reducing cell proliferation, and thus, safeguarding the genetic stability of the endometrium during rapid cyclic remodeling.

Interestingly, the cyclical differences in the expression of the three AR responsive genes (IL1R1, DUSP3, and OIP5) were lost in the endometriosis samples, and the expression levels of these genes were significantly higher in the proliferative phase endometriosis as compared to proliferative healthy endometrium. The increased expression of IL1R1 in endometriosis is in accordance with the previous data (Akoum *et al*, 2007; Lawson *et al*, 2007) and may play a role in the adhesion and invasion of endometriotic cells (Sillem *et al*, 2001; Sillem *et al*, 1999). Interleukin 1, the ligand of IL1R1, has also been shown to inhibit the growth of normal human endometrial stromal cells (Van Le *et al*, 1992). Still, it would be of importance to evaluate the role of the aberrant AR responsive gene expression in the endometriotic cell proliferation.

6.2.4 Estrogen metabolizing HSD17Bs in endometrium and endometriosis

The expression of estrogen metabolizing enzymes in endometriosis is still controversial and no conclusive data in different types of endometriosis lesions have existed (Rizner, 2009). Thus, in this study, the expression of 14 different HSD17B-enzymes was evaluated in various types of endometriosis tissue by microarray, and verified the most interesting data by qRT-PCR analysis and immunohistochemistry. The present data show that of the HSD17B enzymes, the expression of HSD17B2 and HSD17B6 display the most marked differences between endometrium and endometriosis specimens. HSD17B2 is markedly decreased in all endometriosis lesion types and HSD17B6 is greatly increased in deep infiltrating and peritoneal endometriosis as compared with endometrium of the patients or healthy controls.

The HSD17B2 is considered as one of the major enzymes inactivating E2 to E1. Aberrant expression of HSD17B2 has been previously reported in deep endometriosis (Dassen *et al*, 2007; Matsuzaki *et al*, 2006b), ovarian endometriotic cysts (Banu *et al*, 2008; Cheng *et al*, 2007; Matsuzaki *et al*, 2006a), and inadequately described endometriosis (Absenger *et al*, 2004; Zeitoun *et al*, 1998), while no differences have been detected in proliferative phase samples (Carneiro *et al*, 2007; Smuc *et al*, 2007). The present data evidently show an aberrant HSD17B2 mRNA expression comparably in ovarian, peritoneal and deep infiltrating endometriosis. Accordingly, as compared with normal endometrium, the inactivation of E2 to E1 has recently been shown to be decreased in endometriosis lesions (Delvoux *et al*, 2009). It has been hypothesized that the decreased local inactivation of E2 by the decreased HSD17B2 expression would increase the local

concentration of E2, which is most probably maintained by the counteracting HSD17B1 enzyme. That would, thus, increase the proliferation of endometriosis tissue.

The relevance of the other HSD17B enzymes in E2 inactivation and pathophysiology of endometriosis is not known. Our study suggests that other E2 inactivating HSD17B enzymes, namely HSD17B4, 8, and 10 are expressed in both endometrium and endometriosis and the expression is not remarkably altered in endometriosis lesions as compared with the expression in the eutopic endometrium. As an exception, the expression level of HSD17B14 was moderately but significantly up-regulated in endometriosis vs. patient endometrium. Interestingly, its expression also possessed negative correlation with serum E2 level, which suggests the E2 regulation of HSD17B14 in human endometrium.

While in the healthy endometrium the expression of HSD17B2 is increased during the secretory phase, presumably through a progesterone-dependent mechanism, no such increase is apparent in endometriosis (Cheng *et al*, 2007). This may to be due to progesterone resistance of endometriosis (Bulun *et al*, 2006; Burney *et al*, 2007). Our data well supports the above conclusion, as there was a significant correlation between the circulating P4 concentration and HSD17B2 expression in control endometrium, while already less in patient endometrium and altogether lost in the endometriosis lesions.

There is evidence indicating that HSD17B2 may also be involved in retinoid action. Transgenic mice over-expressing human HSD17B2 present with phenotype, which mimics retinoid excess (Rantakari *et al*, 2008; Zhongyi *et al*, 2007). Retinoic acid (RA) also increases the HSD17B2 mRNA expression in human endometrium (Cheng *et al*, 2008). In endometrium, there is a cross talk between estrogen and retinoic actions. RA suppresses estrogen-dependent proliferation of endometrial cells whilst estrogen up-regulates RA production and signaling in the human endometrium (Deng *et al*, 2003). Interestingly, in the present study the expression of HSD17B2 possessed highest correlation with CYP26A1, an enzyme involved in RA inactivation. Previous studies have indicated that similar to HSD17B2, CYP26A1 is regulated by progesterone in endometrial epithelial cells (Deng *et al*, 2003; Fritzsche *et al*, 2007). The progesterone resistance in endometriosis may, thus, cause aberrant expression of both estradiol and retinol metabolizing enzymes leading to increased retinoid and estrogen actions. Therefore, while the progesterone resistance in endometriosis may cause increased estrogen-derived endometriosis cell proliferation the concurrent increase in retinoid action may partly counterbalance this effect.

While HSD17B2 is considered to have a central role in the inactivation of E2 to E1, HSD17B1 is considered to have a major role with the highest catalytic activity (Rizner, 2009) in activating E1 to E2. While the total HSD17B activity converting E1 to E2 appears to be increased in the endometriosis as compared with endometrium (Delvoux *et al*, 2009), the data on HSD17B1 expression is inconclusive. The enzyme has been identified both in mRNA and protein levels in the endometriosis lesions (Borghese

et al, 2008; Dassen *et al*, 2007; Smuc *et al*, 2007; Zeitoun *et al*, 1998), while some studies have indicated increased expression in endometriosis (e.g. Borghese *et al*, 2008; Smuc *et al*, 2007). In the present study, we did not detect any major difference in the mRNA expression of HSD17B1 between sample groups in either of the cycle phases. The increased expression of HSD17B1 in ovarian endometriosis should be assessed with concern as the enzyme is highly expressed in ovarian granulosa cells, which may be present also in endometrioma specimen analyzed. Granulosa cells are an important source of E2, which, in theory, may affect on the growth of the nearby endometrioma in paracrine mechanism. The recent report (Vercellini *et al,* 2008) indicated that ovulation may be critical for the recurrence of ovarian endometriotic cysts. However, the mechanism of the ovarian function or the role of paracrine E2 action on the growth of endometrioma has not been characterized. Nevetherless, the presence of HSD17B1 in endometriosis specimens together with the decreased HSD17B2 activity emphasizes the role of HSD17Bs in the regulation of local E2 level.

In addition to the differential expression of HSD17B2, a marked increase in the expression of HSD17B6 in endometriosis lesions was identified as compared with the endometrium. The greatest increase was detected in deep rectovaginal endometriosis followed by deep endometriosis lesions in uterosacral ligament and intestine as well as peritoneal endometriosis lesions. No cyclical changes were observed in any of the studied tissues. HSD17B6, which is also known as 3(α→β) hydroxysteroid epimerase and oxidative 3α-hydroxysteroid dehydrogenase, is a multifunctional enzyme. It recognizes both retinoids and 3α-hydroxysteroids as substrates and possesses both oxidoreductase and 3(α→β) epimerase activities (Chetyrkin *et al*, 2001; Huang & Luu-The, 2000).

The highest expression of human HSD17B6 has been detected in the liver, while the enzyme is also expressed at least in the adrenal, brain, spleen, uterus, and prostate (Chetyrkin *et al*, 2001; Huang & Luu-The, 2000). The expression of HSD17B6 in the brain provides evidence for the possible involvement of the enzyme in the regulation of neurosteroid levels, and further, of hormonal changes in the brain (Chetyrkin *et al*, 2001; Huang & Luu-The, 2000). However, the physiological role of HSD17B6 in human health and disease is unknown. As androgens counteract the effect of estrogens on endometrial cell proliferation the metabolism of androgens by HSD17B6 (Chetyrkin *et al*, 2001) further characterization; Huang LuuThe 2000 molecular) in endometriosis may affect endometriosis cell proliferation. On the other hand, neurosteroids allopregnanolone and androsterone, which are inactivated by HSD17B6, are allosteric modulators of the inhibitory gamma-aminobutyric acid (GABA) –receptors, and thus, the effect of increased HSD17B6 may be involved in increased pain signaling. Interestingly, GABRP expression was down-regulated in endometriosis specimens *(III: Fig. 2)* as compared with CE or PE. GABRP transcript has been detected in several human tissues, and is particularly abundant in the uterus (Hedblom & Kirkness, 1997). Within our microarray analysis, the most highly correlating gene with HSD17B6 was proapoptotic PRUNE2, which is also known as BMCC1 or BNIP-XL. Interestingly, a high expression level of PRUNE2 has been detected in the human nervous system (Machida *et al*, 2006). Its expression is

increased in prostate cancer under androgen regulation (Clarke *et al*, 2009). PRUNE2 has been suggested to play an important role in regulating differentiation, survival and aggressiveness of the neuroblastoma cells (Machida *et al*, 2006). Whether the similar increase in expression of HSD17B6 and PRUNE2 in endometriosis is due to similar regulation or an increased amount of nerve fibers in endometriosis lesions (Tokushige *et al*, 2006) is not known. It also remains to be evaluated if the increased activity of HSD17B6 in endometriosis is involved in the pathophysiology of endometriosis and / or in the generation of endometriosis related pain, and if the effect is mediated by changes in androgen, neurosteroid and/or retinol metabolism.

6.3 SERUM HE4 AND CA125 CONCENTRATIONS IN DIFFERENTIAL DIAGNOSIS OF OVARIAN ENDOMETRIOTIC CYSTS FROM OVARIAN CANCER

HE4 is a novel serological marker used especially for ovarian cancer diagnosis (e.g. (Gagnon & Ye, 2008; Hellstrom & Hellstrom, 2008; Hellström *et al*, 2003; Moore *et al*, 2008b). Because of its high sensitivity it is useful also for detecting stage I ovarian cancer (Havrilesky *et al*, 2008; Moore *et al*, 2008b). Furthermore, HE4 has been suggested as a biomarker for the diagnosis of endometrial cancer (Moore *et al*, 2008a). Currently, several biomarker panels are being evaluated in order to increase the sensitivity and specificity of ovarian cancer diagnosis. The combination of CA125 and HE4 with, or without, other biomarkers such as Glycodelin, Plau-R, MUC-1, PAI-1 (Havrilesky *et al*, 2008), SMRP (Hellström & Hellström, 2008; Moore *et al*, 2008a), CA72-4 and osteopontin (Moore *et al*, 2008b) have been evaluated to improve ovarian cancer diagnosis. The data suggest that by combining these markers the predictive accuracy in ovarian malignancy is better than by applying any of the markers alone. The panel of biomarkers including HE4 has been evaluated also for monitoring the recurrence of ovarian cancer (Havrilesky *et al*, 2008; Moore *et al*, 2009).

In female tissues, HE4 immunoreactivity has been shown to be highest in glandular epithelium of the genital tract including endocervical glands, endometrial glands, fallopian tubes, and Bartholin's glands (Drapkin *et al*, 2005; Galgano *et al*, 2006). In contrast to the normal ovarian surface epithelium, which does not express HE4, cortical inclusion cysts lined by metaplastic Müllerian epithelium have been shown to express the protein abundantly (Drapkin *et al*, 2005). The expression of HE4 protein in ovarian tumors is highest in serous carcinomas but immunostaining has been detected also in the vast majority of ovarian endometrioid and clear cell carcinomas (Drapkin *et al*, 2005; Galgano *et al*, 2006). In addition to ovarian carcinoma, some pulmonary, endometrial, and breast adenocarcinomas have been shown to express HE4 (Galgano *et al*, 2006). Although the protein has been detected in both normal and malignant endometrium, the expression of HE4 in the endometriotic lesions is only superficially known. Recently, Moore and co-workers (Moore *et al*, 2008b) analysed HE4 and eight other biomarkers in the sera of 166 patients with ovarian cancer or with several other kinds of pelvic masses,

of whom 29 had endometriosis. They showed that the HE4 and CA125 concentrations were the best combination of biomarkers to distinguish ovarian cancer patients from those with other pelvic masses. However, the types of endometriosis lesions in these patients were not described.

In agreement with other recent studies, (e.g. Gagnon & Ye, 2008; Hellstrom & Hellstrom, 2008; Hellström *et al*, 2003; Moore *et al*, 2008b) we detected increased HE4 concentration in patients with ovarian and endometrial cancer. The present data demonstrate that neither the expression of HE4-encoding gene in the endometriotic lesions nor serum HE4 concentration in the endometriosis patients with any types of endometriosis is increased. It is of specific interest to note that HE4 is not increased even in patients with ovarian endometriosis. In contrast, the serum level of CA125 was increased in patients with advanced endometriosis and ovarian endometriomas, as expected. It should be noted that endometriosis is typically diagnosed at young adult age (25-35 years) and often disappears after menopause, while the incidence of ovarian cancer increases in older women (highest incidence at the age of 50-60 years). The age difference between the patient groups may, however, affect the results. The healthy controls of the study were also premenopausal (mean age 38.5) although older than the endometriosis patients. However, more important aspect is that all the study subjects were laparoscopically diagnosed to have endometriosis or ovarian cancer, or to be free from both. Interestingly, it has been reported that the concentration of HE4 increases with age in healthy postmenopausal women, while CA125 does not (Lowe *et al*, 2008), emphasizing the use of their combination.

Thus, measuring both HE4 and CA125 together, rather than either of them alone, provides a more accurate tool for differential diagnosis of patients with ovarian cancer and ovarian endometriotic cysts from healthy subjects. It may also help clinicians in the follow-up of patients suffering from advanced endometriosis when considering the possibility of malignant transformation of the lesions. Within the patients with an ultrasound-detected ovarian mass, the high serum HE4 with high CA125 would suggest the presence of ovarian cancer while elevated CA125 without elevated HE4 would direct towards advanced or ovarian endometrioma or other benign conditions. Furthermore, the elevated serum HE4 concentration with normal CA125 concentration would suggest either the presence of ovarian or possibly other type of cancer, *e.g.* endometrial cancer.

The greatest benefit of highly specific differentiation between ovarian cancer and endometriosis may well be found in the identification of ovarian cancer in the early non-symptomatic stage. A high proportion of ovarian cancers are diagnosed at an advanced stage with a dismal survival rate. In contrast, the 5 year survival rate for stage I disease with the malignancy confined to the ovary is above 90%. This emphasizes the importance of detecting the ovarian cancers at their early stage in order to improve the mortality rate.

7. SUMMARY AND CONCLUSIONS

Endometriosis lesions are classified to peritoneal, deep and ovarian diseases with suggested divergent etiology. These subtypes also differ in the associated symptoms, recurrency and response to treatment. However, a comparable genome-wide expression analysis of all the main lesions types has not been reported. In the present study, the transcriptomic profiles of 335 tissue samples, including different types of endometriosis, eutopic endometrium and unaffected peritoneum of the women with endometriosis and healthy controls, were generated and utilized to define the molecular subtypes of endometriosis. Similarly, the genome-wide expression analyses were exploited in evaluation of hormone actions in endometriosis and health endometrium, and to identify novel biomarkers for endometriosis. The main conclusions of the present study are the following:

The clustering of endometriosis, unaffected peritoneum, and eutopic endometrium specimens based on their genome-wide expression profiles groups the samples into tissue and cycle-phase specific clusters. The expression profiles of the unaffected peritoneums of women with endometriosis and healthy controls are diverged, suggesting that the morphologically normal patient peritoneum may be functionally altered. It would be important to further evaluate whether these putative changes are involved in the pathogenesis of the disease. The ability of expression profiling to define endometriosis subtypes encourages further analysis of the deregulated pathways between the subtypes. Moreover, it can be utilized to identify the putative biomarkers for prediction of endometriosis-associated infertility and the recurrence of the disease.

Human endometrial stromal cells become increasingly responsive to androgens upon differentiation due to attenuation of the AR sumoylation. The androgen receptor governs the expression of a limited decidual gene pool, responsible for cytoskeletal organization and inhibition of cell motility and proliferation. These changes in cell functions may be critical for coordinated trophoblast invasion and placental development. However, the possible role of these findings in the pathogenesis of endometriosis remains to be identified.

Messenger RNA expression of HSD17B6 enzyme is highly up-regulated in endometriosis as compared to eutopic endometrium of women with or without endometriosis. Due to the ability of HSD17B6 to metabolize androgens and neurosteroids, the enzyme may be involved in the regulation of the endometriotic cell proliferation and / or in pain generation.

The serum concentration of HE4, a novel biomarker for ovarian cancer, is not increased in patients with ovarian endometrioma or any other types of endometriosis, while the serum CA125 concentration was increased in advanced endometriosis. The results suggest that the serum HE4 concentration is a valuable marker to better distinguishing patients with ovarian malignancies from those suffering from the benign ovarian endometriotic cysts.

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