Estrogen Metabolism in Endometrial Cancer. 17beta-Hydroxysteroid Dehydrogenases

New Anti-Estrogenic Therapeutics for Endometrial Cancer?

Laura Visconti

promotor :
dr. A. ROMANO

Universiteit Maastricht

Masterproef voorgedragen tot het bekomen van de graad van
master in de biomedische wetenschappen

universiteit hasselt

Contents

Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
17β-HSD1, -2, -5, -7, -12	17β-hydroxysteroid dehydrogenase type 1, 2, 5, 7, 12
ACN	Acetonitrile
AKR	Aldo-keto reductase
BMI	Body mass index
BrdU	5-bromo-2'-deoxyuridine
CDB	2-(4-carboxy-phenyl)-5,6-dimethylbenzimidazole
cDNA	Complementary deoxyribonucleic acid
DAB	3,3'-diaminobenzidine
DABCO	1,4-diazabicyclo-(2,2,2)octane
DAPI	4'-6-diamidino-2-phenylindole
E_1	Estrone
E_2	17β-estradiol
EDC	1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride
ELISA	Enzyme-Linked Immunosorbent Assay
ERE	Estrogen responsive element
EST	Sulpho-transferases
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
HPLC	High-Pressure Liquid Chromatography
HRP	Horse-raddish peroxidase
LH	Luteinizing hormone
mRNA	Messenger RNA
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
$NAD(P)^+$	Nicotinamide adenine dinucleotide
PBS	Phosphate buffered saline
PEI	Polyethylenimine
SDR	Short-chain alcohol dehydrogenase reductase
SDS	Sodium dodecyl sulphate
STS	Sulphatases
TFF-1	Trefoil factor 1
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Room temperature

Acknowledgements

This work was supported by the Department of Gynaecology & Obstetrics at the University of Maastricht, the Netherlands. First of all, I would like to thank my supervisor Andrea Romano for his guidance, teaching and support. Additionally, I want to thank him for reviewing my thesis.
Next, I want to thank Bert Delvoux, for his teaching and support with the HPLC analysis.
I would also like to thank Karlijn Cornel, a medicine student who took a year off to do research, for her guidance during the HPLC analysis. I also want to thank Wilfred Germeraad for his suggestions on the proliferation assays, which unfortunately could not be applied during this study, but will be kept in mind for the future and I would also like to thank him for reviewing my thesis. Finally, I would also like to thank my family for their support.

Abstract

Background – Endometrial cancer (EC) is the most frequent type of cancer occurring in the female genital tract in Western Europe and the United States. About 53,913 women are affected in the European Union and 12,737 of them die every year. The incidence of EC will keep rising due to an increasing life-expectancy. Although the exact cause of EC is unknown, excessive exposure to increased levels of unopposed estrogens, in particular the biologically active form, 17β-estradiol (E_2), is considered to play an important role. Previous studies indicate that the expression of 17β-hydroxysteroid dehydrogenases (17β-HSDs), converting estrone, E_1, to E_2 and *vice versa*, greatly contributes to the *in situ* availability of E_2. Still it is not known which of the different 17β-HSDs are responsible for the high concentration of E_2 in EC tissue.

Hypothesis & objectives – We hypothesized that ***17β-HSDs are aberrantly expressed in EC, which leads to a hyper-estrogenic environment***. To test this hypothesis, it was first investigated which of these 17β-HSDs are aberrantly expressed, by comparing their expression between EC tissues and postmenopausal control tissues. Secondly, clinical sample results were confirmed *in vitro* by investigating the role of an aberrant expression of 17β-HSDs on E_2 synthesis. Thirdly, *in vitro* observations were reconfirmed by measuring the effect of an aberrant expression of 17β-HSDs on cell proliferation. Finally, during *in vitro* confirmation of clinical sample results, aromatase was found to have some implication in E_2 synthesis. To confirm these findings, the effect of aromatase inhibition was investigated via similar *in vitro* approaches.

Materials & Methods – Gene expression of 17β-HSDs was determined by qRT-PCR analysis. The effect of an aberrant expression of 17β-HSDs (1) on E_2 synthesis was determined in EC cell lines (ECC-1) using a luciferase assay and (2) on cell proliferation was measured in ECC-1 cells by means of a BrdU incorporation assay. The influence of aromatase inhibitors (1) on estrogenic activity of aromatase was determined via luciferase assay and (2) on aromatase-induced cell proliferation was determined via equal proliferation assay.

Results – 17β-HSD type 1 (17β-HSD1) gene expression is significantly increased in EC tissue compared to postmenopausal control tissue. *In vitro* over-expression of 17β-HSD1 significantly induces conversion of E_1 to E_2, increasing the availability of E_2. This is further supported by the increased cell proliferation measured when over-expressing 17β-HSD1 *in vitro*. To evaluate the role of aromatase, aromatase inhibitors were used, which significantly decreased the stimulating effect of aromatase on E_2 synthesis.

Conclusion – Taken together, these observations support our hypothesis. We confirmed an aberrant expression of 17β-HSD1 in EC tissue and *in vitro* this aberrant expression led to increased levels of E_2. And although aromatase was found to have some activity in EC cells, the role of aromatase in EC has to be further investigated.

Achtergrond – Endometriale carcinoma (EC) is de meest voorkomende vorm van kanker in het vrouwelijk genitale stelsel in zowel West-Europa als de Verenigde Staten van Amerika. Ongeveer 53.913 vrouwen worden getroffen in de Europese Unie en 12.737 hiervan sterven ieder jaar. De incidentie van EC zal blijven stijgen door een toenemende levensverwachting. Hoewel de exacte oorzaak van EC nog niet gekend is, blijkt een overvloedige blootstelling aan ongehinderde oestrogenen, voornamelijk aan 17β-oestradiol (E_2), een belangrijke rol te spelen. Andere studies gaven aan dat de expressie van 17β-hydroxysteroid dehydrogenases (17β-HSDs), die estron (E_1) omzetten naar E_2 en *vice versa*, sterk bijdragen aan de aanwezigheid van E_2 in het weefsel. Het is nochtans niet bekend welk van deze 17β-HSDs verantwoordelijk zijn voor de hoge E_2 concentratie in EC weefsel.

Hypothese & doelstellingen – We veronderstellen dat ***17β-HSDs een afwijkende expressie vertonen in EC, wat leidt tot een hyper-oestrogene omgeving.*** Om deze hypothese the testen, werd ten eerste onderzocht welk van deze 17β-HSDs een afwijkende expressie vertonen, door hun expressie te vergelijken tussen EC weefsels en post-menopausaal controle weefsels. Ten tweede werden de bevonden resultaten *in vitro* bevestigd door de rol van een afwijkende expressie van 17β-HSDs in de synthese van E_2 te achterhalen. Ten derde werden de *in vitro* bevindingen herbevestigd door het effect van een afwijkende expressie 17β-HSDs op celproliferatie te achterhalen. Ten laatste werd er tijdens *in vitro* experimenten gevonden dat aromatase ook een rol speelt in de synthese van E_2. Om deze bevindingen te bevestigen werd het effect van aromatase inhibitie onderzocht via dezelfde *in vitro* benaderingen.

Materiaal & Methoden – Genexpressie van 17β-HSDs werd bepaald via qRT-PCR analyse. Het effect van een afwijkende expressie van 17β-HSDs (1) op de synthese van E_2 werd achterhaald in EC cellijnen (ECC-1) met behulp van een luciferase test en (2) op celproliferatie werd gemeten in ECC-1 cellen via een BrdU incorporatie test. De invloed van aromatase inhibitoren (1) op de oestrogene activiteit van aromatase werd achterhaald via een luciferase test en (2) op aromatase-geïnduceerde celproliferatie was achterhaald via dezelfde proliferatie test.

Resultaten – 17β-HSD type 1 (17β-HSD1) genexpressie is significant verhoogd in EC weefsel vergeleken met post-menopausaal controle weefsel. *In vitro* overexpressie van 17β-HSD1 induceert op een significante manier de omzetting van E_1 naar E_2, wat de aanwezigheid van E_2 verhoogt. Dit werd verder ondersteund door een toename in celproliferatie bij overexpressie van 17β-HSD1 *in vitro*. Om de rol van aromatase te achterhalen werden aromatase inhibitoren gebruikt, die het stimulerend effect van aromatase op de synthese van E_2 verhinderen.

Conclusie – Onze bevindingen ondersteunen onze veronderstellingen. We bevestigden dat er een afwijkende expressie van 17β-HSD1 is in EC weefsel en dat deze afwijkende expressie *in vitro* leidt to verhoogde E_2 niveaus. En hoewel men gevonden heeft dat aromatase enige activiteit heeft in EC cellen, zal de rol van aromatase in EC verder onderzocht moeten worden.

Introduction

This thesis focuses on the metabolism of estrogens in endometrial cancer. In order to investigate the role of estrogen metabolism in the pathogenesis of endometrial cancer, background on the function of the normal endometrium and its menstrual cycle is important. Based on this information, and based on literature findings about estrogen metabolism in endometrial cancer, the research described in this thesis was set-up.

Endometrium and the menstrual cycle

The endometrium is the inner layer of the uterus that lines the uterine cavity (*Figure 1*). The endometrium consists of two distinct layers, the basal and the functional layer. The basal layer is in direct contact with the myometrium, which is the muscle layer of the uterus, and undergoes only minor changes during the menstrual cycle. The functional layer, which surrounds the lumen of the uterine cavity, consists of two different cell types, the glandular cells forming the endometrial glands, and the surrounding stroma cells [1].

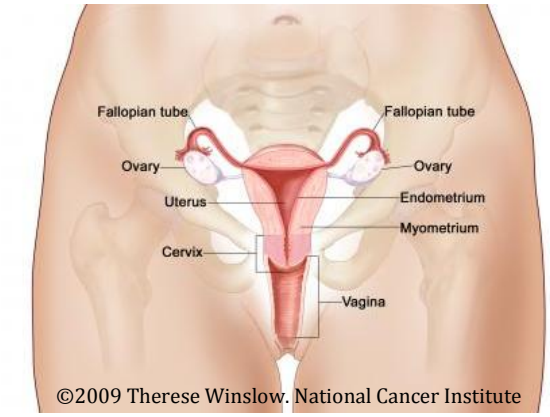

Figure 1. Anatomy of the female reproductive system. The internal female reproductive organs are the vagina, cervix, uterus, fallopian tubes and ovaries. The female reproductive system consists of two main parts: the ovaries and the uterus. The uterine cavity is lined by the endometrium.

Starting at menarche (first menstruation), the uterus undergoes monthly cyclic changes controlled by differential production and secretion of the ovarian hormones, estrogen en progesterone (*Figure 2*). The menstrual cycle can be divided into three stages: (1) the follicular or proliferative phase, (2) the luteal or secretory phase and (3) the menstrual phase [2]. During the follicular phase, the hypophyse secretes follicle stimulating hormone (FSH), which induces the maturation and growth of the follicles. FSH will stimulate the follicles within the ovarium to produce estradiol, inducing proliferation of the endometrium and leading to a relative hypertrophy of the uterine mucosa [2]. Estrogens induce formation and proliferation of glands. Stroma cells and vascular endothelial cells form, leading to vascular growth of the functional layer of the endometrium. Around day 14 of the menstrual cycle, a surge in the luteinizing

hormone (LH) secreted by the hypophyse induces ovulation, accompanied by a reduction in the circulating estrogen level. At this point, the secretory phase starts, which is characterized by high levels of progesterone, produced by the corpus luteum. This progesterone stimulates the differentiation of the glandular cells of the endometrium. When no pregnancy occurs, the corpus luteum regresses, which leads to a drop in circulating levels of estrogen and progesterone. In response to the decrease in hormone levels, the arteries in the endometrium constrict, leading to ischemia of the tissue. This eventually leads to discharge of the functional layer of the endometrium, known as menstruation or menses.

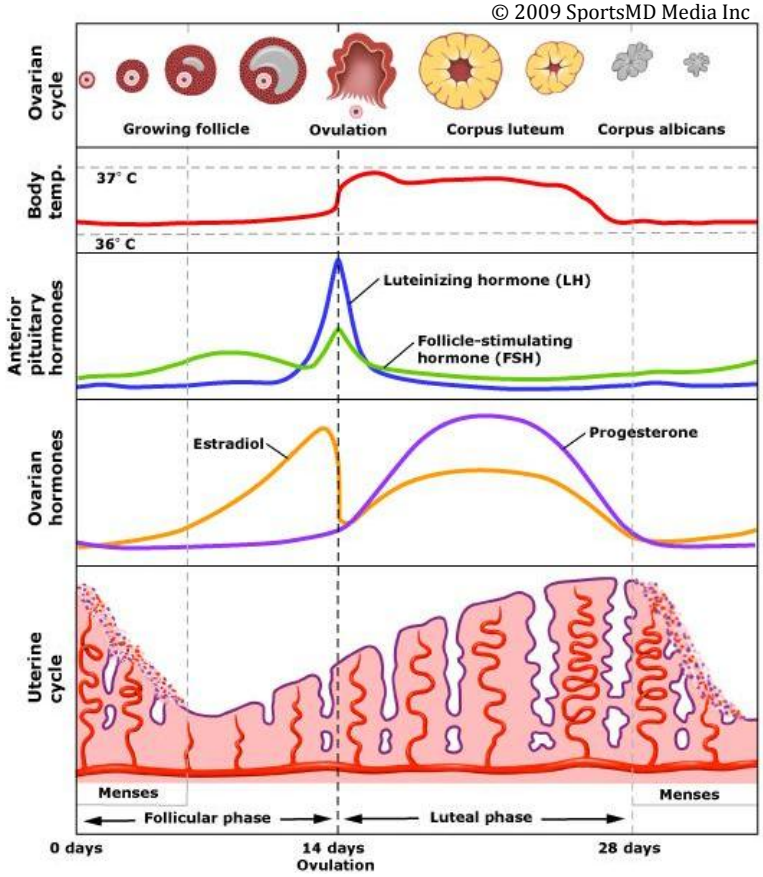

Figure 2. Schematic representation of the menstrual cycle. Changes in the endometrium (uterine cycle) are related to changes in the ovarian cycle and are under the influence of variations in hormone levels.

Around the age of 50, the menstrual cycle becomes irregular and ovulation eventually stops completely, also known as menopause. This leads to a strong decline in the production of estrogen and progesterone by the ovaries and eventually to a regression of the endometrium. Postmenopausal women are characterized by the absence of menstrual bleedings for at least one year. However, in some postmenopausal women, local estrogen level remains relatively high and the endometrium tends to proliferate again. Due to the absence of progesterone, unopposed estrogen exposure predisposes these women to the development of endometrial hyperplasia or even endometrial cancer [3, 4].

Endometrial hyperplasia

Endometrial hyperplasia is characterized by the abnormal thickening of the endometrial wall, associated with an overgrowth of both endometrial glands and stroma. It is most often diagnosed in postmenopausal women, although women of every age exposed to high levels of unopposed estrogen are at increased risk of developing endometrial hyperplasia [5]. Abnormal uterine bleeding is the most common presenting symptom of endometrial hyperplasia and it can be treated with either progestins to antagonize the estrogenic effects or with surgery, namely hysterectomy [6]. Atypical hyperplasia represents the greatest risk for progression to endometrial carcinoma [5]. Without treatment, 25% of the patients with this atypical hyperplasia will develop endometrial cancer [5].

Endometrial cancer

Endometrial cancer frequently arises on a background of endometrial hyperplasia and is defined as the abnormal growth of cells in the inner lining of the uterus, called the endometrium [7].

Epidemiology and pathogenesis

Endometrial cancer is the most frequent type of cancer occurring in the female genital tract in Western Europe and the United States [7-9] . About 53,913 women are affected in the European Union, and 12,737 of them die every year [10, 11]. In the United States, 40,100 new endometrial cancer cases, and 7,470 new death cases were estimated in the year 2008 [12]. In 2009, the amount of estimated new endometrial cancer cases rose to 42,160 cases, and 7,780 new death cases were estimated for that year [13]. The incidence of endometrial cancer will keep rising due to an increasing life expectancy [7].

Endometrial cancer mainly occurs in postmenopausal women (90% of endometrial cancer cases) with the highest incidence between the ages of 60 and 80 years [14]. Under the age of 40, endometrial cancer is rare. About 25% of patients are diagnosed before menopause [14-16].

There are two clinical settings in which endometrial carcinomas arise: (1) in perimenopausal women with estrogen excess and (2) in older women with endometrial atrophy. Both are correlated with differences in histology: endometrioid and serous carcinoma of the endometrium, respectively [7, 17]. Endometrioid carcinoma (type I) is the most common type of endometrial cancer, accounting for 80% of the patients. These types of lesions are associated with estrogen excess and endometrial hyperplasia, are low grade tumours and have an excellent prognosis [18]. Serous carcinoma (type II) of the endometrium arises in older women, are usually associated with endometrial atrophy and are of high grade [7, 18].

Normally, during fertile life, the proliferative properties of estrogens are counterbalanced by progesterone, which has differentiating properties. After menopause, a decline in serum levels of

estrogen and progesterone is observed because of the absence of follicle recruitment in the ovary (follicular phase of the menstrual cycle). This consequently results in an atrophic or inactive state of the endometrium. However, in some women, increased levels of local estrogens (endo- or exogenous) are present, which will stimulate the proliferation of the endometrium and may result in the development of endometrial cancer [4, 19].

Symptoms

The most common symptom of endometrial cancer is abnormal vaginal/uterine bleeding, followed by anemia due to the chronic loss of blood. Women also experience lower abdominal pain or pelvic cramping [18].

Risk-factors

Several well-defined risk factors can increase the incidence of endometrial cancer, especially of the endometrioid type. Epidemiological studies show that an increased exposure to unopposed estrogen levels, either endogenous (i.e. late onset of menopause or estrogen-producing tumors) or exogenous (estrogen-only hormone replacement therapy) can increase the risk for endometrial carcinoma [4, 7, 20-22]. Obesity (Body Mass Index (BMI) ≥ 30 kg/m^2) is also known as a main risk factor for the development of endometrial cancer due to an increased synthesis of estrogens in fat deposits and from adrenal and ovarian precursors [7, 15, 18]. Other risk factors are physical inactivity, early menstruation and late menopause, infertility often resulting in nulliparity, diabetes mellitus and hypertension [7, 23]. Most of these risk factors are related to prolonged, unopposed estrogen action or increased estrogen levels [20, 22].

Therapy

The most important therapy for endometrial cancer is surgery. Besides this, radiotherapy can also be chosen as a therapy for endometrial cancer. Radiation can be delivered externally to the pelvis, as vaginal brachytherapy, or as a combination. Systemic treatment of the cancer is also a possible option for therapy. However, in surgical type I or type II endometrial cancer, there is currently no proof that adjuvant hormone therapy or chemotherapy results in a better outcome [18].

Estrogen metabolism in endometrial cancer

The endometrium is the inner layer of the uterus. Starting at menarche, the uterus undergoes monthly cyclic changes, known as the menstrual cycle. Around the age of 50, menopause starts. From this point, the menstrual cycle becomes irregular and ovaries eventually stop producing estrogen and progesterone. In some postmenopausal women, however, levels of estrogens stay elevated and are not counterbalanced by progesterone, which eventually may lead to the

development of endometrial hyperplasia and even endometrial cancer. In most post-menopausal women, serum estrogen levels are not elevated [21, 24]. This means that the diseased tissue itself is the source of estrogens in these patients. Endometrial carcinoma tissues contain the enzyme systems required for local biosynthesis of estrogen [21]. The primary enzymes involved in the formation of biologically active estrogen are steroid sulphatase (STS), estrogen sulphotransferase (EST), aromatase and 17*β*-hydroxysteroid dehydrogenases (17*β*-HSDs) (*Figure 3*).

Sulphatases (STS) and sulpho-transferases (EST)

Sulphatases (STS) and sulpho-transferases (EST) activate and inactivate sulphated estrogens, respectively. Sulphated estrogens are the most abundant estrogens and the most stable form of circulating estrogens [21].

Aromatase

Aromatase is also known to be responsible for the synthesis of estrogens, by converting the circulating androgens (mainly androstenedione and testosterone) into estrogens (mainly estrone, E_1 and 17*β*-estradiol, E_2) [25]. Androgens are the most abundant circulating steroids produced by the adrenal-cortex and partially by the ovaries. Aromatase (or Cyp19) converts androstenedione to E_1 and testosterone to E_2. There is still a lot of controversy about the role of the aromatase pathway in endometrial cancer.

17β-hydroxysteroid dehydrogenases (17β-HSDs)

The 17*β*-hydroxysteroid dehydrogenases (17*β*-HSDs) are cofactor dependent and are responsible for the conversion of low-active estrogens (E_1) to active compounds (E_2), and *vice versa* [26]. Via reduction and oxidation of estrogens, 17*β*-HSDs regulate the amount of active compound available to bind to its specific receptor. Receptor activation leads to the transcription of target genes (i.e. *TFF-1*) that ultimately stimulate cell proliferation [27, 28]. At present, there are twelve known isoforms of the 17*β*-HSD family [29]. All isoforms belong to the short-chain alcohol dehydrogenase reductase (SDR) superfamily, except for type 5, which belongs to the aldo-keto reductase (AKR) superfamily [26, 27].

17*β*-HSDs type 1, 5, 7 and 12 catalyze the reduction of E_1 to E_2 using NAD(P)H as a substrate, yielding $NAD(P)^+$ as a reaction product [26, 28, 30]. The inactivation of E_2 to E_1 is catalyzed by 17*β*-HSD type 2, 4 and 8 using $NAD(P)^+$ as a substrate, yielding NAD(P)H as a reaction product [26]. The balance between the reduction and oxidation of estrogens in healthy endometrium guarantees that the tissue is exposed to the correct estrogen levels. Besides reducing E_1 to E_2, 17*β*-HSD type 5 is also described to be responsible for the interconversion of androgens, more

specifically for the conversion of androstenedione to testosterone (*Figure 3*) [27]. Besides oxidizing E_2 to E_2, 17β-HSD type 2, 4 and 8 are described to be responsible for the conversion of testosterone to androstenedione [24, 29].

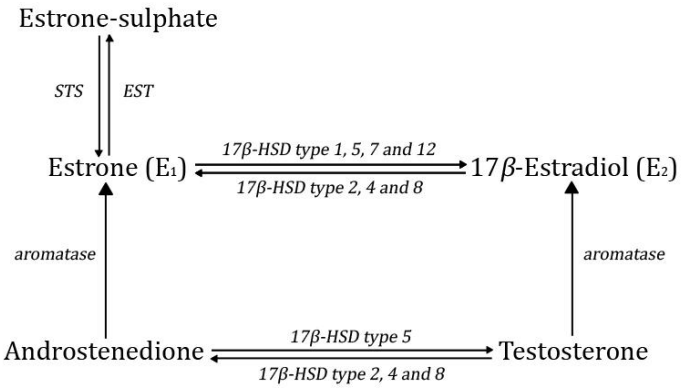

Figure 3: Metabolism of estrogens (and androgens) in human endometrial carcinoma tissues. 17β-estradiol (E_2) can be synthesized via two major pathways: the sulphastase/sulpho-transferase pathway or the aromatase pathway. Both pathways require 17β-hydroxysteroid dehydrogenases (17β-HSDs) to convert low-active estrogen (E_1) or androgens (androstenedione and testosterone) into biologically active estrogen (E_2).

Each year, endometrial cancer develops in about 142,000 women worldwide, and an estimated 42,000 women die from it [18]. Although the tumor is confined to the uterus in most cases, in approximately 25% of endometrial cancer cases, the tumor has already spread beyond the uterus upon surgery and eventually these patients die of metastasis. The exact cause of endometrial cancer is still unknown, but excessive and/or prolonged exposure to increased levels of unopposed estrogens, in particular the biologically active form, E_2, is considered to play an important role in the pathogenesis and development of human endometrial carcinoma [4, 21, 24]. Most estrogen-dependent endometrial carcinomas develop after menopause, when ovaries have ceased to produce estrogens [24]. Nevertheless, the majority of post-menopausal women show no elevation in serum estrogen levels, which suggests that the diseased tissue itself is the source of estrogens in these patients [21, 24, 31]. Previous studies indicate that the expression of 17β-HSDs greatly contributes to the *in situ* availability of biologically active estrogen (E_2) [21, 24, 31]. Recently, 17β-HSDs have been shown to be aberrantly expressed in breast tumors and endometriotic lesions, which leads to increased levels of E_2 [31-33]. Moreover, preliminary data from our lab, obtained using High-Pressure Liquid Chromatography (HPLC; [34]), showed that 17β-HSDs metabolism is disturbed in endometrial cancer (*Figure 4*). Consequently, the inhibition of local 17β-HSD activity provides a potential therapeutic approach to treat endometrial cancer by reducing biologically active estrogen in endometrial cancer tissue. Still, it is not known which of the different 17β-HSDs are responsible for the high concentration of E_2 in endometrial cancer tissue.

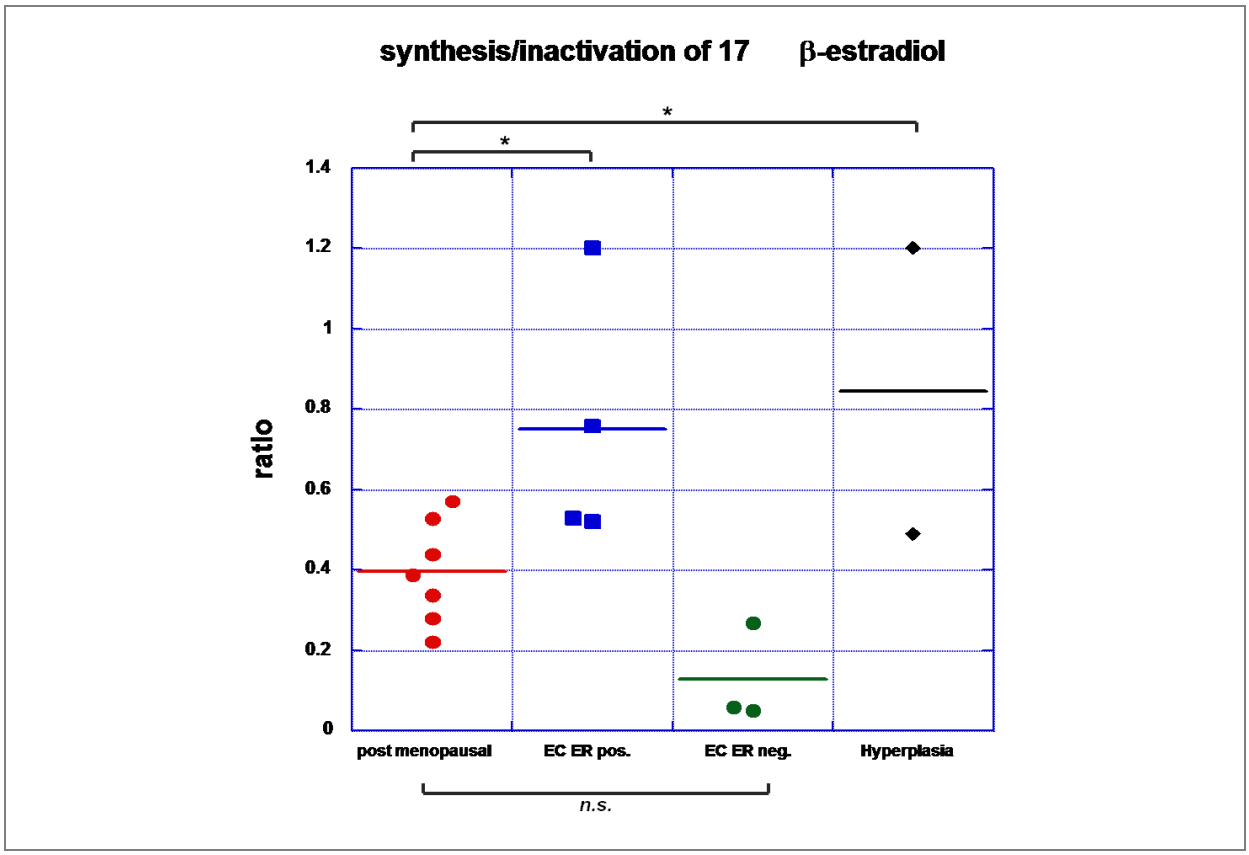

Figure 4. Preliminary data showing the ratio between the reducing/synthesizing 17*β*-hydroxysteroid dehydrogenases (17*β*-HSD1, -5, -7, -12) and the oxidizing/inactivating 17*β*-HSD (17*β*-HSD-2, -4 and -8) to be increased in endometrial hyperplasia tissue and endometrial cancer tissue samples. Postmenopausal tissues were used as a positive control. Estrogen receptor positive (ER^+) endometrial cancer tissue and endometrial hyperplasia tissue show a significant (*P-value* < 0.05) increase in ratio compared to postmenopausal control tissue. ER^- tumor tissue shows no significant difference in ratio compared to postmenopausal control tissue. Statistical significance was defined as *P-value* < 0.05 and is represented by an asterisk. Non-significant results are shown as n.s.

17*β*-HSD type 1 (17*β*-HSD1) is the most well characterized of the 17*β*-HSDs and catalyzes the reaction of E_1 to E_2 [27]. In breast cancer and endometriosis, 17*β*-HSD1 was already found to be the responsible enzyme for the conversion of E_1 to E_2, leading to a high concentration of E_2 in the diseases tissue [35-37]. The same is expected for endometrial cancer. In this way, inhibition of local 17*β*-HSD1 production would provide a potential therapeutic approach to treat endometrial cancer by reducing the level of E_2 in the endometrial tissue.

Besides 17*β*-HSD1, 17*β*-HSD5 also draw the attention, since Ito et al. declared 17*β*-HSD5 as one of the key enzymes in the regulation of local estrogen concentration in endometrial malignancy [21].

Research question, hypothesis, objectives and aims

Concerning the role of estrogen metabolism in endometrial cancer, the following question was addressed: "**What causes the disturbed estrogen metabolism in endometrial cancer?**".

Consequently, it was hypothesized that ***17β-HSDs are aberrantly expressed in endometrial cancer, which leads to a hyper-estrogenic environment in the endometrial tissue.*** To test this hypothesis, it was first investigated which of these 17*β*-HSDs were aberrantly expressed in endometrial cancer by comparing their expression between human endometrial cancer biopsy tissues and postmenopausal endometrial control tissues using quantitative real-time PCR (qRT-PCR). Secondly, clinical sample results, indicating an aberrant 17*β*-HSD1 expression in endometrial cancer tissue, were confirmed *in vitro*, more specifically in endometrial cancer (ECC-1) cell lines. The role of an aberrant 17*β*-HSD1 expression on E_2 synthesis was investigated *in vitro* using a luciferase assay, which indirectly measures the level of available E_2 by assessing the activation of an estrogen-responsive-luciferase construct. Thirdly, *in vitro* findings were reconfirmed in ECC-1 cells using a proliferation assay. The role of an aberrant 17*β*-HSD1 expression on the potential carcinogenesis of the endometrium was determined by investigating cell proliferation. To this end, S-phase entry of the cell cycle was measured using a BrdU incorporation assay. Finally, during *in vitro* confirmation of clinical sample results, aromatase was found to have some implication in E_2 synthesis. To confirm these interesting observations, the effect of aromatase inhibition was investigated via similar *in vitro* approaches.

Materials and methods

Tissues

Endometrial cancer tissue was collected from post-menopausal women of 48 – 92 years of age (n = 18), who underwent surgery for removal of the uterus (hysterectomy). Endometrial control tissue was collected from post-menopausal women of 58 – 80 years of age (n = 5), who underwent hysterectomy for non-malignant indications. The tissue was collected from residue hysterectomy specimens. After inspection of the uteri by a pathologist, normal and pathological endometrial tissues were brought on ice within 30 minutes of hysterectomy. Histological examination showed that eleven endometrial cancer tissues were grade I tumors, four tissues were grade II tumors and three were grade III tumors. Histological examination also confirmed postmenopausal endometrial control tissue to be non-malignant tissue. Tissues were deposited in an 1.5 ml Eppendorf tube on ice, or imbedded in Optimal Cutting Temperature compound (Tissue-Tek® O.C.T.™; Sakura Finetek Europe b.v., Zoeterwoude, the Netherlands). One part of the endometrial cancer tissues and postmenopausal control tissues were frozen in TRIsure Isolation Reagent TM (Roche, Basel, Switserland) at -80°C until mRNA isolation. Another part of the biopsies was fixed in formalin and imbedded in paraffin, for use in immunohistochemistry.

Immunohistochemical staining of 17β-HSD1

17*β*-HSD1 expression in endometrial cancer tissue sections and postmenopausal control tissue sections was assayed using immunohistochemistry on paraffin embedded tissue. First, to confirm that paraffin sections were tumor tissue, sections were stained with haematoxylin and counterstained with eosin. Sections were prepared for mounting by dehydration in a series of ethanol and xylene and were mounted on glass slides using Entellan mounting solution (Merck KgaA, Darmstadt, Germany). Haematoxylin/eosin stained cryosections were analyzed using a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan) and photographed with a Nikon digital Camera DXM 1200. Secondly, before incubation with primary antibody, paraffin sections were baked during 30 minutes at 80°C. After deparaffinization and rehydratation in a series of xylene and ethanol, the sections were boiled in pepsin with 0.01 N Hydrochloride (HCl; pH = 2) for 30 minutes and cooled slowly to room temperature (RT). Sections were exposed to 1% hydrogen peroxide (H_2O_2) for 20 minutes and washed with 1 x PBS (Phosphate Buffered Saline; 10 x PBS stock: 2.03g NaH_2PO_4, 11.49g Na_2HPO_4, 85g NaCl, pH=7.4). After blocking sections with 5% goat serum in PBS to saturate non-specific binding sites (30 min, RT), slides were incubated overnight with rabbit anti-17*β*-hydroxysteroid dehydrogenase type 1 antibody (anti-17*β*-HSD1 IgG; dilution 1:250; Epitomics, Burlingame, CA, U.S.A.) at 37°C. After

washing with 1 x PBS, sections were incubated (30 min, RT) with labeled polymer-horse-radish peroxidase anti-mouse IgG (Real™ Envision™ DAKO HRP Rabbit/Mouse, ready-to-use, Glostrup, Denmark) and color reaction was performed with 3,3'-diaminobenzidine (liquid DAB+; Dako, Glostrup, Denmark). Sections were counterstained with haematoxylin and dehydrated in a series of ethanol and xylene before mounting with Entellan mounting solution (Merck KgaA, Darmstadt, Germany). Slides were examined using a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan) and photographed with a Nikon digital Camera DXM 1200.

Total RNA isolation and cDNA synthesis.

Total RNA from endometrial cancer tissues and postmenopausal control tissues was extracted using the TRIsure method (Bioline GmbH, Luckenwalde, Germany), according to the manufacturer's protocol. In short, the tissue was thawed at 4°C prior to RNA isolation and homogenized in TRIsure reagent using a Ultra Turrax® T25 basic homogenizer (IKA-WERKE®; Straufen, Germany) with a 8-mm knife. RNAse-free chloroform was added. Samples were thoroughly mixed, incubated (3 min, RT) and centrifuged (12,000 g, 15 minutes). The upper aqueous phase was collected in a new 1.5 ml Eppendorf tube and RNAse-free isopropanol was added to precipitate the RNA. After inversion, samples were incubated (20 min, RT) and centrifuged (12,000 g, 10 min). Pellets were washed with RNAse-free 75% ethanol, air-dried, and collected in RNase-free water. After incubation on ice during 2 hours, RNA quantity and quality was assessed using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA) at 260-nm and 280-nm wavelengths. Finally, mRNA was frozen at -20°C until cDNA synthesis. Five hundred nanograms of total RNA were further subjected to complementary DNA (cDNA) synthesis reaction using iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA, USA) following the instructions of the manufacturer. In short, complete reaction mix (*per sample:* 4 µl 5x iScript reaction mix, 1 µl iScript™ reverse transcriptase and 500 ng RNA supplemented with sterile H_2O to an end volume of 20 µl) was incubated 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C.

Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) analysis

Total RNA isolated from endometrial cancer tissue and postmenopausal control tissue was subjected to quantitative amplification using quantitative real-time PCR (qRT-PCR). Analysis was performed using the SensiMix™ SYBR & Fluorescein Kit (Bioline GmbH, Germany) according to the manufacturer's protocol. Standard 3-step cycling was used with the following conditions: 95°C for 10 min repeated for one cycle to activate the polymerase added to the reaction, 95°C for 15 s, 60°C for 15 s and 72°C for 15 s repeated for 40 cycles. qRT-PCR was performed using the

Bio-Rad MyIQ apparatus. Oligonucleotides used for quantitative PCR amplification were purchased from MWG-Biotech AG (Ebersberg, Bayern, Germany) and are listed in *Table 1*.

Table 1. Oligonucleotides used for qRT-PCR analysis. F indicates forward primer and R indicates reverse primer.

Primer	Forward sequence	Reverse Sequence
17*β*-HSD1	5'-GTT TAT TGC GCC AGC AAG TT-3'	5'-CCC AAC ACC TTC TCC ATG A-3'
17*β*-HSD2	5'-ACC TGT GGA TCA GAA GGC AG-3'	5'-TCA AAA CTC CGG CAA ATA CC-3'
17*β*-HSD5	5'-AAT GAG CAG CCC ATC AGA C-3'	5'-GGG TGG CTA GCA AAA CTA TCA-3'
17*β*-HSD7	5'-ACC ACT GGC TTT GGA AGA AAT-3'	5'-TGA CCC TAA TGT GCT TTT CCA-3'
17*β*-HSD12	5'-GTC TGG GGA GTG GGG AAT-3'	5'-CCT TCA TTC CAT GCT TTG CT-3'
cyclophilin A	5'-CCG TGT TCT TCG ACA TTG CCG T-3'	5'-AAT CCT TTC TCT CCA GTG CTC AGA-3'
β-actin	5'-GCC AAC CGC GAG AAG ATG AC-3'	5'-GAT GGG CAC AGT GTG GGT GAC-3'

Human cyclophilin A and *β*-actin were used as house-keeping genes to normalize the differences in the amount of cDNA added to each qRT-PCR reaction and were purchased from Applied Biosystems (Foster City, CA, USA). All data were processed using the delta-Rn method, which permits to compare the expression level of distinct genes. Briefly, the delta-Ct (ΔCt), meaning the cycle threshold (Ct) of the studied gene minus the Ct of its corresponding house-keeping gene (cyclophilin A and *β*-actin), was first calculated. Next, the delta-delta Ct (ΔΔCt) was determined, which represents the difference between the ΔCt of the sample minus the ΔCt of the lowest expressing sample for the same gene, namely the average of the control samples. Finally, the relative expression level (fold change) was assessed with this formula: $2^{-(\Delta\Delta Ct)}$. Fold changes are represented graphically on a logarithmic scale using the GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) software program and statistical analysis was performed using the KaleidaGraph (Synergy Software, Reading, PA) software package.

In vitro cell model

The ECC-1 cell line is derived from a well-differentiated adenocarcinoma of a human endometrium transplanted into nude mice, and was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA; ATCC® number: CRL-2923™). Cells were routinely maintained in phenol-red containing Roswell Park Memorial Institute medium (RPMI 1640; Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 2mM Sodium Pyruvate, 100 U/l penicillin and 100 mg/l streptomycin in a humidified incubator at 5% CO_2 and at 37°C. For all experiments involving hormonal stimulation, ECC-1 cells were cultured for minimum three days prior to, and during the experiments in RPMI 1640 without phenol-red supplemented with 5% hormone-stripped serum, 2 mM L-glutamine, 2 mM Sodium Pyruvate, 100 U/l penicillin and 100 mg/ml

streptomycin in a humidified incubator at 5% CO_2 and at 37°C. For transfection experiments, cells were plated at 50% confluency per well in 6-well plates. For stimulatory experiments, cells were plated at 50% confluency per well in 24-well plates.

Proliferation assay: BrdU incorporation

ECC-1 cells (ATCC, Rockville, MD, USA) were cultured for minimum three days in RPMI 1640 without phenol-red supplemented with 5% hormone-stripped serum, 2 mM L-glutamine, 2 mM Sodium Pyruvate, 100 U/l penicillin and 100 mg/ml streptomycin in a humidified incubator at 5% CO_2 and at 37°C. Cells were seeded at 50% confluency on glass coverslips in 6-well plates prior co-incubation of 5-bromo-2'-deoxyuridine (BrdU) with estrone (E_1; 0.01 nM – 10 nM), 17β-estradiol (E_2; 1 nM), testosterone (T; 0.1 nM – 10 nM) or anastrozole (aromatase inhibitor; 5 µM). After removal of growth medium, cells were fixed overnight using 100% ice-cold methanol (MeOH). Cells were washed with 1 x PBS and permeabilized using 0.2% Triton X-100 dissolved in 1 x PBS (15 min, RT). After permeabilization of the cells, DNA was denaturated by incubating cells in 0.2 N hydrogen chloride (HCl) (20 min, 37°C). Acid was neutralized by immersing glass coverslips in 0.1 M Sodium Tetraborate (pH = 8.5). Cells were incubated with BrdU monoclonal antibody (dilution 1:200; Becton, Dickinson and Company, Heidelberg, Germany) during 2 hours at 37°C, followed by incubation with goat anti-mouse TexasRed IgG antibody (dilution 1:100; Jackson Immunoresearch Laboratories Inc, Cambridge, UK) or goat anti-mouse Cy3 IgG antibody (dilution 1:500; Jackson Immunoresearch Laboratories Inc, Cambridge, UK) during 1 hour at 37°C. Cells were mounted onto slides using the nuclear staining DAPI (4'-6-diamidino-2-phenylindole; DABCO in glycerol, supplemented with 1 M Tris-HCl (pH 7.5) and adjusted to pH 8.0). Glass slides were inspected using DAPI (for nuclear visualization) and Cy3 (for visualization of BrdU) filter settings and images were acquired with an SD200 SpectraCube system (Applied Spectral Imaging, Carlsbad, CA) mounted on a Leica DM RBE microscope (Leica Microsystems, Wetzlar, Germany). The number of BrdU-positive ECC-1 cells was evaluated quantitatively using a 40X oil immersion objective.

Transient transfection

17β-HSD1, -5 and -12 cDNA expression plasmids were a generous gift from Joanna Day (London, U.K.). DNA (± 200 ng) was amplified in *Escherichia coli* XL2-blue cells by heat-shock. Plasmids for transfection were prepared using the GENElute mini-prep kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Plasmid concentration was determined spectrophotometrically and by agarose gel electrophoresis. The ERE-tk-LUC construct was a generous gift from Prof Schuele, and contains the estrogen-responsive element gene promoter fused upstream of the luciferase reporter as described previously [38, 39]. For transient

transfection assays, ECC-1 cells were seeded in 6-well plates at 50% confluency per well. After 24h, ECC-1 cells were transfected with either an empty vector, a pCEP4 vector containing 17β-HSD1, a pcDNA3.1 vector containing 17β-HSD5 or a pCEP4 vector containing 17β-HSD12 using JetPEI™ (PEI: polyethylenimine, Q-BioGene Molecular Biology, Heidelberg, Germany) according to the manufacturer's instructions with minor modifications based on experience of my co-workers.

Preparation of protein extracts and western immunoblotting

Cell lysates were prepared using RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0,25% sodiumdeoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with complete proteinase inhibitor (Mini Protease Inhibitor Cocktail Tablets supplied in EASYpack; Roche Applied Science, Mannheim, Germany). Protein concentrations were determined using the BC Assay Protein Quantitation Kit (Uptima, Interchim, France). Proteins were boiled with Laemmli sample buffer (1:1; Bio-Rad Laboratories, Hercules, CA, USA) during 5 minutes and subsequently separated by SDS-PAGE on a 10% Sodium dodecyl sulfate (SDS) polyacrylamide gel (200V, 40 min, RT). After separation, proteins were transferred to nitrocellulose membranes (Protran Nitrocellulose transfer membrane, Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) (100V, 1h, 4°C). Equal sample loading and transfer were confirmed by Ponceau S (Sigma Aldrich) staining before incubation of membranes with 5% non-fat milk (Difco™ Skim Milk, Becton, Dickson and company, Sparks, MD, USA) during 1 hour, to saturate the non-specific binding sites. Membranes were incubated overnight at 4°C with rabbit anti-17β-HSD type 1 (anti-17β HSD1; dilution 1:10,000; Epitomics, Burlingame, CA, USA) to visualize 17β-HSD1 proteins. After washing with 1 x TBS-T (10 x stock: 24.23g Tris base, 87g HCl, pH = 7.6), membranes were incubated with the peroxidase-conjugated AffiniPure goat anti-rabbit IgG antibody (dilution 1:50,000; Jackson Immunoresearch Laboratories Inc., Cambridge, UK) during 1 hour at RT. After washing with 1 x TBS-T, proteins were visualized using the Western Lightning® Ultra chemiluminescence substrate (dilution 1:1; Perkin Elmer, Waltham, MA, USA) according to the manufacturer's instructions. Western blot density was analyzed using a Kodak Biomax Cassette (Eastman Kodak Company, Rochester, New York) and the appropriate Super RX photo developing film.

Luciferase assay

Prior to and during luciferase assay, ECC-1 cells were cultured for minimum three days in RPMI 1640 without phenol-red supplemented with 5% hormone-stripped serum, 2 mM L-glutamine, 2 mM Sodium Pyruvate, 100 U/l penicillin and 100 mg/ml streptomycin in a humidified incubator at 5% CO_2 and at 37°C. ECC-1 cells were seeded at 50% confluency per well onto 6-well plates in equal medium and incubated overnight. In each well, cells were transfected with

different expression plasmids (empty vector, 17*β*-HSD1, -5, and -12; 2.8 µg) along with the *ERE-tk-LUC* (1.2 µg) using jetPEI™ (QBioGene Molecular Biology, Heidelberg, Germany) according to the manufacturer's instructions with minor modifications based on the experience of my co-workers. Cells transfected with an empty vector were used as a control.

The *ERE-tk-LUC* construct is used as a reporter, which contains two estrogen-responsive elements (EREs), one herpes simplex thymidine kinase minimal promoter and one firefly luciferase reporter gene. ECC-1 cells are estrogen receptor-alpha (ERα) positive. Therefore, when E_2 is synthesized in the cells, it binds to and activates the ERα. Activated ERα binds to the *ERE-tk-LUC* and activates the promoter, driving the expression of the luciferase gene in an estrogen-dependent manner. The principle behind this is the following: the more E_2 is synthesized, the more activated ERα will bind to the *ERE-tk-LUC* construct and the more luciferase will be expressed.

After transfection, cells were detached, and seeded into 96-well plates. Twelve hours after transfer, stimuli were added to the growth medium. ECC-1 cells were cultured for 24 hours in the presence of ethanol, increasing concentrations of estrone (E_1; 0.01 nM – 10 nM), 17*β*-estradiol (E_2; 0.01 nM – 10 nM), androstenedione (A; 0.01 nM – 10 nM), testosterone (T; 0.01 nM – 10 nM), or anastrozole (aromatase inhibitor; 5 µM). Each treatment was performed in triplicate. After 24 hours of stimulation, cells were harvested in lysis buffer (0.1 M Tris pH 7.8, 0.5% Triton X-100). During 15 minutes of incubation, 96-well reading plate for luciferase assay was prepared following instructions provided by the manufacturer. After incubation, cell lysate was transferred to the reading plate and luciferase activity was measured in a GloMax® 96 microplate luminometer (Promega Gmbh, Mannheim, Germany) using the Steady Glo™ Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luciferase that is present in the cell extract will induce the oxidation of the Steady Glo™ luciferine substrate, leading to the emission of a bioluminescent signal, that can be measured using a luminometer. The intensity of the light emission is linearly related to the amount of firefly luciferase present in the cell extract. Measurements were repeated in triplicate, and standard deviations were calculated from these three measurements.

Chemicals

All steroid hormones used (estrone (E_1), 17*β*-estradiol (E_2), androstenedione and testosterone) were purchased from Sigma Aldrich Chemie BV, the Netherlands. All hormones were dissolved in ethanol at a stock concentration of 10^{-5} M. Anastrozole (aromatase inhibitor) and other chemicals used during the experiments were purchased from Sigma Aldrich.

Culture supplementations were purchased from Gibco® BRL (Grand Island, New York, USA).

Statistical analysis

For qRT-PCR results, statistical significance was compared between endometrial cancer tissue samples and post-menopausal control tissues using the non-parametric Wilcoxon signed rank test (unpaired) via the KaleidaGraph (Synergy Software, Reading, PA) software package. Values were expressed as the average gene expression level. Statistical significance was defined as *P-value* < 0.05 and is represented by an asterisk. Non-significant results are indicated as n.s. For luciferase assays and BrdU incorporation assay, statistical significance was calculated using the student's *t*-test. Values are expressed as mean ± SD.

Results

The main purpose of this research was to investigate which of the different 17β-HSDs are responsible for the high concentration of E_2 in endometrial cancer tissue. As an aberrant expression of 17β-HSD1 was already found to be the responsible enzyme for increased E_2 levels in breast cancer and endometriosis, other estrogen-dependent diseases, the question arose whether this is also true in endometrial cancer. This question is addressed using the specific approach described in *Figure 5*.

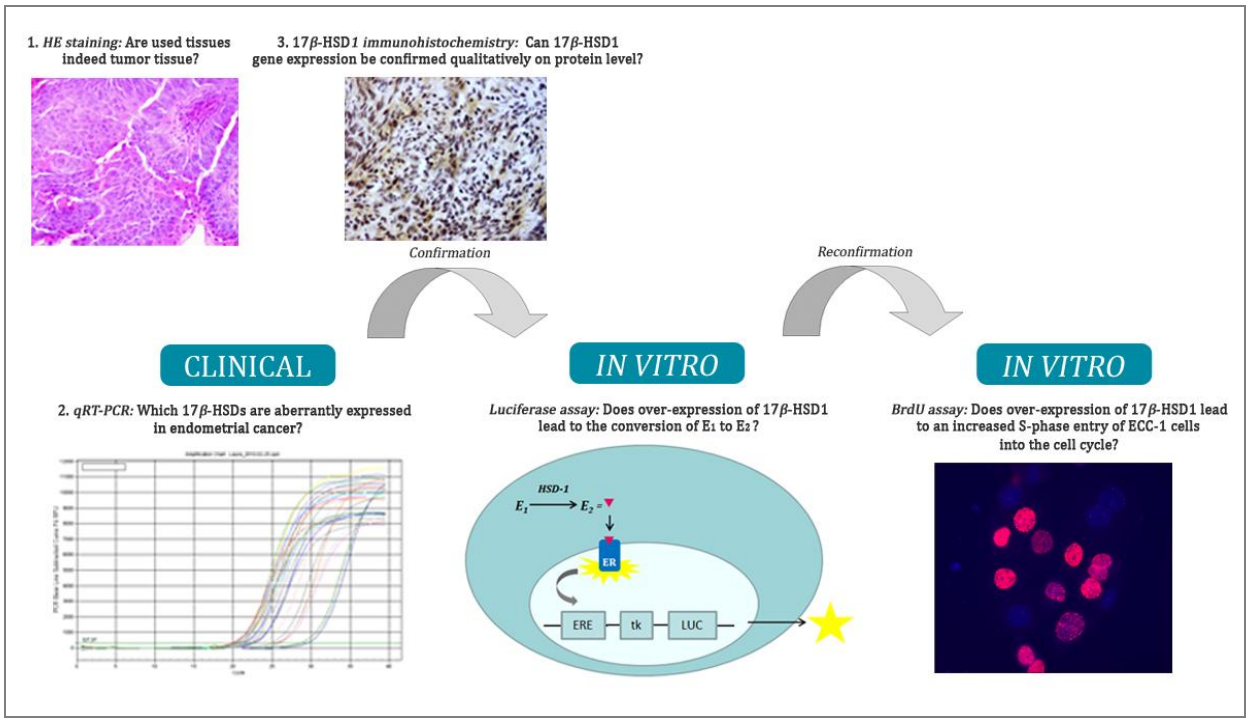

Figure 5. Approach used to test the hypothesis that 17β-hydroxysteroid dehydrogenases (17β-HSDs) are aberrantly expressed in endometrial cancer, which leads to a hyper-estrogenic environment. From left to right: the experiments performed with clinical samples (HE staining, qRT-PCR, and 17β-HSD1 staining), *in vitro* confirmation of the found results (luciferase assay), *in vitro* reconfirmation of these results (BrdU incorporation assay).

Initially, clinical samples are used to address this main question. To ensure that tissue samples used for the experiments were endometrial cancer tissues or postmenopausal control tissues, 5 µm slices from the paraffin embedded tissues were used for haematoxylin/eosin staining and were evaluated by the pathologist. Histological examination confirmed endometrial cancer tissue to be malignant tissue and postmenopausal endometrial control tissue to be non-malignant tissue. Next, it was investigated which of the different 17β-HSDs are aberrantly expressed in endometrial cancer by comparing the expression of the different enzymes between endometrial cancer tissue and postmenopausal control tissue using qRT-PCR. An increase in

17*β*-HSD1 gene expression was detected in endometrial cancer tissue. Expression of 17*β*-HSD1 on protein level and its localization was confirmed by qualitative evaluation using immunohistochemical staining, which showed a more pronounced immunoreactivity in endometrial cancer tissue.

Secondly, clinical sample results were confirmed *in vitro*, more specifically in endometrial cancer (ECC-1) cell lines. The role of an aberrant 17*β*-HSD1 expression on E_2 synthesis was investigated *in vitro* using a luciferase assay, which indirectly measures the level of available E_2 by assessing the activation of an estrogen-responsive luciferase construct. When over-expressing 17*β*-HSD1 in ECC-1 cells, its role in the conversion of E_1 to E_2 was demonstrated, confirming that increased 17*β*-HSD1 expression stimulates E_2 synthesis.

Thirdly, *in vitro* findings were reconfirmed in ECC-1 cells using a proliferation assay. The effect of an aberrant 17*β*-HSD1 expression in the potential carcinogenesis of the endometrium was determined. To this end, S-phase entry of the cell cycle was measured using a BrdU incorporation assay. ECC-1 cells were found to have an increased incorporation of BrdU after stimulation with E_2, compared to untreated cells, whereas cells stimulated with E_1 were not affected. However, in cells over-expressing 17*β*-HSD1, E_1 had proliferative effects similar to those observed for E_2.

Finally, during luciferase assays, it was observed that testosterone was able to activate the estrogen-responsive reporter, indicating a possible aromatase activity. To confirm these interesting observations, the effect of aromatase inhibitors was investigated via both luciferase assays and BrdU incorporation assays. When ECC-1 cells were stimulated with testosterone, which is converted to E_2 by aromatase, an increased luciferase activity was measured, and this effect was impaired by the use of an aromatase inhibitor. In addition, testosterone also induces cell proliferation, and this effect is inhibited by an aromatase inhibitor. These results suggest that at least *in vitro*, aromatase plays an important role in E_2 synthesis.

Expression of 17β-hydroxysteroid dehydrogenase type 1 is increased in endometrial cancer tissue

It is well known that 17*β*-HSDs play a role in the metabolism of estrogens. The 17*β*-HSD1, -5, -7 and -12 catalyze the reduction of the low-active estrone (E_1) to the highly estrogenic 17*β*-estradiol (E_2). The inactivation of E_2 to E_1 is catalyzed by 17*β*-HSD2, -4 and -8 (*Figure 6A*).

It was investigated which of the different 17*β*-HSDs was responsible for the high concentration of E_2 in endometrial cancer tissue. To this end, the expression of the different enzymes was compared between endometrial cancer biopsies and postmenopausal control tissues using qRT-PCR. Only 17*β*-HSD1 gene expression is found to be significantly (*P-value* < 0.05) increased in endometrial cancer biopsies compared to postmenopausal control tissues (*Figure 6B*). Besides

this, all three grades of endometrial tumor tissue show no difference in gene expression level, which means all tumors have an equal gene expression level of 17β-HSD1.

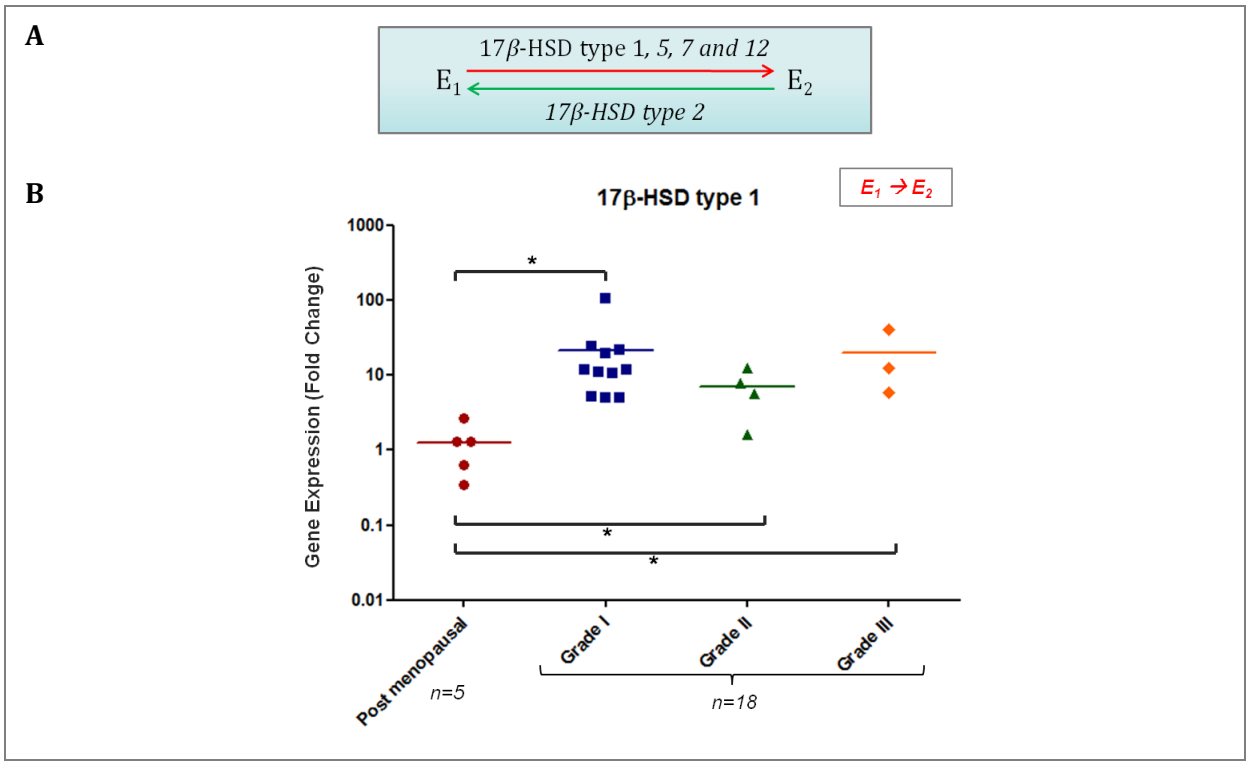

Figure 6. Gene expression level of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) is significantly increased in endometrial cancer biopsies compared to postmenopausal control biopsies. A. Conversion of low-active estrogen (estrone, E_1) to biologically active estrogen (17β-estradiol, E_2) by 17β-HSDs. **B.** Gene expression level of 17β-HSD1 is significantly (*P-value* < 0.05) increased in endometrial cancer tissues (grade I – grade III; n_{tot}=18) compared with the postmenopausal control tissues (n=5). Differences in expression levels between grade I, II or III tumors are not significant. The average gene expression level of postmenopausal control tissues was set at a level of one. Values were expressed as the average gene expression levels and are given as fold changes on a logarithmic scale. Statistical significance was defined as *P-value* < 0.05 and is represented by an asterisk. Non-significant results are indicated as n.s.

For the other reducing enzymes (17β-HSD-5, -7 and -12), no significant difference in expression level is detected between endometrial cancer tissues and postmenopausal control tissues (*Figure 7*). Besides reducing 17β-HSDs, oxidizing 17β-HSDs (17β-HSD2, -4, and -8) may also play a role in endometrial cancer. Literature already showed 17β-HSD4 and 17β-HSD8 not to be involved in the conversion of E_2 to E_1 in endometrial cancer, which led us to focus on 17β-HSD2 [29, 40-42]. The gene expression of 17β-HSD2 is not significantly different between both tissues (*Figure 7*). Taken together, these data indicate that 17β-HSD1, and not 17β-HSD2, -5, -7, or -12 gene expression is significantly increased in endometrial cancer tissue compared to postmenopausal control tissue.

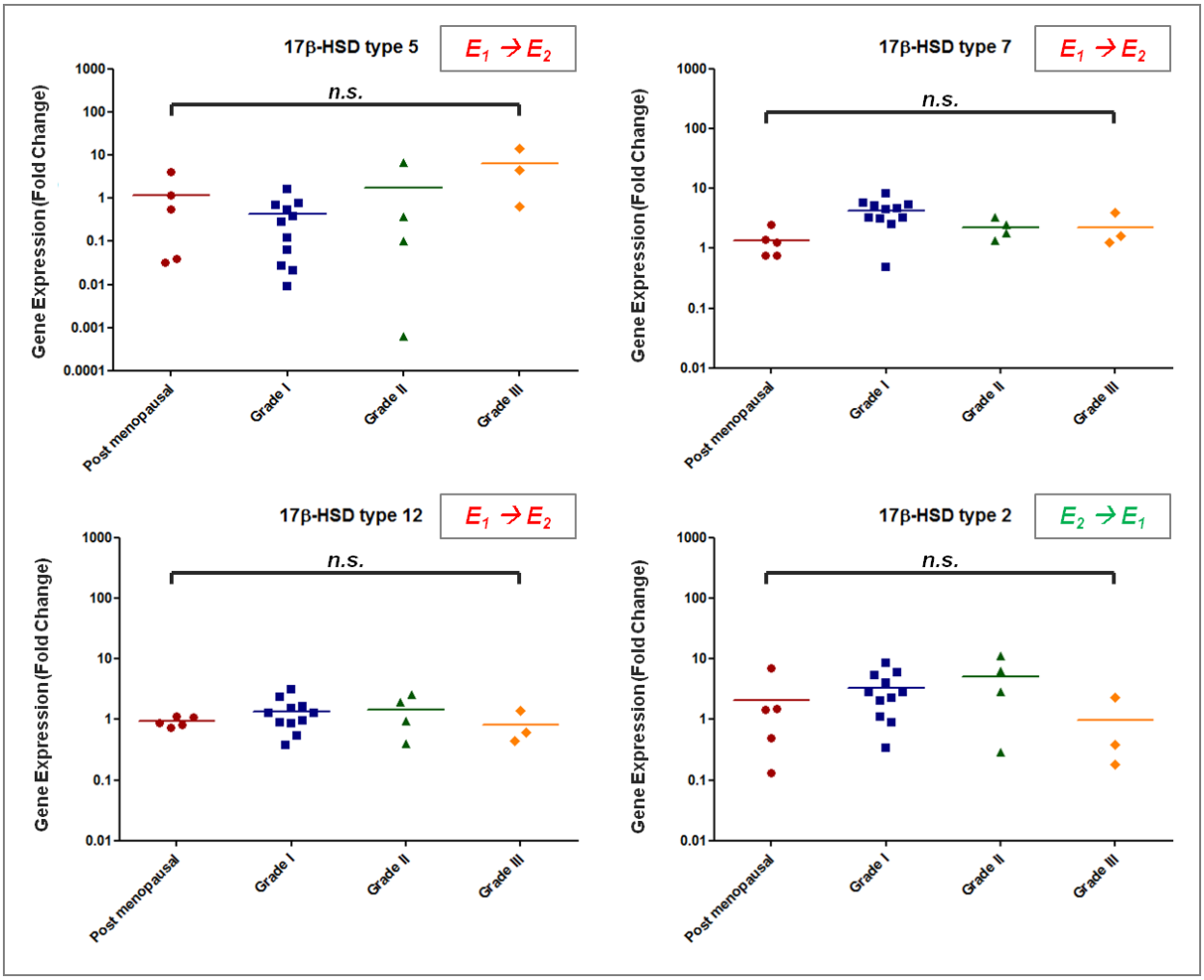

Figure 7. Gene expression level of other reducing enzymes (17*β*-HSD5, -7 and -12) and gene expression level of 17*β*-HSD2 (oxidizing enzyme) is not increased in endometrial cancer biopsies compared to postmenopausal control biopsies. Gene expression level of 17*β*-HSD5, -7 and -12 is not significantly different between endometrial cancer tissue and postmenopausal control tissue. The same is true for 17*β*-HSD2. The average gene expression level of postmenopausal control tissues was set at a level of one. Values were expressed as the average gene expression levels and are given as fold changes on a logarithmic scale. Statistical significance was defined as *P-value* < 0.05 and is represented by an asterisk. Non-significant results are indicated as n.s.

As this research is part of a larger research project about estrogen metabolism in endometrial cancer, the results found after the qRT-PCR experiments can be correlated with results found by a medical student at the department of Gynaecology & Obstetrics. Protein activity of 17*β*-HSD1 was measured in the same endometrial cancer tissues as those used for qRT-PCR experiments. Both activity and gene expression of 17*β*-HSD1 are found to correlate (*Figure 8*).

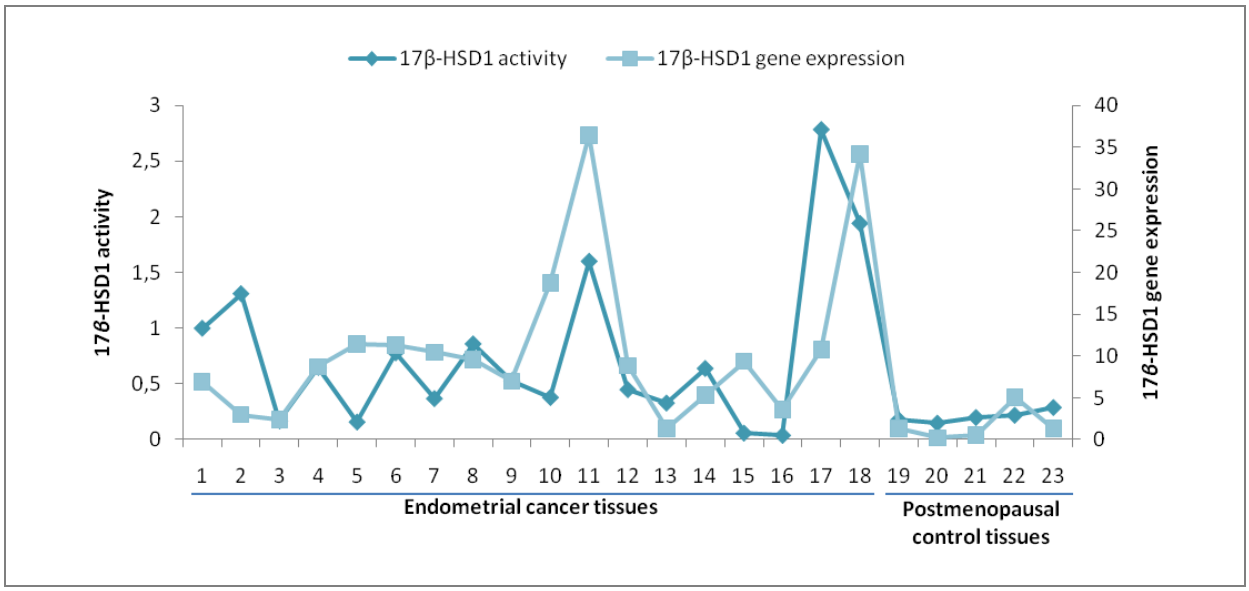

Figure 8. Gene expression and protein activity of 17β-HSD1 correlate in both endometrial cancer tissues and postmenopausal control tissues. 17β-HSD1 gene expression is given in fold change values and 17β-HSD1 protein activity is given as the ratio between the reducing and the oxidizing 17β-HSD enzymes.

To further confirm these results on protein level, qualitative immunohistochemical stainings were performed. Immunohistochemical stainings were performed on paraffin embedded sections from endometrial cancer tissues and postmenopausal control tissues to examine whether 17β-HSD1 expression could be detected on protein level and whether its localization could be confirmed. After preparation of paraffin embedded sections in pepsin, sections were stained for 17β-HSD1. All endometrial cancer tissues show a higher expression of 17β-HSD1, though this was only evaluated qualitatively (*Figure 9-EC*). No staining for 17β-HSD1 is detected in postmenopausal control tissues (*Figure 9-N*). Placental tissue was used as a positive control, and high 17β-HSD1 immunoreactivity is detected (*Figure 9-P*). Immunoreactivity for 17β-HSD1 is represented by a brown staining and is detected primarily in cytoplasm of carcinoma cells. These results demonstrate a higher expression of 17β-HSD1 on protein level in endometrial cancer tissue compared to postmenopausal control tissues, confirming results found with qRT-PCR analysis.

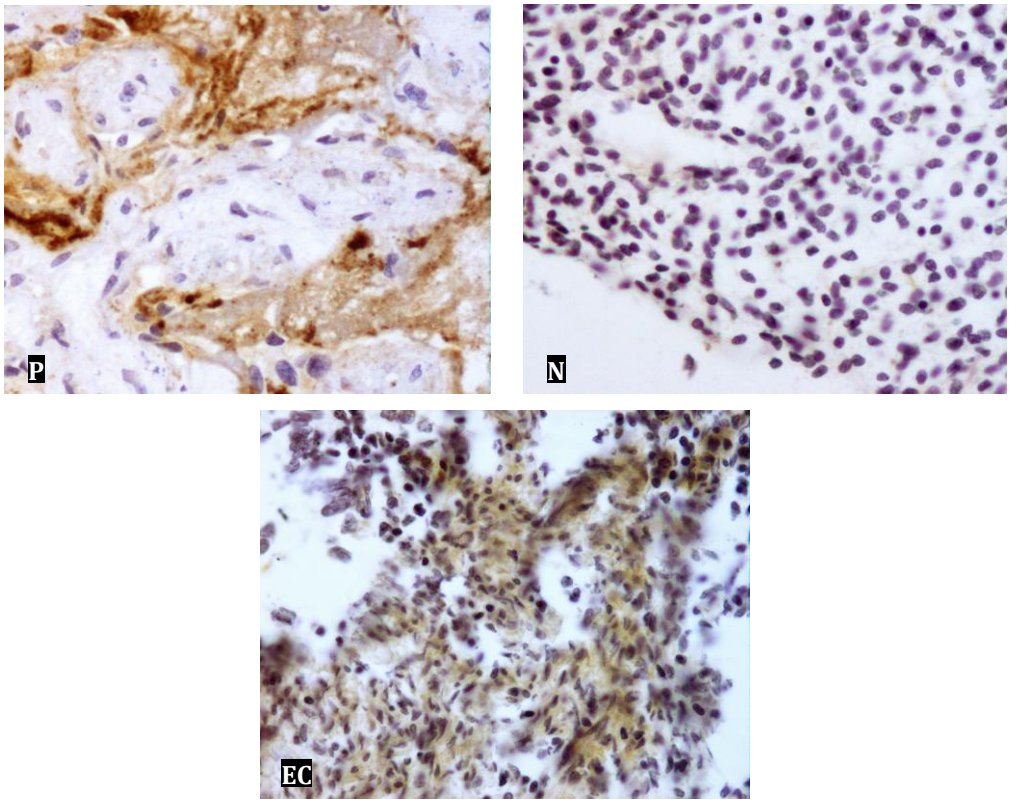

Figure 9. Expression of 17*β*-HSD1 on protein level is increased in endometrial cancer tissue compared with postmenopausal control tissue. Immunohistochemical staining for 17*β*-HSD1 in paraffin embedded endometrial cancer tissue and postmenopausal control tissue. **P** stands for placenta tissue, used as a positive control, showing a high immunoreactivity represented by the brown staining. **N** stands for postmenopausal control tissue, which shows no immunoreactivity and **EC** stands for endometrial cancer tissue, showing immunoreactivity for 17*β*-HSD1 in the cytoplasm of the cells. Paraffin embedded tissues were obtained from the same patients whose material was used for qRT-PCR analysis. Images were visualized using a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan) and they were photographed using a Nikon digital Camera DXM 1200. Images were evaluated using a 40X objective.

Over-expression of 17β-HSD1 increases estrogen synthesis by converting estrone, E_1, to 17β-estradiol, E_2, using in vitro model systems

Results found with the clinical samples, showing that 17*β*-HSD1 gene expression is increased in endometrial cancer tissue, are confirmed *in vitro* by investigating the role of an aberrant expression of 17*β*-HSD1 on estrogen synthesis. To this end, endometrial cancer cell (ECC-1) lines are used, that do not express 17*β*-HSD1 at basal level, making them the ideal cell line to use for these experiments. Prior to stimulatory experiments, ECC-1 cells are transfected with 17*β*-HSD1. An efficient over-expression of 17*β*-HSD1 in transfected ECC-1 cells was confirmed via western blotting (*Figure 10*). Placental protein lysates were used as a positive control and express the 17*β*-HSD1 protein with a size of 35 kDa (*Figure 10, lane 3*). ECC-1 cells not over-expressing 17*β*-HSD1 were used as a negative control and show no signal (*Figure 10, lane 1*). For ECC-1 cells

over-expressing 17*β*-HSD1 by transfection, 17*β*-HSD1 protein is detected (*Figure 10, lane 2*). Western blot results indicate that transfections were performed successfully.

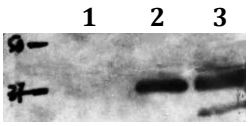

Figure 10. ECC-1 cells do not express 17*β*-HSD1 at basal level but show expression of the enzyme when transfected with 17*β*-HSD1. Lane 1: ECC-1 cells expressing no 17*β*-HSD1 used as a negative control. **Lane 2:** ECC-1 cells over-expressing 17*β*-HSD1 (35 kDa). **Lane 3:** Placental cell lysate used as a positive control for 17*β*-HSD1 expression.

Via a luciferase reporter assay the effect of an aberrant expression of 17*β*-HSD1 on estrogen synthesis was investigated. ECC-1 cells are estrogen receptor-alpha (ERα) positive, which makes them ideal to use for this specific luciferase assay. The level of available E_2 was indirectly measured by assessing the activation of an estrogen-responsive luciferase construct. This construct was transfected in ECC-1 cells together with an empty vector (negative control) or with 17*β*-HSD1. After transfection, cells were cultured in the presence of ethanol, E_1 (0.01 nM – 1 nM) or E_2 (0.01 nM – 1 nM). Low-active estrogen, E_1, has less estrogenic action compared to biologically active estrogen, E_2, which reflects in a lower estrogenic/luciferase activity measured by the luciferase assay. Nevertheless, when E_1 is converted to E_2, estrogenic action increases, which will be measured as an increase in luciferase activity.

When ECC-1 cells transfected with an empty vector are stimulated with E_2, luciferase activity is significantly (*P-value* < 0.05) increased compared to untreated cells, whereas stimulation with E_1 has no significant effect on luciferase activity (*Figure 11A*). In contrast, when ECC-1 cells over-expressing 17*β*-HSD1 are stimulated with E_1, a significant (*P-value* < 0.05) increase in luciferase activity was measured compared to untreated cells (*Figure 11B*). In this case, the estrogenic activity measured after stimulation with E_1 is comparable to that measured when cells are stimulated with E_2. These results indicate that E_1 is completely converted to E_2 by 17*β*-HSD1 *in vitro*. These findings confirm the relevant role of 17*β*-HSD1 in the synthesis of E_2.

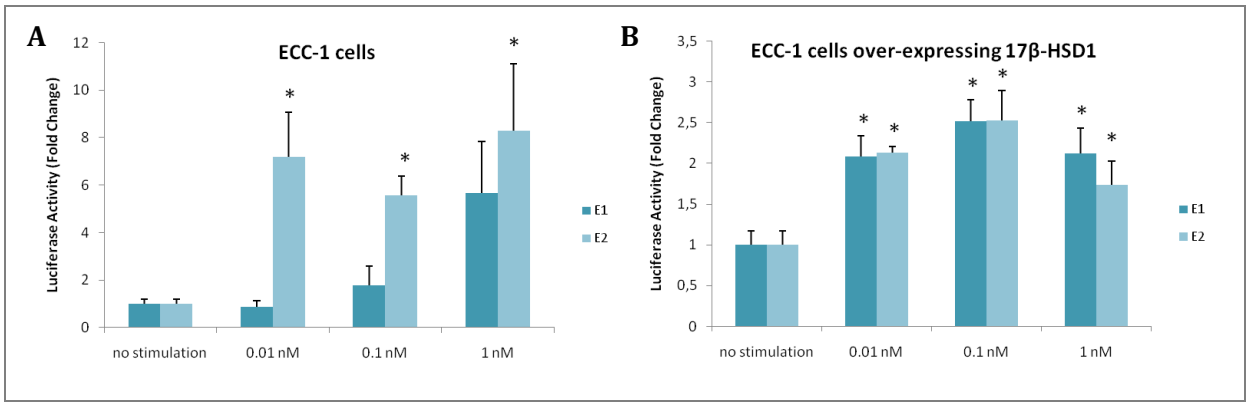

Figure 11. Over-expression of 17*β*-HSD1 stimulates the conversion of E_1 to E_2. A. When ECC-1 cells are transfected with an empty vector (negative control), stimulation with E_2 (0.01 nM – 1 nM) induces a significant (*P-value* < 0.05) increase in luciferase activity compared to untreated cells, while stimulation with E_1 (0.01 nM – 1 nM) has no significant effect on luciferase activity. **B.** When ECC-1 cells over-expressing 17*β*-HSD1 are stimulated with E_1 (0.01 nM – 1 nM), a significant (*P-value* < 0.05) increase in luciferase activity was measured compared to untreated cells and this luciferase activity is comparable to the activity measured when cells are stimulated with E_2 (0.01 nM – 1 nM). Thus, 17*β*-HSD1 is active and converts E_1 into E_2. The non stimulated condition was fixed at a value of one. Values are expressed as the average luciferase activity (mean ± SD) of three replicate measurements and are given as fold changes. Results were reproduced in two independent experiments. Luciferase activity reflects estrogen responsive activity. Statistical significance was calculated against untreated conditions, is defined as *P-value* < 0.05 (*t*-test) and is represented by an asterisk. Non-significant results are not indicated.

Luciferase activity was also measured after over-expressing other reducing enzymes (17*β*-HSD5 and 17*β*-HSD12). When over-expressing these enzymes in ECC-1 cells, luciferase activity was not significantly different than that measured in ECC-1 cells over-expressing an empty vector after stimulation with either E_1 or E_2 (data not shown). This indicates that both 17*β*-HSD5 and 17*β*-HSD12 do not play a role in the conversion of E_1 to E_2, which confirms qRT-PCR results.

Over-expression of 17β-HSD1 increases cell proliferation by converting E_1 to E_2 using in vitro model systems

Results found with the luciferase reporter system indicate that 17*β*-HSD1 plays an important role in the conversion of E_1 to E_2 *in vitro*. To validate the physiological relevance of these observations and to confirm the role of an aberrant 17*β*-HSD1 expression in the potential carcinogenesis of the endometrium, *in vitro* cell proliferation was measured using a BrdU incorporation assay. Bromodeoxyuridine (BrdU) is a synthetic thymidine analog that incorporates into the cell's DNA when the cell is dividing (S-phase of cell cycle) and can be detected via immunofluorescence. This method measures cell proliferation by analyzing the entry of the cells in the S-phase of the cell cycle. Due to starvation of cells in RPMI 1640 without phenol-red supplemented with 5% hormone-stripped serum for a duration of three days prior experiments, cells are synchronized in the G1 phase of the cell cycle, just before entering the

synthesis phase of the cell cycle (S-phase). Adding different stimuli to the cell culture can induce these cells to proliferate again.

When ECC-1 cells transfected with an empty vector are stimulated with E_2, a significant (*P-value* < 0.05) increase in BrdU incorporation is observed compared to untreated cells, whereas stimulation with E_1 does not influence S-phase entry of the cells (*Figure 12*). In contrast, when ECC-1 cells over-expressing 17β-HSD1 are stimulated with E_1, the percentage of BrdU incorporation is increased compared to untreated cells (*Figure 12*). In this case, the percentage of BrdU staining measured after stimulation with E_1 is comparable to that measured when cells are stimulated with E_2. Taken together, these results confirm the role of 17β-HSD1 in the synthesis of E_2 and, additionally, these results indicate that E_2 plays a role in the proliferation of endometrial cancer cells.

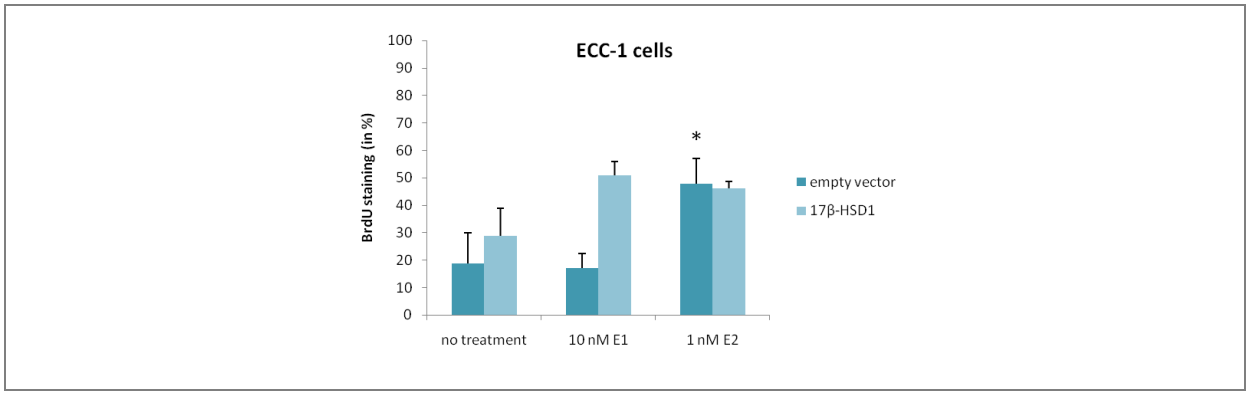

Figure 12. 17β-HSD1 plays a role in the conversion of E_1 to E_2 and in this way stimulates cell proliferation. When ECC-1 cells are transfected with an empty vector, stimulation with E_2 (1 nM) induces a significant (*P-value* < 0.05) increase in BrdU incorporation compared to untreated cells, while stimulation with E_1 (10 nM) has no effect on BrdU incorporation. When ECC-1 over-expressing 17β-HSD1 are stimulated with E_1 (10 nM), a increase in BrdU incorporation was measured compared to untreated cells and this incorporation is comparable to that measured when cells are stimulated with E_2 (1 nM). Thus, 17β-HSD1 is active and converts E_1 into E_2. Incubation of ECC-1 cells with E_2 (1 nM) induces a comparable BrdU incorporation to cells incubated with E_1 (10 nM). This confirms that E_2 plays a role in the proliferation of endometrial cancer cells. The non stimulated condition was fixed at a value of one. Values are expressed as the average BrdU staining (mean ± SD) of two replicate measurements and are given as fold changes. Results were reproduced in two independent experiments. Percentage of BrdU staining is representative for the number of BrdU-positive cells. Statistical significance was calculated against untreated conditions, is defined as *P-value* < 0.05 (*t*-test) and is represented by an asterisk. Non-significant results are not indicated.

The aromatase pathway plays a role in the availability of E_2 in endometrial cancer

The role of the aromatase pathway in the pathogenesis of endometrial cancer is still unclear. Aromatase is known to convert androstenedione to E_1 and testosterone to E_2 and the conversion of androstenedione to testosterone is catalyzed by 17β-HSD5 (*Figure 3*). Both of these enzymes may be involved in E_2 synthesis in endometrial cancer.

Aromatase plays a role in the availability of E_2 but 17β-HSD type 5 does not play a role in the conversion of androstenedione to testosterone in endometrial cancer

Despite the fact that 17β-HSD5 was already found earlier, using a luciferase assay, not to be involved in the conversion of E_1 to E_2, the role of 17β-HSD5 in the conversion of androstenedione to testosterone could still be relevant in endometrial cancer. Earlier, Ito et al. found the expression and immunoreactivity of 17β-HSD5 to be significantly increased in endometrial carcinoma tissue [24]. Moreover, 17β-HSD5 was declared as one of the key enzymes for estrogen concentrations in endometrial malignancies [31]. To assess the roles of aromatase and 17β-HSD5, the same approach of luciferase assay was used and ECC-1 cells were stimulated with either androstenedione or testosterone.

When ECC-1 cells transfected with an empty vector are stimulated with testosterone, an increase (*P-value* = 0.09) in luciferase activity is detected compared to untreated cells, whereas stimulation with androstenedione has no significant effect on luciferase activity (*Figure 13A*). This indicates that testosterone is converted to E_2 by aromatase. Additionally, this implies that ECC-1 cells do not have intrinsic 17β-HSD5 activity, which would have resulted in androstenedione to testosterone conversion, followed by aromatization to E_2.

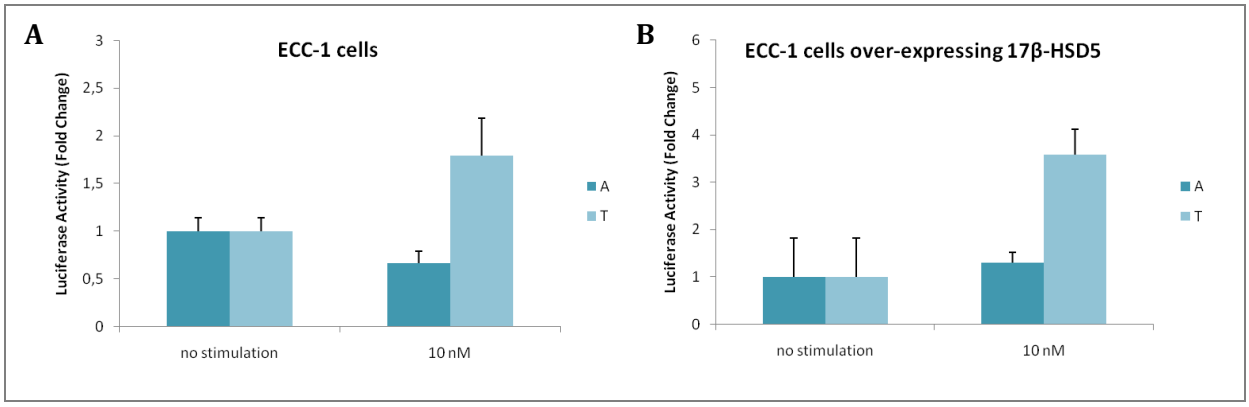

Figure 13. Over-expression of 17β-HSD5 plays no role in the conversion of androstenedione (A) to testosterone (T). A. When ECC-1 cells are transfected with an empty vector (negative control), stimulation with T (10 nM) induces an increase (*P-value* = 0.09) in luciferase activity compared to untreated cells, while stimulation with A (10 nM) has no significant effect on luciferase activity. This indicates that testosterone is converted to E_2 by aromatase. Moreover, this implies that ECC-1 cells have no intrinsic 17β-HSD5 activity. **B.** When ECC-1 cells over-expressing 17β-HSD5 are stimulated with either A (10 nM) or T (10 nM), observed results are comparable (*P-value* = 0.07) to those for cells not over-expressing 17β-HSD5. This indicates that 17β-HSD5 does not play an active role in the conversion of A to T in, at least, this endometrial cancer cell line (ECC-1). The non stimulated condition was fixed at a value of one. Values are expressed as the average luciferase activity (mean ± SD) of three replicate measurements and are given as fold changes. Results were reproduced in two independent experiments. Luciferase activity reflects estrogen responsive activity. Statistical significance was calculated against untreated conditions, is defined as *P-value* < 0.05 (*t*-test) and is represented by an asterisk. Non-significant results are not indicated. *P-values* were of borderline significance, due to large standard deviations of untreated samples.

Subsequently, ECC-1 cells were also transfected with 17β-HSD5 and stimulated with either androstenedione or testosterone to measure estrogenic activity. When ECC-1 cells over-expressing 17β-HSD5 are stimulated with either androstenedione or testosterone, the same results are observed compared to ECC-1 cells not over-expressing 17β-HSD5 (*Figure 13B*). This indicates that 17β-HSD5 plays no role in the conversion of androstenedione to testosterone in, at least, this endometrial cancer cell line (ECC-1). It should be however noted that an efficient over-expression of 17β-HSD5 in transfected ECC-1 cells was not confirmed via western blotting, due to the fact that antibodies for this protein were not available at the lab.
Taken together, these data indicate that aromatase plays a role in the availability of E_2, but that 17β-HSD5 does not play a role in the conversion of androstenedione to testosterone in endometrial cancer.

To further investigate the role of aromatase in the estrogen metabolism in endometrial cancer, the luciferase reporter system was used to investigate the effect of aromatase inhibitors on the estrogenic activity in ECC-1 cells.
ECC-1 cells, either transfected with an empty vector or over-expressing 17β-HSD5, were stimulated with anastrozole (5 µM), an aromatase inhibitor together with androstenedione (10 nM) or testosterone (10 nM). When ECC-1 cells transfected with an empty vector are co-stimulated with androstenedione and anastrozole, no significant difference in luciferase activity was observed compared to untreated cells (*Figure 14A*). The same is observed when cells are co-stimulated with testosterone and anastrozole (*Figure 14A*). Interestingly, when ECC-1 cells transfected with an empty vector are co-stimulated with testosterone and anastrozole, a trend towards a decrease in luciferase activity is observed compared to cells stimulated with testosterone alone (*Figure 14A*). On the other hand, when co-stimulated with androstenedione and anastrozole, this decreasing trend was not observed compared to cells stimulated with androstenedione alone (*Figure 14A*). In contrast, when ECC-1 cells over-expressing 17β-HSD5 are stimulated with anastrozole, together with either androstenedione or testosterone, the same observations are found compared to those found for cells transfected with an empty vector (*Figure 14B*).
These results re-confirm that 17β-HSD5 does not play a role in the conversion of androstenedione to testosterone and these results also confirm that aromatase, indeed, plays a role in the conversion of testosterone to E_2.

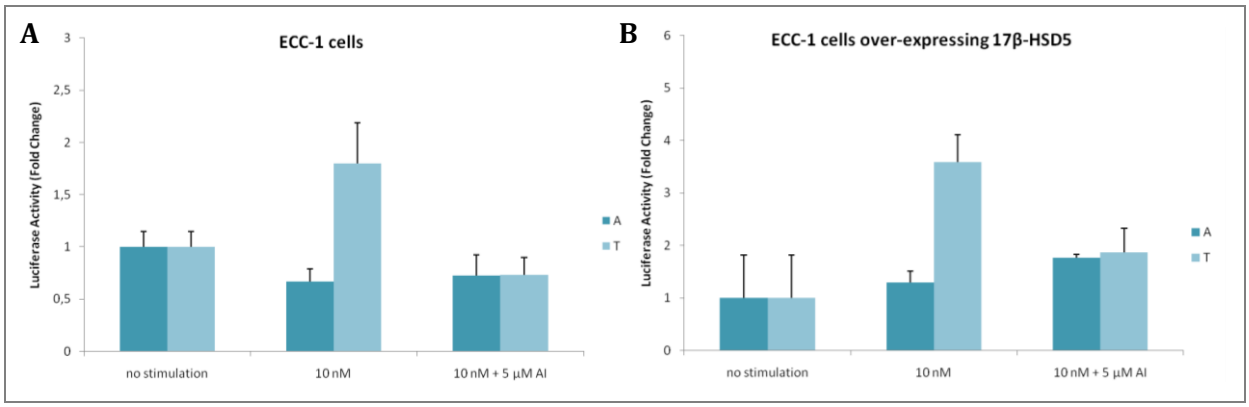

Figure 14. Aromatase plays a role in the availability of E_2 in endometrial cancer cells due to the conversion of testosterone in E_2. Over-expression of 17β-HSD5 plays no role in the conversion of androstenedione to testosterone in endometrial cancer cells and stimulation with anastrozole (aromatase inhibitor; AI) reduces testosterone-induced estrogenic activity. **A.** When ECC-1 cells are transfected with an empty vector, co-stimulation with A (10 nM) and AI (5 µM) induces no significant difference in luciferase activity compared to untreated cells. The same is observed when cells are co-stimulated with T (10 nM) and AI (5 µM). Interestingly, when ECC-1 cells transfected with an empty vector are co-stimulated with T (10 nM) and AI (5 µM), a trend towards a decrease in luciferase activity is observed compared to cells stimulated with T (10 nM) alone. On the other hand, co-stimulation with A (10 nM) and AI (5 µM) does not induce this decreasing trend. This indicates that aromatase plays an important role in the conversion of T to E_2. **B.** When ECC-1 cells over-expressing 17β-HSD5 are co-stimulated with either A (10 nM) or T (10 nM) and AI (5 µM), the same observations are found compared to those found for cells transfected with an empty vector. This indicates that 17β-HSD5 does not play an active role in the conversion of A into T in ECC-1 cells and that this enzyme does not influence the function of anastrozole. The non stimulated condition was fixed at a value of one. Values are expressed as the average luciferase activity (mean ± SD) of three replicate measurements and are given as fold changes. Results were reproduced in two independent experiments. Luciferase activity reflects estrogen responsive activity. Statistical significance was calculated against untreated conditions, is defined as *P-value* < 0.05 (*t*-test) and is represented by an asterisk. Non-significant results are not indicated.

Testosterone increases cell proliferation in vitro via conversion to E_2 by aromatase

To reconfirm the findings that testosterone increases cell proliferation *in vitro* via conversion to E_2 by aromatase, the same approach of BrdU incorporation assay was used.

When ECC-1 cells transfected with an empty vector are stimulated with testosterone, an increase in BrdU incorporation is observed compared to untreated cells (*Figure 15A*). When these cells are co-stimulated with testosterone and anastrozole, no significant difference in BrdU incorporation was observed compared to untreated cells, whereas a trend towards a decrease in S-phase entry is observed compared to cells stimulated with testosterone alone (*Figure 15A*). Moreover, when ECC-1 cells transfected with an empty vector are stimulated with testosterone (10 nM), the percentage of BrdU incorporation is comparable to that measured when these cells are stimulated with E_2 (*Figure 15B*). These observations reconfirm that aromatase is active in ECC-1 cells and plays a role in the conversion of testosterone to E_2.

In contrast, when ECC-1 cells over-expressing 17β-HSD1 are stimulated with testosterone alone or co-stimulated with both testosterone and anastrozole, the same observations are found

compared to those for cells transfected with an empty vector (*Figure 15A*). In addition, the comparative increase in BrdU incorporation induced by testosterone and E_2 is also observed when ECC-1 cells over-express 17*β*-HSD1 (*Figure 15B*). This indicates that 17*β*-HSD1 is not involved in the testosterone-induced cell proliferation and does not influence the function of anastrozole.

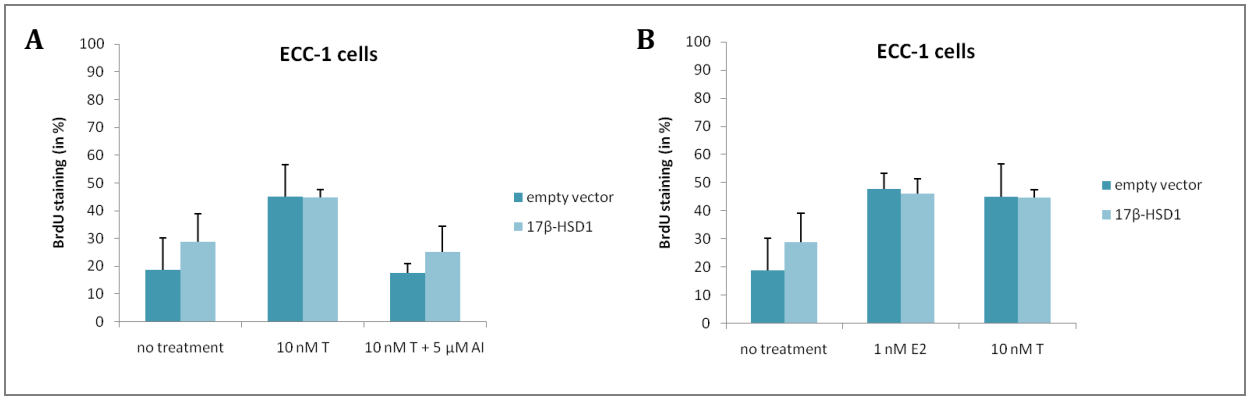

Figure 15. Testosterone increases cell proliferation due to its conversion to E_2 by aromatase. A. When ECC-1 cells are transfected with an empty vector, stimulation with T (10 nM) induces an increase in BrdU incorporation compared to untreated cells. When these cells are co-stimulated with T (10 nM) and AI (5 µM), no significant difference in BrdU incorporation was observed compared to untreated cells, whereas a trend towards a decrease in S-phase entry is observed compared to cells stimulated with testosterone (10 nM) alone. This reconfirms that aromatase is active in ECC-1 cells and converts testosterone to E_2. When ECC-1 cells over-expressing 17*β*-HSD1 are stimulated with T (10 nM) alone or co-stimulated with both T (10 nM) and AI (5 µM), the same observations are found compared to those for cells transfected with an empty vector. This indicates that 17*β*-HSD1 is not involved in the testosterone-induced cell proliferation and does not influence the function of anastrozole. **B.** When ECC-1 cells transfected with an empty vector are stimulated with testosterone (10 nM), the percentage of BrdU incorporation is comparable to that measured when these cells are stimulated with E_2 (1 nM). These observations reconfirm that aromatase is active in ECC-1 cells and plays a role in the conversion of testosterone to E_2. In addition, the comparative increase in BrdU incorporation induced by testosterone (10 nM) and E_2 (1 nM) is also observed when ECC-1 cells over-express 17*β*-HSD1. This indicates that 17*β*-HSD1 is not involved in the testosterone-induced cell proliferation and does not influence the function of anastrozole. The non stimulated condition was fixed at a value of one. Values are expressed as the average BrdU staining (mean ± SD) of two replicate measurements and are given as fold changes. Results were reproduced in two independent experiments. Percentage of BrdU staining is representative for the number of BrdU-positive cells. Statistical significance was calculated against untreated conditions, is defined as *P-value* < 0.05 (*t*-test) and is represented by an asterisk. Non-significant results are not indicated.

Taken together, these observations confirm that aromatase is active in, at least, ECC-1 cells and that it is responsible for the conversion of testosterone to E_2, which increases cell proliferation.

Discussion

Endometrial cancer is the most frequent type of cancer occurring in the female genital tract in Western Europe and it will increase due to an increasing life-expectancy [8, 9, 15, 18]. Although the exact cause of endometrial cancer is still unknown, excessive and/or prolonged exposure to increased levels of unopposed estrogens, in particular the biologically active form (17β-estradiol, E_2), is considered to play an important role in the pathogenesis and development of human endometrial carcinoma [4, 21, 24]. Most estrogen-dependent endometrial carcinomas develop after menopause, when ovaries have ceased to produce estrogens [24]. Nevertheless, the majority of post-menopausal women show no increase in serum estrogen levels, suggesting that the diseased tissue itself is the source of estrogens in these patients [21, 24, 31]. Previous studies indicated that the expression of the 17β-hydroxysteroid dehydrogenases (17β-HSDs) greatly contribute to the *in situ* availability of biologically active estrogen (E_2) [24, 31]. Recently, 17β-HSDs have been shown to be aberrantly expressed in breast tumors and endometriotic lesions, leading to increased levels of E_2 [31-33]. Moreover, preliminary data from our lab showed that 17β-HSDs metabolism is disturbed in endometrial cancer (*Figure 4*). Consequently, the inhibition of local 17β-HSD activity provides a potential therapeutic approach to treat endometrial cancer by reducing biologically active estrogen in the endometrial tissue. So far, no reports indicate which of the different 17β-HSDs are responsible for the high concentration of E_2 in endometrial cancer tissue. Therefore, in this study, we hypothesized that ***17β-HSDs are aberrantly expressed in endometrial cancer, which leads to a hyper-estrogenic environment in the endometrial tissue.*** A detailed testing of this hypothesis is of particular interest for a better understanding of the role of these enzymes in the pathogenesis of endometrial cancer. First, it was investigated which of these 17β-HSDs are aberrantly expressed by comparing their expression between endometrial cancer tissues and postmenopausal control tissues. Secondly, clinical sample results were confirmed *in vitro* by investigating the effect of an aberrant expression of 17β-HSDs on E_2 synthesis. Thirdly, *in vitro* observations were reconfirmed by investigating the role of an aberrant expression of 17β-HSDs in cell proliferation. Finally, during *in vitro* confirmation of clinical sample results, aromatase was found to have some implication in E_2 synthesis. To confirm these interesting observations, the effect of aromatase inhibition was investigated via similar *in vitro* approaches. Our results provide some interesting observations.

Increased expression of 17β-HSD1 plays an important role in the pathogenesis of endometrial cancer

17β-HSD1 gene expression is significantly increased in endometrial cancer tissue

Considering that in breast cancer and endometriosis, other estrogen-dependent diseases, 17*β*-HSD1 is already known to be important in the conversion of E_1 to E_2, it was tempting to speculate whether the same is true for endometrial cancer [31-33]. Hence, an important observation in our study is the significant increase in 17*β*-HSD1 gene expression in endometrial cancer tissue compared to postmenopausal control tissue. This is of particular interest since previous studies were not able to detect 17*β*-HSD1 gene expression in endometrial cancer biopsies [21]. Gene expression of other reducing 17*β*-HSDs (i.e. 17*β*-HSD5, -7 and -12) and gene expression of 17*β*-HSD2 (oxidizing enzyme) were compared between endometrial cancer tissue and postmenopausal control tissue using qRT-PCR. For these 17*β*-HSDs, no significant difference in gene expression was observed between endometrial cancer tissue and postmenopausal control tissue.

In addition, past studies detected higher 17*β*-HSD1 immunoreactivity in both breast cancer tissue and endometriosis tissue [43, 44]. Nevertheless, attempts to measure immunoreactive protein in endometrial cancer biopsies failed previously [24, 45]. Therefore, it is interesting that in our study, 17*β*-HSD1 immunoreactivity was found to be predominantly present in endometrial cancer tissue compared to postmenopausal control tissue, though immunoreactivity was only assessed qualitatively due to lack of time. As we expected, 17*β*-HSD1 immunoreactivity is detected primarily in cytoplasm of carcinoma cells, as 17*β*-HSD1 is known to be cytosolic enzyme [46].

In vitro over-expression of 17β-HSD1 increases estrogen synthesis by converting E_1 to E_2

To confirm our observations in the clinical samples (qRT-PCR), the relevant role of 17*β*-HSD1 in E_2 synthesis was investigated *in vitro*, more specifically in endometrial cancer (ECC-1) cell lines. This well-differentiated cell line has high expression of the estrogen-receptor-alpha (ERα), making it a perfect model system to study estrogen responses. At the same time, this cell line does not express 17*β*-HSD1 under basal conditions, which makes it an ideal cell line to perform over-expression experiments.

After over-expression of 17*β*-HSD1 in ECC-1 cells, an efficient transfection was determined using western blot analysis and estrogenic activity was measured using a luciferase assay. When ECC-1 cells over-expressing 17*β*-HSD1 are stimulated with E_1, the measured estrogenic activity is comparable to that measured when cells are stimulated with E_2, whereas this is not observed

in cells transfected with an empty vector. This means E_1 is completely converted to E_2 by 17β-HSD1 *in vitro*. These findings confirm the relevant role of 17β-HSD1 in the conversion of E_1 to E_2. To further confirm that 17β-HSD1 is the important enzyme in the synthesis of E_2, the same approach of luciferase assay was used to measure the estrogenic activity after over-expressing other reducing enzymes (17β-HSD5 and 17β-HSD12) in ECC-1 cells and stimulating them with either E_1 or E_2. In both ECC-1 cells transfected with an empty vector and ECC-1 cells transfected with 17β-HSD5 or 17β-HSD12, stimulation with E_2 induced an increased luciferase activity compared to non-stimulated cells. Stimulation with E_1 did not increase luciferase activity compared to non-stimulated cells. This indicates that both 17β-HSD5 and 17β-HSD12 do not play a role in the availability of E_2, confirming the important role of 17β-HSD1 in the conversion of E_1 to E_2.

It was unknown if ECC-1 cells express these enzymes under basal conditions, but transfection of the cells with an empty vector (negative control) indicated that the basal level of these enzymes is low and not sufficient to convert E_1 to E_2 in ECC-1 cells transfected with the empty vector.

Nevertheless, it should be noted that an efficient transfection of ECC-1 cells with 17β-HSD5 and 17β-HSD12 could not be confirmed via western blotting due to unavailability of 17β-HSD5 antibodies and 17β-HSD12 antibodies at our lab. In addition, luciferase activity was not measured after over-expressing 17β-HSD7, another reducing 17β-HSD, due to unavailability of vectors containing this gene at our lab. Anyway, recent investigations have already excluded its activity [27].

Besides over-expression of 17β-HSD1, down-regulation of the enzyme in ECC-1 cells using siRNA was also a possibility, but ECC-1 cells have no basal expression of 17β-HSD1, which makes is pointless to down-regulated it in these cells. Instead of ECC-1 cells, other endometrial cancer cell lines (RL-952 or Ishikawa) could be used, but western blot analysis showed 17β-HSD1 protein expression to be absent in these cells. This was confirmed earlier by Smuc et al., who were not able to detect 17β-HSD1 protein expression in Ishikawa cells either [47].

Based on previous experiments, where a luciferase reporter system was used, 17β-HSD1 was found to play an important role in the conversion of E_1 to E_2 in endometrial cancer cells. To validate the physiological relevance of these observations and to confirm the role of aberrant 17β-HSD1 expression in cell growth, *in vitro* proliferation of ECC-1 cells was measured, using a BrdU incorporation assay. The effect of an aberrant 17β-HSD1 expression on cell proliferation was determined. To this end, S-phase entry of the cell cycle was measured. Due to a pre-incubation of minimum three days in RPMI 1640 medium without phenol-red supplemented with 5% hormone-stripped serum, cells were synchronized in the G1 phase of the cell cycle,

which stops their proliferation. When ECC-1 cells over-expressing 17β-HSD1 are stimulated with E_1, the measured BrdU incorporation is comparable to that measured when cells are stimulated with E_2. This finding is of particular interest since it reconfirms the *in vitro* observations found by using the luciferase assay, indicating that 17β-HSD1 is responsible for the conversion of E_1 to E_2.

Taken together, these observations indicate that 17β-HSD1 is the key enzyme responsible for the conversion of E_1 to E_2 in endometrial cancer, and that an increase in E_2 finally results in increased cell proliferation *in vitro*.

The aromatase pathway plays a role in the availability of E_2 in endometrial cancer

Since the role of 17β-HSD1 in the conversion of E_1 to E_2 was already proven in other estrogen-dependent diseases, the main intention of this study was to investigate the role of 17β-HSD1 in the pathogenesis of endometrial cancer. Nevertheless, in one of their reviews, Ito et al. declared 17β-HSD5 as one of the key enzymes for estrogen concentrations in endometrial malignancies [31]. As 17β-HSD5 was already observed not to play a role in the conversion of E_1 to E_2, it was tempting to speculate that 17β-HSD5 could play a role in the conversion of androstenedione to testosterone, since it is already described to catalyze this reaction in normal endometrium [24, 42, 48, 49]. The same approach of luciferase assay, as described earlier, was used to investigate this.

An important observation is that when stimulated with testosterone, ECC-1 cells transfected with an empty vector show high luciferase activity, indicating an intrinsic aromatase action. If 17β-HSD5 would be involved in converting androstenedione to testosterone, stimulation with androstenedione would result in similar luciferase activity as when cells are stimulated with testosterone. However, in ECC-1 cells over-expressing 17β-HSD5, stimulation with androstenedione did not induce any luciferase activity.

This indicates that 17β-HSD5 does not play a role in the conversion of androstenedione to testosterone in endometrial cancer tissue. Nevertheless, an efficient over-expression of 17β-HSD5 in transfected ECC-1 cells was not confirmed via western blotting, due to unavailability of 17β-HSD5 antibodies at our lab. Future work will be aimed to repeat these same experiments after confirmation of efficient transfection.

The observation that testosterone is able to induce luciferase activity indicates that some activity of aromatase is present *in vitro*. This is of particular interest since intratumoral testosterone concentration was already found to be inversely correlated with aromatase mRNA

level in endometrial carcinoma, suggesting that the amount of testosterone produced in the tumor tissue may be finally converted to E_2 by aromatase [24, 31].

And although there is a lot of controversy about the role of aromatase in the pathogenesis of endometrial cancer, it was already found to be over-expressed in endometrial cancer tissues using RT-PCR [3, 31, 50, 51]. Moreover, preliminary data from our lab observed that aromatase gene expression was increased in endometrial pathologies, including endometrial cancer, but its expression was relatively low.

To further confirm these findings, the same approach of luciferase assay was initiated to investigate whether aromatase is active and plays a role in the availability of E_2 in endometrial cancer by using aromatase inhibitors. When co-incubating ECC-1 cells with both testosterone and an aromatase inhibitor (anastrozole), the effect of testosterone to increase estrogenic activity was repressed. These results indicate that aromatase is indeed active *in vitro* and that the availability of E_2 is influenced by aromatase. In addition, luciferase results were reconfirmed with the same proliferation assay as described earlier. When ECC-1 cells transfected with an empty vector are stimulated with testosterone, a significant increase in BrdU incorporation was observed, which reflects an increase in cell proliferation. Interestingly, co-incubation of ECC-1 cells with both testosterone and anastrozole, repressed the induction of BrdU incorporation by testosterone.

Taken together, these observations indicate that aromatase is involved in estrogen metabolism *in vitro* and that it plays a role in the availability of E_2. These results are of particular interest as aromatase has already been considered as a key enzyme in the synthesis of estrogen in endometrial carcinoma as well as in breast cancer and aromatase inhibitors are currently used for treatment of breast cancer [3, 21]. Interestingly, the possibility to identify endometrial cancer patients who may benefit from aromatase inhibitor treatment is particularly attractive since aromatase inhibitors are already approved for clinical use. However, it is important to note that the role of aromatase in endometrial carcinoma is still in its infancy and this has to be further investigated [50, 52].

Besides the role of the aromatase pathway, where both aromatase and 17β-HSD1 play a role in estrogen metabolism in endometrial cancer, another pathway may be involved (*Figure 3*). Although, the sulphatase (STS)/sulphotransferase (EST) pathway is also suggested to play a role in endometrial cancer, investigations about this pathway are limited. At our lab, attempts to measure the activity of STS and EST by means of HPLC have already been performed, with disappointing results, since EST action was difficult to measure. This method is currently being optimized and will be used in the future.

Future directions

Endometrial cancer is the most common type of uterine cancer in Western Europe and since the incidence of endometrial cancer will increase due to the increasing life-expectancy, a good understanding of the pathogenesis of endometrial cancer is important in order to develop effective treatment for this type of cancer. There has already been a lot of interest about 17*β*-HSD enzymes, as they are important in the conversion of E_1 to E_2 and *vice versa*.
Since 17*β*-HSD1 is already suggested as the most important enzyme responsible for the high concentration of E_2 in breast cancer and endometriotic tissue, targeting this enzyme might form a good therapeutic treatment for endometrial cancer treatment. During this study, interesting and important observations were found concerning the role of 17*β*-HSD1 in estrogen metabolism in endometrial cancer. Surprisingly, we also found aromatase to be active *in vitro* and to play a role in the availability of E_2. This finding about aromatase has to be confirmed and further investigated in the future.

During our study, 17*β*-HSD1 gene expression was found to be increased in endometrial cancer tissue compared to postmenopausal control tissue using qRT-PCR. Although these results, obtained using 18 tumor tissue samples and 5 control tissue samples, are promising, patient groups need to be enlarged. Recently, sample size was enlarged, using an improved method, which guarantees that tissues used for biochemical and molecular analyses are identical to the tissues inspected by the pathologists after immunohistochemical staining. To this end, endometrial cancer tissues embedded in Optimal Cutting Temperature compound (Tissue-Tek® O.C.T.™; Sakura Finetek Europe b.v., the Netherlands) were sectioned in a microtome-cryostat according to a specific protocol. In short, five slices of 5 µm were taken at the beginning and at the end of the tissue to perform immunohistochemical staining. The central tissue was used for HPLC analysis (10 x 25 µm) and qRT-PCR analysis (2 x 25 µm). Total RNA has already been isolated and cDNA synthesis is already synthesized for qRT-PCR analysis. Due to lack of time, qRT-PCR analysis for 17*β*-HSD1, -2, -5, -7, and -12 could not be repeated using these samples. In the future, these analyses will be performed to enlarge the clinical sample groups.
The cryosections for HPLC were already lysated in RIPA buffer and HPLC analysis has already been performed (see Appendix for method). To our regret, we were only able to detect useful retention times for the oxidizing enzymes (specifically 17*β*-HSD2), but not for the reducing enzymes (specifically 17*β*-HSD1) due to technical problems. In the future, HPLC analysis will be repeated and results will be used to examine whether gene expression level of 17*β*-HSD1 (qRT-PCR, cryosections) can be correlated to the protein activity of 17*β*-HSD1 (HPLC, cryosections) in the same way as shown previously in this study (*Figure 7*).

Immunohistochemical stainings on cryosections were already performed, but results were disappointing. Therefore, the used protocol for cryosections has to be optimized and will be used in the future for immunohistochemical stainings.
Besides enlarging the clinical sample groups, we would also like to use matched samples, where endometrial cancer tissue and postmenopausal control tissue originate from the same patient. We did not have this opportunity during this study, but when available at the pathology department, these matched tissues will be used to repeat qRT-PCR experiments.

To confirm the observations found with qRT-PCR, luciferase assays and proliferation assays were performed using transient transfections of different expression plasmids. Although the results found with this experimental set-up are already promising, results are more robust when stable transfections are performed prior to stimulatory experiments. We have observed that transient transfections might cause problems interpreting data due to differences in absolute luciferase activity (relative light units) and fold changes between cells transfected with different plasmid preparations and incubated with different stimuli. This problem can be solved by using cells stably expressing the gene of interest.
During this study, transfected cells were already selected for stable expression of 17β-HSD1 plasmid over several weeks by addition of 160 µg/ml hygromycin B to the growth medium. To our regret, we were not able to isolate monoclonal ECC-1 cells stably expressing the 17β-HSD1 cDNA. The reason for this can be that the concentration of the selection reagent (hygromycin B) was too high, though the used concentration was based on sensitivity of most other cell types. Moreover, Day et al. (2008), who provided us with the expression plasmids, used even a higher concentration (250 µg/ml) of hygromycin B to retrieve stably transfected cells [48]. An attempt to culture stably transfected cells will be repeated in the future to use them for new luciferase and proliferation assays.

Another important aspect about the luciferase assays performed during this study was that some expression plasmids or antibodies were not available at the lab. For example, we were not able to confirm that 17β-HSD7 is not involved in estrogen metabolism *in vitro* due to unavailability of this expression plasmid. In addition, efficient transfection of 17β-HSD5 and 17β-HSD12 could not be determined before incubation of cells with different stimuli due to unavailability of specific antibodies for western blot analysis. In the future, 17β-HSD7 cDNA expression plasmid and 17β-HSD5, -7, and -12 antibodies will be ordered and experiments will be performed as planned. In this way, all 17β-HSDs thought to play a role in estrogen metabolism can be investigated and the study can be completed.

Moreover, another technique will be used in the future to investigate the amount of E_2 *in vitro*. This method is based on the same principle as a luciferase assay, although the estrogenic activity will not be measured via the activity of the firefly luciferase, but via measurement of estrogen-responsive genes, such as *TFF-1*. *TFF-1* gene expression will be investigated in ECC-1 cells stimulated with different stimuli using qRT-PCR. This technique was already used at our lab to compare the expression of *TFF-1* between endometriotic tissue and eutopic endometrial tissue of the same patient [33]. Since they were able to find a significant difference in expression of the estrogen-responsive gene between diseased and normal tissue of the same patient, we expect the same to be true for endometrial cancer tissue and postmenopausal control tissue. Therefore, measuring estrogen-responsive gene activity is something that will be done in the future.

Besides a luciferase assay, we also used a BrdU incorporation assay to reconfirm our observations and to measure cell proliferation. The choice for this assay was based on the fact that it is an easy assay to indirectly measure cell proliferation. Due to lack of time, we had to exclude other techniques that will be used in the future. One of these techniques, CFSE-based proliferation assay, was recommended by W. Germeraad of the Department of Immunology (Maastricht University, the Netherlands). Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that can be used to measure cell proliferation using flow cytometry. Another technique is BrdU-based FACS, which is more time consuming, but more sensitive and more accurate. Both techniques will be used in the future.

Concerning luciferase assays and proliferation assays that were used to indicate that aromatase plays a role in the availability of E_2, it is important to note that the concentration of anastrazole used during these experiments (5 μM) was chosen based on literature indications and that, although the results found during this study are already promising, anastrozole concentration might need to be elevated to obtain significant inhibition of aromatase activity. Different concentrations will be tested in the future when the role of aromatase in endometrial cancer is further investigated.

Finally, in the future, we will try to culture endometrial cancer tissue, with the intention to perform experiments on tissue explants cultures. The approach consists of chopping the tissue in little pieces, transfer it to a 6-well plate and cultivate it during 24 hours in RPMI 1640 medium without phenol-red supplemented with 5% hormone-stripped serum. To quantify the amount of tissue, the cultivation plate will be weighted before transferring the tissue and reweighted after transfer. Medium before and after cultivation of the tissue will be used for further experiments. An experiment that has been planned to be performed when endometrial tissue culture is a

success, is an estrogen-specific Enzyme-Linked Immunosorbent Assay (ELISA). Cultured tissue will be stimulated with either E_1 or E_2 and the conversion of E_1 to E_2 and *vice versa* will be measured using the ELISA-kit. Besides this, cultured endometrial tissue will also be used to repeat luciferase assays, BrdU incorporation assays and possible alternatives described above.

Generally, the results from this study indicate that 17β-HSD1 and aromatase play a role in the availability of E_2 in the endometrial cancer tissue. As already stated earlier, aromatase is important in both the conversion of androstenedione to E_1 and of testosterone to E_2 (*Figure 3*). Yet, testosterone is a male sex hormone that is produced only in small quantities in the ovaries and its production decreases during the menopause [53]. Which makes the attention turn towards the conversion of androstenedione (precursor of testosterone) to E_1 by aromatase, since androstenedione is the most abundant estrogen in postmenopausal women. But then, still, 17β-HSD1 is needed to convert E_1 to E_2, which induces the pathogenic effects in endometrial tissue. This suggests that 17β-HSD1 is the key enzyme in the pathogenesis of endometrial cancer, making it a perfect target for therapeutic treatment. Therefore, the next step is to test whether 17β-HSD1 inhibitors can revert the found results in this study (luciferase assay and proliferation assay). 17β-HSD1 inhibitors (B10735564 and B10721802) were already developed within a collaborative initiative between our research group and the Pharmaceutical Company Solvay (2004-2006). Novel collaborative initiatives with Bayer-Schering (Berlin) have been recently started and aim at the development of novel 17β-HSD1 inhibitors, and their application in endometriosis. In the future, these inhibitors will also be available for application in endometrial cancer and these novel therapeutics will be used to restore the impaired estrogen metabolism in endometrial cancer.

Conclusion

Taken together, the observations made during this study support our hypothesis that ***17β-HSDs are aberrantly expressed in endometrial cancer, leading to a hyper-estrogenic environment***.
An aberrant expression of 17*β*-HSD1 was confirmed in endometrial cancer tissues and *in vitro*, this aberrant expression led to increased levels of E_2. Aromatase was also found to have some activity in endometrial cancer cells.

These results are of particular interest as they have implications with respect to the development of novel therapeutic approaches for endometrial cancer. More specifically, inhibition of 17*β*-HSD1 alone or in combination with an aromatase inhibitor, which is possible in a patient-tailored manner, represents potential therapy for the future. In this context, these therapies are expected to be well tolerated by the patients, as this is true for other already described endocrine treatments. Therefore, both 17*β*-HSD1 inhibitors and aromatase inhibitors may easily be prescribed for endometrial cancer as adjuvant treatment or in stabilization settings. Furthermore, both therapeutics would provide a good alternative to hysterectomy in case of fertility-preserving purposes.

Still, the role of aromatase in endometrial cancer has to be further investigated in the future.

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Appendix

Oxidative and reductive 17β-HSD activity measurements: High-Performance Liquid Chromatography (HPLC)

Preparation of cryosections for enzyme activity studies

Endometrial cancer cryosections (10 x 25 µm) were frozen immediately in 1.5 ml Eppendorf tubes and were stored at -80 °C until further use. Protein lysates were prepared from these frozen cyrosections by homogenization in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0,25% sodiumdeoxycholate, 150 mM NaCl, 1 mM EDTA) using glass beads. Non-dissolved remnants were removed through centrifugation at 13,200 g and the supernatant was stored at -80 °C. Protein concentration was determined using the BC Assay Protein Quantitation Kit (Uptima, Interchim, France).

Derivatization and HPLC analysis

To determine the amounts of estrone and/or 17*β*-estradiol in the reaction mixture we used a HPLC method our lab has recently developed with minor modifications [34]. After adding the internal standard (IS) butyl-4-hydroxybenzoate (2.50 pg) to the reaction mixture a reversed phase cartridge (SPE cartridge Chromafix S-C18, Machery-Nagel, Dueren, Germany) was used for clean up. After washing the cartridge with H_2O and hexane, estrogens were eluted with dichloromethane. The eluted organic phase was evaporated under nitrogen at 45 °C and estrogens were derivatized with 2-(4-carboxy-phenyl)-5,6-dimethylbenzimidazole (CDB; 0.05%, w/v in pyridine) in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC; Pierce, Rockford, IL, USA) dissolved in acetonitrile (ACN) at 50 °C for 20 minutes. Of this 200 µl reaction mix, 10 to 2.5 µl was injected in the HPLC. The 2-(4-carboxy-phenyl)-5,6-dimethylbenzimidazole (CDB) which is not commercially available, was synthesized as described earlier [34].

HPLC was carried out using a Hewlett Packard series 1050 solvent delivery system. The HPLC system was connected to a Jasco fluorescent detector model 821-FP (excitation wavelength 336 nm, emission wavelength 440 nm). The derivatized steroids were injected into a LiChroCART 250-4 RP 18 column (Merck, Darmstadt, Germany) and eluted with a gradient of methanol/H_2O.

Reduction of estrone (E_1) to 17*β*-estradiol (E_2) (synthesizing 17*β*-HSDs) was carried out as follows. A 500 µl reaction mixture containing 2.6 mM NADP+, 3.3 mM glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 10 nmol estrone in 100 mM potassium phosphate buffer (pH 7.4) was incubated with 10 to 20 µg of the protein lysate at 37 °C for 24 hours.

Oxidation of 17*β*-estradiol to estrone (inactivating 17*β*-HSDs) was measured in a 500 µl reaction mixture containing 5 mM NADP+, 10 nmol 17*β*-estradiol in 100 mM potassium phosphate buffer (pH 7.4) and 10 to 20 µg of the protein lysate incubated at 37 °C for 24 hours.

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