Aus der Klinik für Frauenheilkunde und Geburtshilfe

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RELATIVE EXPRESSION OF 1,25-DIHYDROXYVITAMIN D3 RECEPTOR, VITAMIN D 1ALPHA-HYDROXYLASE, VITAMIN D 24-HYDROXYLASE AND VITAMIN D 25-HYDROXYLASE IN ENDOMETRIOSIS AND GYNECOLOGIC CANCERS

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ABBREVIATIONS

CPP	Chronic pelvic pain
BMI	Body mass index
AFS	American Fertility Society
NSAIDs	Nonsteroidal anti-inflammatory drugs
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
MPA	Medroxyprogesterone acetate
TNF-α	Tumor necrosis factor-α
1, 25 (OH)$_2$ D3	1, 25 dihydroxy vitamin D3
1, 25 D	1, 25 dihydroxy vitamin D3
UVB	Ultraviolet B radiation
24-OHase	25-hydroxyvitamin D-24-hydroxylase
25 D	25-hydroxyvitamin D3
1α-OH	1α-hydroxylase
PTH	Parathyroid hormone
VDR	Vitamine D receptor
RXR	Retinoid-X receptor
RAR	Retinoid-A receptor
VDRE	Vitamin D response element
PLCg	Protein lipase Cg
MAPK	Mitogenactivated protein kinase
VSCC	Ca^{2+}-regulated voltage-sensitive chanel
VICC	Ca^{2+}-regulated voltage-insensitive chanel
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
FCS	Fetal calf serum
PSG	Penicillin streptomycin gentamycin
HPRT	Hypoxanthine phosphoribosyl transferase
cDNA	Complementary DNA

RT	Reverse transcription
DTT	Dithiothreitol
HPLC	High pressure liquid chromatography
TBE	Tris-borate/EDTA
EDTA	Ethylenediaminetetraacetic acid
UDG	Uracil-DNA glycosylase
SE	Standard error
Eo	Endometriosis
Co	Control
Carc	Carcinoma
Endom	Endometrium
Ovar	Ovary
epi	Epithelium
str	Stroma
DBP	Vitamin D binding protein

1   INTRODUCTION

1.1   Endometriosis

Endometriosis is a common benign gynecologic disease defined as the growth of endometrial tissue outside the cavum uteri, which implies a chronic inflammatory reaction. The most common location of endometrial ectopic tissue (endometriotic tissue) is on the ovaries and other pelvic sites (fallopian tubes, uterus or bladder). After these, the abdomen (omentum, bowel, appendix, anterior abdominal wall, surgical scars) is the next most usual location of endometriotic tissue. Other sites, such as diaphragm, pleura, pericardium, muscles and central nervous system, are rarities. This endometriotic tissue is reacting in the same way to regular hormonal changes during the menstrual cycle as eutopic endometrial tissue, causing pain and dyspareunia (pain associated with sexual intercourse).

1.1.1   Epidemiology and pathogenesis of endometriosis

Endometriosis was described as a disease process over 300 years ago (Knapp, 1999). However, although it has been extensively investigated, the cause of growth of ectopic endometrial tissue outside the uterus remained undefined.

1.1.1.1   Prevalence, incidence and risk factors

Concerning the differences between authors in defining endometriosis and in the design of studies, as well as invasive nature of diagnosis, determining the prevalence, incidence and risk factors for endometriosis is difficult.

Published estimates of prevalence have varied by mode of diagnosis. Among women seeking tubal ligation, the prevalence of endometriosis was found in two studies to range from 2 to 18% (Strathy et al., 1982; Moen, 1994). The prevalence within the infertile population has been reported to range from 5 to 50% (Peterson, 1970; Duignan, 1972; Liston, 1972; Kleppinger, 1976; Hasson, 1976; Hornstein and Barbieri, 1999). The range of prevalence for women admitted

to a hospital because of pelvic pain is 5-21% (Duignan, 1972; Liston, 1972; Kleppinger, 1976; Hasson, 1976). In any case, there is an agreement that the prevalence of endometriosis is approximatly 10% of all women in their reproduction phase of life (Rothman et al., 1998).

The majority of risk factors for endometriosis belong to one of the following groups: menstrual and reproductive factors, body habitus, lifestyle, environmetal factors and immune disorder comorbidity (Missmer and Cramer, 2005). The risk of endometriosis appears to be increased in the presence of reproductive factors associated with increased exposure to menstruation, such as earlier age at menarche, shorter menstrual cycle length, longer duration of flow, greater menstrual volume of flow, and reduced parity (Eskenazi and Warner, 1997). Weak inverse associations between endometriosis and BMI (body mass index, kg/m^2) have been found (Signorello et al., 1997). Conversely, an increased risk with greater height has been reported for endometriosis (Signorello et al., 1997). Regular exercise has been associated with a reduced risk of endometriosis (Cramer et al., 1986; Signorello et al., 1997).

1.1.1.2 Pathogenic hypotheses

There are several theories about the pathogenesis of endometriosis including retrograde menstruation/ transplantation, coelomic metaplasia, induction theory, altered cellular immunity, lymphatic and vascular metastasis, composite theory, genetic basis, environmental basis and other multifactorial modes (Giudice and Kao, 2004; Witz, 2005). But the most widely accepted view of the pathogenesis of endometriosis is retrograde menstruation/ transplantation, with coexisting abnormal immune function. This theory, usually referred to as Sampson's theory, proposes that endometrial tissue passes through the fallopian tubes during menstruation, then attaches and proliferates at ectopic sites in the peritoneal cavity (Sampson, 1927). Additionally, components of the immune system contribute to the pathophysiology of endometriosis.

1.1.1.3 Similarities to cancer

Endometriosis has several not only pathophysiological similarities to malignant diseases such as reduced apoptosis, invasion of endometrial tissue in other organs (bowel, bladder) and increased angiogenesis (Varma et al., 2004) , but also clinical: recurrence (Donnez et al., 2002) and need of

several surgical interventions. In addition to these similarities, endometriosis patiens have a doubled increased risk to develope ovarian cancer in comparison to woman without endometriosis. If these patients suffer additionaly from longterm infertility, the incidence rate for ovarian cancer is even increased more than four times (Oral et al., 2003).

1.1.2 Diagnostics and classification of endometriosis

Endometriosis is a disease that often presents with an array of clinical symptoms, including pain, dysmenorhea, dyspareunia, cyclic pain, abnormal uterine bleeding, and infertility. Of all the above symptoms, pain is the most commonly associated with endometriosis (Mathias et al., 1996). Chronic pelvic pain (CPP) is typically confined to the lower abdomen and below the umbilicus, interfering with normal daily function.

The diagnosis of endometriosis is made on the symptoms, history, physical examination, laboratory testing and imaging techniques (the most commonly performed is ultrasonography). These investigations can not always confirm a diagnosis of endometriosis. Ultrasonography may detect ovarian cysts, but this is not specific for endometriotic cysts (endometriomas). Laparoscopy is the 'gold standard' for confirming the diagnosis by direct inspection and biopsy (Brosens, 1997).

Several classification systems for endometriosis have been proposed. In 1978, a committee created by the American Fertility Society system (AFS) established uniform classification of endometriosis. This AFS classification was revised several times until 1996 (Medcine, 1996). The AFS classification system stages the disease based on a cumulative score of an arbitrary value system. Judgment of the severity of disease was based on the involvement of peritoneum, ovaries and fallopian tubes with endometriosis or adhesion.

1.1.3 Therapy of endometriosis

The therapy of endometriosis can be medical, surgical and a combination of medical and surgical therapy. The choice of treatment depends on the presence and severity of symptoms, pregnancy plans, age, and the therapeutic goal.

1.1.3.1   Medical therapy of endometriosis

Medical therapy can be focused only on symptoms, providing analgesia, or can be attempt to influence the pathological basis of the disease- hormonal therapy. Preferred therapeutics for pain relief are nonsteroidal anti-inflammatory drugs (NSAIDs) (Olive et al., 1993; Bergqvist, 1999). NSAIDs are often the first choice because they inhibit the inflammatory processes that occur during endometriosis. If NSAIDs are poorly tolerated or contraindicated, paracetamol might be an alternative.

The main goal of hormonal therapy for endometriosis is to inhibit ovulation, suppress menstruation and induce atrophy of the ectopic endometrium. Established hormonal therapy includes oral contraceptives, danazol, progestogens, GnRH agonists and gastrinone. The combination of estrogen and progestogen (oral contraceptives) for the treatment of endometriosis - the so-called 'pseudopregnancy' regimen – is believed to produce initial decidualization and growth of endometrial tissue, followed in several months by atrophy. Today, oral contraceptives are the most commonly prescribed treatment for endometriosis symptoms. Danazol is an isoxazol derivative of 17α-ethinyl testosterone and acts primarily by suppressing the midcycle luteinizing hormone (LH) surge, creating a chronic anovulatory state (Göbel and Rjosk, 1977; Floyd, 1980). Progestagens are a class of compounds that produce progesterone-like effects on endometrial tissue. A large number of progestagens exist, but medroxyprogesterone acetate (MPA), norethindrone, norgestrel and levonorgestrel are mostly used. The proposed mechanism of action of these compounds is initial decidualisation of endometrial tissue followed by eventual atrophy (Olive, 2005). Recent evidence suggests that another mechanism of action on the molecular level is the suppression of matrix metalloproteinases, enzymes important for the implantation and growth of ectopic endometrium (Bruner et al., 1999). Gonadotropin-releasing hormone agonists (GnRH agonists: buserelin, goserelin, nafarelin, leuprorelin and triptorelin) are analogs of the hormone GnRH. They bind to the pituitary receptors and remain for a lengthy period. They are identified by the pituitary and, after initial stimulation of FSH and LH secretion, result in a shut-down (downregulation) of the pituitary and missing stimulation of the ovary. This inducts a hypoestrogenic state producing endometrial atrophy and amenorrhea. Gestrinone (ethylnorgestrienone, R2323) is an antiprogestational steroid believed to act by inducting a progesterone withdrawal effect at the endometrial cellular level, thus enhancing lysosomal

degradation of the cell culture. Other therapies, such as mifepristone and selective progesterone receptor modulators, GnRH antagonists, aromatase inhibitor, TNF-α inhibitors, angiogenesis inhibitors, matrix metalloproteinase inhibitors, pentoxifylline and other immunomodulators, and estrogen receptor β agonists are still experimental treatment. Further studies should be done to possibly confirm them as successful therapeutics.

There are two major problems in hormonal therapy. First, hormonal therapy of endometriosis is not fitted to the wishes of many patients since they suffer from adverse effects during long duration of treatment. Adverse effects are most commonly irregular bleeding, bloating, mood changes, and weight gain in progestogens and occasionally deepening of the voice in danazol treatment. Adverse effects of GnRH analogues include hot flushes, insomnia, reduced libido, vaginal dryness, headache and reduced bone mineral density. The second problem of hormonal therapies of endometriosis is the recurrence, which occurs commonly after discontinuation of hormonal treatment, sometimes even during treatment. It is likely that between 25-60% of women will have recurrence of the disease within 1 year after finishing their treatment (Edmonds, 1999; Winkel and Scialli, 2001).

1.1.3.2 Surgical therapy of endometriosis

The surgical treatments for women with endometriosis may be classified generally as either conservative or radical.

Conservative surgical procedures are most often accomplished today via laparoscopy approach that aims to conserve the functional capacity of uterus, fallopian tubes, and ovaries. The main conservative surgical techniques performed by laparoscopy are thermal or laser ablation, excision, ovarian cystectomy (surgical management of ovarian endometriomas) and ancillary surgical procedures (denervation procedures). A retrospective review found that 74% of women reported improvement or resolution of pain after surgery (DTB, 1999).

The radical approach is total hysterectomy and bilateral salpingoooophorectomy. It's often called 'definitive' surgery in spite of the fact that women who have undergone this type of procedure sometimes experience recurrence of endometriosis and of symptoms (Redwine, 1994, Namnoum et al., 1995).

1.1.4 Prognosis of endometriosis

It has been suggested that only about 30% of women experience progressive disease while other either remain in a steady state or deteriorate (Farquhar, 2004). Recurrence is common once hormonal treatment is discontinued. In trials of treatment with GnRH analogues or danazol, about 10-20% of women required further treatment within 12 months. Uncontrolled cohort studies reported recurrence rates of about 50% at 5 years. Cohort studies of surgical treatment of endometriosis report 20% relapse at 5 years after surgery. But extended follow-up trials are required to determine the likelihood of recurrence in the long term (Farquhar and Sutton, 1998).

1.2 Vitamin D

The active metabolite of vitamin D, $1, 25(OH)_2 D_3$, is a steroid, lipophilic hormone. It is well known for its role in the regulation of calcium and phosphate homeostasis. However, a number of studies have shown that vitamin D metabolites also regulate growth and differentiation of other cell types.

1.2.1 Metabolism of vitamin D

The immediate precursor in the vitamin D cholesterol biosynthetic pathway is 7-dehydrocholesterol (provitamin D3). 7-Dehydrocholesterol is produced in relatively large quantities in the skin of many vertebrate animals, including humans. During exposure to sunlight, the 7-dehydrocholesterol in the epidermal and dermal cells absorbs ultraviolet B (UVB) radiation with wavelengths of 290–315 nm. The absorption of this radiation results in a rearrangement of the 5, 7-diene in the B-ring that causes a break in the B-ring to form the 9,10-secosterol, previtamin D3. Previtamin D3 is thermodynamically unstable, and it rearranges its double bonds to form the more thermodynamically stable vitamin D3 (cholecalciferol) structure (Holick, 2003). A smaller part of vitamin D3 content of human body is obtained from the diet (Malloy and Feldman, 1999). In the liver, the enzyme 25-hydroxylase converts vitamin D3 to 25-hydroxyvitamin D3 (25D), the major circulating form of vitamin D3. 25D is further hydroxylated

in the kidney by the enzyme 1α hydroxylase to form the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25D) (**Figure 1**). While the kidney is the primary site of 1,25D production, 1α hydroxylase is also expressed in extrarenal sites allowing for local synthesis of 1,25D (Hewison et al., 2000). 1, 25D also induces its own destruction by enhancing the expression of 25-hydroxyvitamin D-24-hydroxylase (24-OHase).

1.2.2 Vitamin D in calcium homeostasis

Maintenance of serum calcium levels in the physiological range is a complex process that reflects the function of - and interaction between - vitamin D, parathyroid hormone (PTH), and the calcium sensing receptor. The calcium sensing receptor is found on the cell surface of tissues such as the parathyroid gland, kidney, and bone. A lowering of ionised calcium in extracellular fluid is detected by this receptor, and leads to enhanced parathyroid hormone (PTH) secretion by the parathyroid chief cell. PTH increases reabsorption of calcium by the distal renal tubule, mobilises calcium reserves from bone and increases renal phosphate excretion. It also enhances 1α hydroxylation of vitamin D to 1, 25D by the kidney. 1, 25D in turn increases intestinal calcium absorption and, together with PTH, mobilises calcium from bone which increases serum calcium levels.

1.2.3 Vitamin D receptor (VDR)

Vitamin D and its analogues exert their activity through both genomic and non-genomic pathways. The classic genomic response is mediated through VDR, a member of the nuclear receptor superfamily (Mangelsdorf et al., 1995). VDRs are present in more than 30 tissues, including intestine, kidney, bone, brain, stomach, heart, pancreas, skin, activated T and B lymphocytes, colon, ovary, breast, and prostate (Berger et al., 1988, Holick, 2003). VDR is a ligand-activated transcription factor that, in combination with the retinoid-X receptor (RXR) and in some cases the retinoid-A receptor (RAR), binds to the vitamin D response element (VDRE) in the promoters of target genes (Kliewer et al., 1992). The high-affinity VDR/RXR receptor hetero-dimer interacts with coactivator complexes that link VDR to the RNA polymerase complex and initiate transcription.
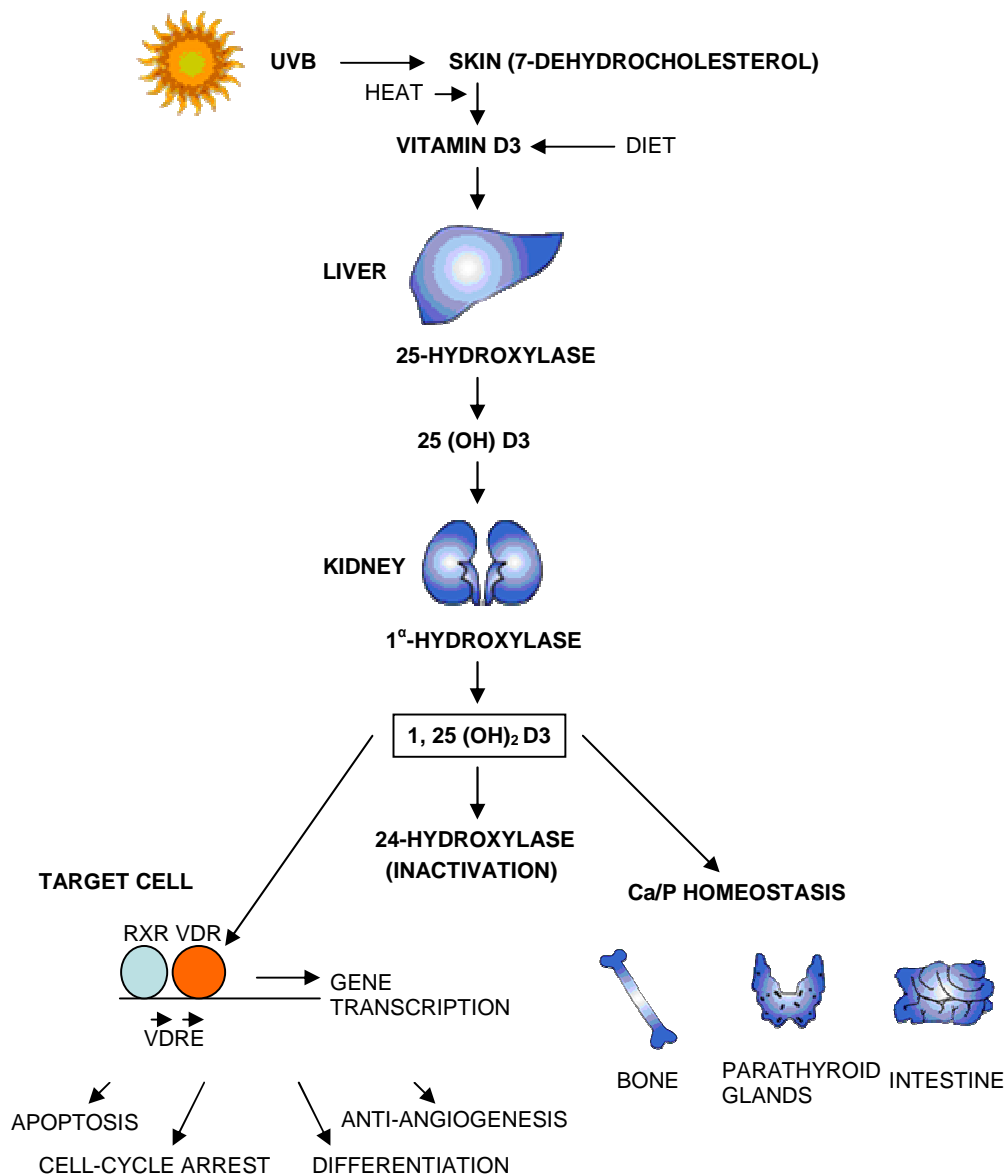
Figure 1. Vitamin D metabolism.

A number of genes are recognized to contain functional vitamin D response elements. These include several bone-related genes [osteocalcin, osteopontin, bone sialoprotein, the calcium binding proteins calbindin-D28k and D9K, fructose 1,6-bisphosphatase, parathyroid hormone, parathyroid hormone-related protein (Osborne and Hutchinson, 2002; Christakos et al., 2003), and human growth hormone (Seoane et al., 2002)], as well as the cell cycle regulator p21 (Liu et al., 1996), the insulin receptor (Maestro et al., 2003), 25(OH)D3 24-hydroxylase (Zierold et al.,

1994), tumor necrosis factor α (Hakim and Bar-Shavit, 2003), urokinase plasminogen activator, protein lipase C g (PLCg), transforming growth factor h2, fibronectin, h3 integrin (Osborne and Hutchinson, 2002), and involucrin (Bikle et al., 2002).

In addition to the classic genomic effects of VDR, vitamin D regulates a number of cytoplasmic signaling pathways through protein kinase C (de Boland et al., 1994; Beno et al., 1995; de Boland et al., 1996), ras and mitogenactivated protein kinase (MAPK) (Beno et al., 1995; Gniadecki, 1996; Park et al., 2000), protein lipase A and prostaglandins (Bellido et al., 1993; Vasquez et al., 1995), cyclic AMP and protein kinase A (Santillan and Boland, 1998), phosphatidyl inositol 3, the ceramide pathway (Bektas et al., 2000), and Ca2+-regulated voltage-sensitive (VSCC) or insensitive (VICC) channels (de Boland and Norman, 1990). Activation of the cytoplasmic signaling pathways often results in rapid changes in intracellular calcium and the activation or deactivation of proteins such as bcl-2 and c-jun. A number of these pathways ultimately affects cellular growth, differentiation, and apoptosis and may cooperate with the classical genomic pathway to transactivate VDR.

1.2.4 Antineoplastic activity of vitamin D

For a long time, vitamin D was well known for its role in the regulation of calcium and phosphate homeostasis (Jones et al., 1998). However, numerous *in vitro* and *in vivo* studies have shown that vitamin D potently inhibits cell proliferation in a wide range of cell types, including carcinomas of the ovaries, breast, prostate, colon, skin, brain, myeloid leukemia cells, and others (Guyton et al., 2003). Based largely on investigations carried out in *in vitro* models of cancer, several mechanisms have been suggested for vitamin D antineoplastic activity. Proposed mechanisms differ between tumor models and experimental conditions, and no unifying hypothesis about the mechanism of activity has emerged. These mechanisms of antineoplastic activity include induction of apoptosis, inhibition of proliferation through cell cycle arrest, induction of differentiation, inhibition of invasion and motility, and reduction of angiogenesis (Trump et al., 2004). Vitamin D-induced apoptosis occurs via the intrinsic apoptotic signaling pathway.

Activation of the intrinsic apoptotic pathway leads to alterations in mitochondrial membrane permeability, which results in the release of cytochrome C into the cytosol and the subsequent activation of downstream caspases (Narvaez and Welsh, 2000). Vitamin D-induced inhibition of proliferation through cell cycle arrest was mediated through stabilization of p27 (tumor suppressor) and G_1 arrest (Pengfei et al., 2004).

1.3 Aim of the study

Concerning several pathophysiological and clinical similarities between endometriosis and cancer we wanted to answer the following two questions. Has endometriosis similarities compared to ovarian cancer in view of the vitamin D system and corresponding hydroxylases?

In the current study, we examined the VDR expression in endometrium and ovaries of patients with endometriosis, healthy controls and endometrial or ovarian cancer by realtime PCR and Westernblot. We also measured the expression of vitamin D related enzymes (1α-hydroxylase, 24-hydroxylase and 25-hydroxylase) in endometrium and ovaries. In endometrial cell culture, by realtime PCR, we examined the expression of VDR in epithelium and stroma. Additionally, expression of VDR in endometrium and ovaries of patients with endometriosis and endometrial cancer was confirmed by immunohistochemistry. 25(OH) vitamin D was measured in peripheral blood of women with and without endometriosis by RIA.

2 MATERIALS AND METHODS

2.1 Patient recruitment and characterization

The study included women undergoing laparoscopy or laparotomy at the Department of Gynecology and Obstetrics, University of Lübeck. All patients had not received hormones or GnRH agonist therapy for at least 6 months before surgery. Each patient had provided written informed consent under a study protocol approved by the Committee on Human Research at the University of Lübeck, Lübeck, Germany. Patients with endometriosis were staged according to the revised American Fertility Society system (American Society for Reproductive Medcine, 1996). Control subjects were women who had no evidence of pelvic pathology despite careful evaluation at laparoscopy. Women with endometrial or ovarian cancer were confirmed histologically.

2.2 Preparation of samples

2.2.1 Preparation of tissue samples

Tissue samples for PCR and immunohistochemistry were collected from patients with endometriosis, healthy controls and patients with endometrial or ovarian cancer undergoing surgery. Endometrial tissue samples were collected using endometrial pipelles (Laboratoire CCD, Paris, France). Immediately after collecting, samples for PCR were placed into liquid RPMI medium (RPMI, Cambrex, Belgium) with 10% FCS and 1% PSG and transported to the laboratory. In the laboratory, samples were washed in DPBS (DPBS, Cambrex, Belgium), cut into adequate size and frozen and stored in liquid nitrogen. Samples for immunohistochemistry were fixed in 4.5 % formaldehyde and embedded in paraffin.

2.2.2 Endometrial cell isolation and culture

Endometrial biopsies were collected under sterile conditions with endometrial pipelles (Laboratoire CCD, Paris, France) and transported to the laboratory in RPMI medium with 10% FCS and 1% PSG (1% penicillin G, 1% streptomycin and 1% gentamycin). The tissue was minced into small pieces, digested with collagenase (2 mg/ml) for 1h at 37°C, and separated using serial filtration. Debris was removed by 100-μm aperture sieves, and epithelial glands were retained on 40- μm aperture sieves and backwashed onto tissue culture dishes. Remaining filtrate with stromal cells was centrifugated and cells were cultured in RPMI medium reconstituted with 10% FCS, 1% penicillin G, 1% streptomycin and 1% gentamycin. All culture dishes were incubated at 37°C; stromal cells for 2h and epithelial cells for 24h. After this time, cells were washed in PBS solution twice and collected in Trizol for RNA isolation.

2.2.3 Preparation of human peripheral blood

For measurement of 25-hydroxy vitamin D in serum, we collected peripheral blood (PB) from patients with and without endometriosis. The study group consisted of 46 women in reproductive age, who had endometriosis diagnosed at laparoscopy. The control group consisted of 33 age-matched women without endometriosis. Blood samples were obtained from peripheral blood of patients before anaesthesia and laparoscopy by a special BD Safety-Lok™ Blood Collection Set (Becton Dickinson and Company, Franklin Lakes, N.J., USA). Samples were centrifugated at 3000 rpm for 5 min and serum was collected in sterile tubes and stored on -20°C.

2.2.4 Instruments and equipments

■ Centrifuge (Hettich)

■ CO_2 Incubator (NUAIRE)

■ Flow (Biohazard)

■ Multi-channel pipette (Biozym)

■ Gamma counter (Wallac)

- Microtome SM 2000 R (Leica)

- Photometer (Eppendorf)

- Realtime PCR (Biozym)

- Vortex mixer (Heidolph)

- Light microscope (Werner Hassa)

- Water bath (Werner Hassa)

2.3   Methods

2.3.1 Immunohistochemistry of VDR

Tissue samples used for immunohistochemistry were: endometriotic tissue of patients with ovarian endometriosis, proliferative and secretory phase endometrium of women with endometriosis and healthy women, and ovarian and endometrial cancer tissue. All samples were fixed in 4.5 % formaldehyde and embedded in paraffin before they were cut to a thickness of 5 µm. Slides were deparaffined with consecutive simmering in alcohol in the following way:

four times for 5 min in Xylol (Merck, Darmstadt, Germany)

two times for 5 min in Isopropylalcohol 100% (Fischer, Saabrücken, Germany)

two times for 5 min in Ethanol 96% (Apotheke, Universität Lübeck, Germany)

two times for 5 min in Ethanol 70% (Apotheke, Universität Lübeck, Germany).

Thereafter, slides were washed two times for 5 min in Aqua dest. (Apotheke, Universität Lübeck, Germany). For preparing slides antigen-free, they were heated in a microwave oven on 600°C with citratbuffer pH 6.0 (Dako, Hamburg, Germany), three times for 5 min. Before washing in Aqua dest. and three times for 5 min in PBS, slides were cooled at room temperature for 20-30 min.

To inhibit unspecific protein binding, the sections were incubated with 1.25 % normal rabbit serum in PBS for 20 min at room temperature. Primary vitamin D receptor (VDR) antibody (Dianova, Hamburg, Germany) was applied at a dilution of 1:200 in a moist chamber overnight at

4°C. After three additional washing steps with PBS for 5 min, biotinylated secondary polyclonal rabbit anti-rat IgG antibody in a dilution of 1:80 (Dako, Hamburg, Germany) was added for 30 min. Sections were washed again for three times with PBS for 5 min and subsequently incubated with avidine-biotine-peroxidase complex for 30 min at room temperature. All slides were washed in PBS three times for 5 min. After an additional washing step in PBS with 0.1 % TritonX-100 (Bio-Rad, München, Germany) for 30 s, the slides were incubated 3 min with peroxidase substrate solution DAB (Vector laboratories Inc., Burlingome, CA). Finally, sections were rinsed three times in Aqua dest. and counterstained with hematoxylin before they were mounted.

2.3.2   Realtime PCR for VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT mRNA

2.3.2.1   Total RNA isolation from tissue samples by the Quiagen kit

Total RNA from endometrial and ovarian tissue samples was isolated by the Quiagen kit for the following cDNA synthesis.

2.3.2.1.1 Reagents used in total RNA isolation by the Quiagen kit

■ Buffer RLT   Quiagen, Hilden, Germany

■ Ethanol (70%)   Merck KGaA, Darmstadt, Germany

■ Buffer RW1   Quiagen, Hilden, Germany

■ DNase   Quiagen, Hilden, Germany

■ Buffer RDD   Quiagen, Hilden, Germany

■ Buffer RPE   Quiagen, Hilden, Germany

■ RNase-free water   Quiagen, Hilden, Germany

2.3.2.1.2 Protocol for total RNA isolation from tissue samples by the Quiagen kit

Tissue samples were disrupted (not more than 30mg), rinsed in Buffer RLT and homogenized in rotor-stator homogenizer to shear genomic DNA and reduce viscosity of lysate. Tissue lysate was centrifuged for 3 min at maximum speed and the supernatant was carefully transferred to a microcentrifuge tube. 70% ethanol was added to the lysate and mixed. The complete newformed sample was applied to the RNeasy mini column placed in a 2 ml collection tube and centrifuged 15 s at 10,000 rpm. Flow-through was discarded. After wash spin with Buffer RW1, DNase on-column digestion was performed. Additional two wash spins with Buffer RPE were made. RNeasy column was transferred to a new 1.5 ml collection tube and RNase-free water was added directly onto the RNeasy silica-gel membrane. The tube was centrifuged for 1 min at 10,000 rpm for elution. The concentration of RNA was determined by measuring absorbance at 260 nm in a spectrophotometer (1:50 dilution). The samples of RNA were stored at -70°C.

2.3.2.2 Total RNA isolation from endometrial cell culture by Trizol

Total RNA from endometrial cell culture (epithelial and stromal cells) was isolated by Trizol reagent.

2.3.2.2.1 Reagents used in total RNA isolation from endometrial cell culture by Trizol

- Trizol reagent   Invitrogen life technology, Karlsruhe, Germany
- Chloroform   Merck KGaA, Darmstadt, Germany
- Isopropylalcohol (100%)   Apotheke, Universität Lübeck, Germany
- Ethanol (100%)   Merck KGaA, Darmstadt, Germany
- 0.1% DEPC H2O   Serva, Germany

2.3.2.2.2 Protocol for total RNA isolation from endometrial cell culture by Trizol

Epithelial and stromal cells were washed with phosphate buffered saline (PBS) and lysed in 1 ml Trizol reagent by repetitive pipetting. The homogenized sample was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added into the homogenized samples and the tube was shaked vigorously by hand for 15 seconds. After incubation for 2 minutes at room temperature, the samples were centrifuged at $12,000 \times g$ for 15 minutes at 4°C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase (RNA phase exclusively). Then the aqueous RNA phase was transferred to a fresh tube carefully. The total RNA from the aqueous phase was precipitated by mixing with 100% isopropylalcohol (0.5 ml). The samples were centrifuged at $12,000 \times g$ for 10 minutes at 4°C after incubation at room temperature for 10 minutes. The RNA, a gel-like pellet on the bottom of the tube was precipitated after centrifugation. The RNA pellet was washed once with 75% ethanol (1 ml) after removing the supernatant. Following vortexing, the sample was centrifuged at $7,500 \times g$ for 5 minutes at 4°C. The ethanol supernatant was removed. The RNA pellet was dissolved in RNase-free water (30 µl) after air-dry for 10 minutes briefly and incubated for 10 minutes at 55 to 60°C. The concentration of RNA was determined by measuring absorbance at 260 nm in a spectrophotometer (1:50 dilution). The samples of RNA were stored at -70°C.

2.3.2.3 Reverse Transcription Reaction

First strand cDNA was synthesized by Reverse Transcription (RT) reaction. Total RNA extracted from treated, untreated U937 cells and human whole blood was utilized as template and specific antisense oligonucleotides described under **2.3.1.3.1** were used as primers.

2.3.2.3.1 Reagents used in Reverse Transcription Reaction

■ Oligo (dT) (500µg/ml)   All from Invitrogen life technology, Karlsruhe,

■ SuperScriptTM II RT   Germany

■ RNaseOUTTM

■ dNTP Mix

■ 0.1 M DTT

■ 5X First-Strand Buffer

2.3.2.3.2 Protocol of Reverse Transcription Reaction

Total RNA (1 µg) was pipetted with HPLC water up to 10µl and 1µl Oligo (dT) (500µg/ml) was added in a steril eppendorf tube. The tubes were heated in 70°C for 10 min in the PCR machine. The RT mixture was prepared as described in **Table 1** and 8 µl RT mixture was pipetted into each tube. 1 µl (200 units) SuperScript^{TM} II RT was added into each tube except the negative control. The sample was incubated at 50°C for 30 min. The cDNA was stored in -20°C and then used as a template for PCR and realtime PCR amplification reactions.

RT mixture	Volumes per reaction
5X First-Strand Buffer	4 µl
0.1 M DTT	2 µl
dNTP Mix (10mM)	1 µl
RNaseOUT^{TM} (40 units/µl)	1 µl
Total volume	8 µl

Table 1: Components of RT mixture

2.3.2.4 PCR of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT

The cDNA first strands were used as templates for polymerase chain reactions (PCR). The appropriate primers were used.

2.3.2.4.1 Reagents used in PCR

■ 10X PCR buffer   Sigma, München,Germany

■ Taq DNA Polymerase   Sigma, München,Germany

■ dNTP MIX (2mM)   Sigma, München,Germany

■ Human VDR, 1α-Hydroxylase,   Metabion, Martinsried, Germany
   24-Hydroxylase and 25-Hydroxylase

   Primer Pairs (**Table 2**

cDNA product size VDR:	383 base pairs (bp)
cDNA product size 1α-Hydroxylase:	226 base pairs (bp)
cDNA product size 24-Hydroxylase:	335 base pairs (bp)
cDNA product size 25-Hydroxylase:	291 base pairs (bp)

■ Hypoxanthine phosphoribosyl transferase   Metabion, Martinsried, Germany
   (HPRT) Primer (**Table 3**)
   cDNA product size : 222 bp

1α-Hydroxylase:	5'-TGTTTGCATTTGCTCAGA-3' upstream
	5'-CCGGGAGAGCTCATACAG-3' downstream
24-Hydroxylase:	5'-GCAGCCTAGTGCAGATTT-3' upstream
	5'-ATTCACCCAGAACTGTTG-3' downstream

25-Hydroxylase:	5'-GGCAAGTACCCAGTACGG-3' upstream
	5'-AGCAAATAGCTTCCAAGG-3' downstream
VDR:	5'-CCAGTTCGTGTGAATGATGG-3' upstream
	5'-GTCGTCCATGGTGAAGGA-3' downstream

Table 2: *Primers used in PCR*

| HPRT: | 5'-CCT GGC GTC GTG ATT AGT GAT-3'upstream |
| | 5'-CCA GCA GGT CAG CAA AGA ATT TA-3'downstream |

Table 3: HPRT primer used in PCR

2.3.2.4.2 Protocol of PCR

The following components were prepared as master mixture for VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase PCR and for HPRT PCR (**Table 4**). The master mixtures were pipetted in a sterile reaction tube for VDR, 1α-Hydroxylase, 24-Hydroxylase and 25-Hydroxylase PCR (50 µl) or HPRT (50.2 µl) PCR respectively. The reactive conditions of PCR were shown hereinafter:

■ Conditions of **VDR, 1α-Hydroxylase,** **24-Hydroxylase and 25-Hydroxylase** PCR	■ Conditions of **HPRT** PCR
94°C for 30 sec	95°C for 1 min
55°C for 30 sec	94°C for 15 sec
72°C for 30 sec	94°C for 15 sec
Repeat for 35 cycles	60°C for 15 sec
	72°C for 15 sec
	Repeat the last 3 steps for 39 cycles
Heated for 10 min in 72°C	Heated for 5 min in 72°C
PCR products were stored at -20°C.	

Reagents in the mixture	Volumes per reaction	
	VDR and enzymes	HPRT
cDNA pool	1 µl	1 µl
10X PCR Puffer	5 µl	5 µl
dNTP (2mM)	5 µl	5 µl
Taq DNA Polymerase	0.2 µl	0.2 µl
VDR, 1α-Hydroxylase, 24-Hydroxylase and 25-Hydroxylase primer pairs (4 µM)	10 µl	
HPRT primer Mix (4 µM)		10 µl
HPLC water	28.8 µl	29 µl
Total volume	50 µl	50.2 µl

Table 4: *Components of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT PCR master mixture*

2.3.2.5 Agarose Gel Electrophoresis

After amplification, the PCR products were separated by gel electrophoresis using 1.5% (w/v) agarose gels. The PCR products of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT were put on agarose gels to show a single amplification fragment located on the expected position and for PCR clean-up gel extraction later.

2.3.2.5.1 Reagents used in Agarose Gel Electrophoresis

■ Agarose   Invitrogen, Karlsruhe, Germany

■ Ethidium Bromide solution (10mg/ml)

■ Marker (100 bp, DNA ladder)   Biolab, Germany

■ Bluestop (6 x)   Apotheke, Universität Lübeck, Germany

■ 10x TBE buffer   Merck KGaA, Darmstadt, Germany

    10x TBE (per liter) contains:

        108g Tris base, 55g boric acid, 40ml 0.5 M EDTA, pH 8.0

2.3.2.5.2 Protocol of Agarose Gel Electrophoresis

To separate and identify DNA fragments, agarose gel electrophoresis was employed. The concentration of agarose was 1.5 % (w/v). The agarose was dissolved in Tris-borate/EDTA (TBE) electrophoresis buffer at 90-100°C for 1-2 min. After cooling down to 60°C, ethidium bromide was added to a final concentration of 0.005 % (v/v) and the gel was poured into a tray with a comb to generate the sample wells. 25 µl of each PCR product was mixed with 5 µl Bluestop buffer (6 x). 20 µl of this mixture above was loaded into one sample well of 1.5% agarose gel. Electrophoresis was carried out with TBE buffer and a constant voltage of 1 V/cm of gel length for approximately 30 min. The bands on agarose gels were visualized under ultraviolet illumination and the results were documented by photography. The PCR products of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT showed a single amplification fragment at the expected position.

2.3.2.6   PCR clean-up Gel extraction

PCR amplified products and restricted DNA fragments were purified by PCR clean-up Gel extraction NucleoSpin$^{®}$ Extract II Kit after isolation by agarose gel electrophoresis.

2.3.2.6.1   Reagents used in PCR clean-up Gel extraction

■ PCR clean-up Gel extraction NucleoSpin®   Macherey-Nagel, Düren, Germany
   Extract II Kit
■ Kit contents: Buffer NT
                 Buffer NT (concentrate)
                 Buffer NE
                 NucleoSpin® Extract II columns
                 NucleoSpin® collecting tubes (2ml)
With the NucleoSpin® Extract II method, DNA binds in the presence of chaotropic salts (buffers NT) to a silica membrane. Afterwards, binding mixtures are loaded directly onto NucleoSpin®

Extract II columns. Contaminations like salts and soluble macromolecular components are removed by a simple washing step with ethanolic buffer NT3. Pure DNA is finally eluted under low ionic strength conditions with slightly alkaline buffer NE (5 mM Tris-Cl, pH 8.5).

2.3.2.6.2 Protocol of PCR clean-up Gel extraction

The DNA fragment were excised from agarose gel by a clean scalpel and transfered to a clean tube. The gel slices were weighed and 3 volumes of dissolving buffer NT was added to 1 volume of gel (to 100 µg agarose gel add 300 µl buffer NT). The sample was incubated at 50°C for 10 min and vortexed briefly every 2-3 min until the gel slices were dissolved completely. A NucleoSpin® Extract II column was placed into a 2 ml collecting tube and the sample was loaded. After centrifugation for 1 min at 11,000 x g, flow was discarded through and the column was placed back into the collecting tube. 600 µl buffer NT3 was added and was centrifuged for 1 min at 11,000 x g. Liquid was removed and the column was placed back into the collecting tube. The sample was centrifuged for 2 min at 11,000 x g to remove buffer NT3 quantitatively. Then the column was transfered into a clean 1.5 ml microcentrifuge tube. 30 µl elution buffer NE was added and incubated for 1 min at room temperature to increase the yield of eluted DNA. The sample was centrifuged for 1 min at 11,000 x g again. The concentration of DNA was determined by measuring absorbance at 260 nm in a spectrophotometer (1:10 dilution) for preparing the standard curve of Realtime PCR. The samples of DNA were stored at -70°C.

2.3.2.7 Realtime PCR of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT

cDNA first strands from endometrial and ovarian tissue samples, as well as from endometrial cell culture, were utilized as template. The appropriate primers were used as described in **2.3.1.4.1**.

2.3.2.7.1 Reagents used in Realtime PCR

■ Human VDR, 1α-Hydroxylase,   Metabion, Martinsried, Germany

  24-Hydroxylase and 25-Hydroxylase

  Primer Pairs (**Table 2**)

■ Hypoxanthine phophoribosyl transferase   Metabion, Martinsried, Germany

  (HPRT) Primer (upstream and downstream)

■ HPLC water   Merck KGaA, Darmstadt, Germany

■ 0.2 ml Low Profile Strip Tubes   Biozym, Postfach, Germany

■ Platinum® SYBR® Green qPCR SuperMix   Invitrogen, Karlsruhe, Germany

  UDG

Platinum® SYBR® Green qPCR SuperMix UDG (2X) contains:

    SYBR® Green I

    60 U/ml Platinum® Taq DNA polymerase

    40 mM Tris-HCL (pH 8.4)

    100 mM KCL, 6 mM $MgCl_2$

    400 µM dGTP, 400 µM dATP, 400 µM dCTP, 400 µM dUTP

    40 U/ml uracil-DNA glycosylase (UDG) and stabilizers.

2.3.2.7.2 Protocol of Realtime PCR

The molecules of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT PCR products after PCR clean-up gel extraction were calculated. All PCR products were diluted into 5 different concentrations: 5×10^6, 5×10^5, 5×10^4, 5×10^3 and 5×10^2 molecules as standard for realtime PCR.

cDNA (1 µl) of each endometrial and ovarian tissue sample, as well as endometrial cell culture sample was pipetted into a steril strip reaction tube. 1 µl HPLC water and 1 µl negative control products after RT PCR was added respectively as two negative controls of Realtime PCR. Master mixture of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase or HPRT Realtime PCR was

prepared as described in **Table 5**. Then 23 µl of the master mixture was pipetted in sterile reaction strip tubes for Realtime PCR as described in cycling program hereinafter:

■ Conditions of **VDR** realtime PCR	■ Conditions of **1α-Hydroxylase**, **24-Hydroxylase** and **25-Hydroxylase** realtime PCR
50°C for 2 min	50°C for 2 min
95°C for 2 min	95°C for 2 min
95°C for 30 sec	95°C for 15 sec
62.4°C for 30 sec	57.3°C for 15 sec
72°C for 30 sec	72°C for 15 sec
Repeat the last 3 steps for 45 times cycling	Repeat the last 3 steps for 45 times cycling

■ Conditions of **HPRT** realtime PCR
50°C for 2 min hold
95°C for 2 min hold
95°C for 15 sec
60°C for 15 sec
72°C for 15 sec
Repeat the last 3 steps for 39 times cycling
50°C for 1 sec. The melting curve was analyzed from 60°C to 95°C and read every 0.3°C for 1 sec.

Reagents in the mixture	Volumes per reaction	
	VDR and enzymes	HPRT
Platinum® SYBR® Green qPCR SuperMix UDG	12.5 µl	12.5 µl
VDR, 1α-Hydroxylase, 24-Hydroxylase and 25-Hydroxylase primer pairs (4 µM)	2.5 µl	
HPRT primer MIX (4 µM)		2.5 µl
HPLC water	8 µl	8 µl
Total volume	23 µl	23 µl

Table 5: Components of master mixture of VDR, 1α-Hydroxylase, 24-Hydroxylase, 25-Hydroxylase and HPRT Realtime PCR

2.3.3 Western blot

Samples from ovarian cancer, endometriotic tissue, endometrium from women with endometriosis and control endometrium were lysed in Tris buffer (50mM Tris/150mM NaCl) and 1% Triton X-100 and 1mM PMSF (Phenylmethylsulfonylfluoride, Sigma) added for 30 minutes at 4°C. Nuclei were removed by centrifugation at 13000g for 30 minutes. The protein concentration was determined according to the method of BCA (Bicinchoninic acid, Pierce Biotechnology, Rockford, USA). The proteins were separated by SDS-polyacrylamide gels (SDS-PAGE), transferred on nitrocellulose membranes and the membranes were blocked in a solution of 5% milkpowder (BD, Heidelberg, Germany) for 1h. Primary antibodies (anti-VDR antibody 1:5000, Dianova, Hamburg, Germany; anti-25(OH)VitaminD3-1a-hydroxylase 1:10000, Biologo, Kronshagen,Germany, CYP27A1(T-19) 1:8000, Santa Cruz Biotechnology, Heidelberg, Germany; CYP24 1:4000, Santa Cruz Biotechnology, Heidelberg, Germany) were diluted in blocking buffer and incubated overnight. After washing, peroxidase-conjugated IgG secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) were added for 1h. For protein load control, anti-β-actin mouse monoclonal antibodies were used. Detection of peroxidase activity was performed with enhanced chemiluminescence kits and hyperfilm (Amersham, Freiburg, Germany).

2.3.4   RIA of vitamin D in peripheral blood

The IDS Gamma-B 25-Hydroxy Vitamin D kit (IDS, Fountan Hills, USA), a complete assay procedure, was used for the extraction and quantification of 25-OH D in serum. To appropriately labelled borosilicate glass tubes with 50 ml of each calibrator, control and sample were added 50 ml of extraction reagent 1. After incubation at 18-25°C for 20 min, 500 ml of extraction reagent 2 was added to each tube for precipitation of serum proteins and extraction of 25-OH D. Following centrifugation at 2000 g for 10 min, 100 ml of ^{125}I 25-Hydroxy Vitamin D was added to 50 ml of each calibrator, control, or sample extract supernatans tubes. After adding 1 ml of antiserum (highly-specific sheep antibody) all tubes were incubated at 18°C for 90 min. Separation of antibody-bound tracer from free is achieved by a short incubation (18-25°C for 20 min) with 100 ml of Sac-Cel, (anti-sheep IgG cellulose) followed by centrifugation (2000 g for 10 min) and decanting. All tubes were counted in a suitable gamma counter for at least 1 min. Bound radioactivity is inversely proportional to the concentration of 25-OH D. Calibration curve covered the range of concentrations found in human serum samples.

2.3.5   Statistical analysis

All experiments were performed in triplicate and all data were expressed as the mean ± SE. All statistical analyses were performed using the GraphPad Prism 4 statistical software package (GraphPad, San Diego, USA). Correlations between all data grouped by a single factor were evaluated using the regular one-way ANOVA with Turkey post test or Kruskal-Wallis statistics. We also used Student's t test to examine two groups of data grouped by one variable. For all analyses, statistical significance was assumed at a *P* level of <0.05.

3   RESULTS

3.1 Immunohistochemical detection of VDR

Immunohistochemistry was performed on samples from human endometrium and endometriotic tissues (**Figure 2**). Each slide was presented as a pair: VDR-positive staining and negative control (staining without VDR antibody). In ovarian endometriosis implants, epithelial cells lining the inside of the endometriotic cyst showed stronger nuclear VDR immunostaining compared with stroma and normal ovarian tissue beneath. VDR immunoreactivity in the proliferative and secretory phase endometrium of patients with endometriosis was markedly increased in the epithelial glands compared to the surrounding stroma. There was even stronger VDR immunostaining of endometrial cells in endometrial cancer compared to cancer stroma.
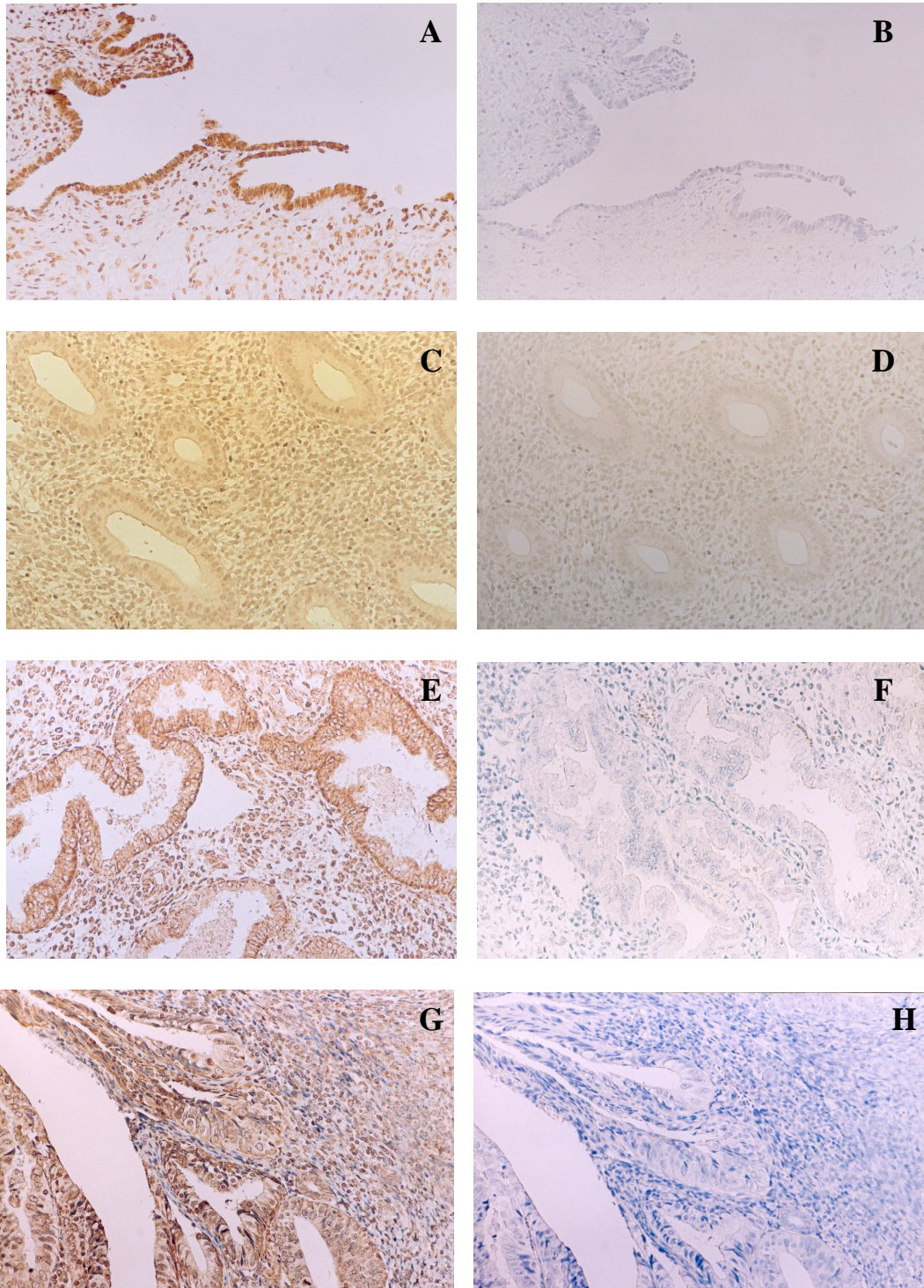
Figure 2. *Immunohistochemical detection of VDR in human endometrial and endometriotic tissues. Epithelial cells lining the inside of the endometriotic cyst of an ovarian endometriosis implant (A) showed stronger nuclear VDR immunostaining compared with stroma and normal ovarian tissue; B- negative control (staining without VDR antibody). VDR immunoreactivity in the proliferative (C) and secretory (E) phase endometrium of patients with endometriosis was markedly increased in the glandular epithelium compared to the surrounding stroma; D and F- negative controls. VDR immunostaining of an endometrial cancer (G) revealed strong immunostaining of endometrial cells; H- negative control.*

3.2 Expression of VDR, 1α-hydroxylase, 24-hydroxylase and 25-hydroxylase in endometrium measured by realtime PCR

Eutopic endometrial VDR mRNA levels, as detected by RT-PCR, were higher in patients with endometriosis (857.3 ± 298.7) compared to the control group (341.3 ± 101.2), but this failed to reach statistical significance ($p=0.10$). However, VDR mRNA was significantly higher in endometrial cancer (1594.0 ± 664.7) compared to control endometrium (341.3 ± 101.2, $p=0.03$) (Figure 3a). The data showed a significantly higher 1α-hydroxylase/HPRT ratio in patients with endometriosis (1.3 ± 0.5) compared to healthy controls (0.3 ± 0.1, $p=0.03$) (Figure 3b). Although levels in cancer were significantly higher than controls, there was no statistical difference between the endometriosis (1.3 ± 0.5) and the cancer group (0.9 ± 0.3, $p=0.68$) in 1α-hydroxylase expression. There was a significantly higher expression of 24-hydroxylase in patients with endometrial cancer (236.3 ± 93.6, $p=0.0109$) than in the control group (24.6 ± 9.6). However, this was not the case with the endometriosis group (203.5 ± 123.8), which showed no statistical difference compared with the cancer ($p=0.8751$) or control group ($p=0.1470$). (Figure 3c).
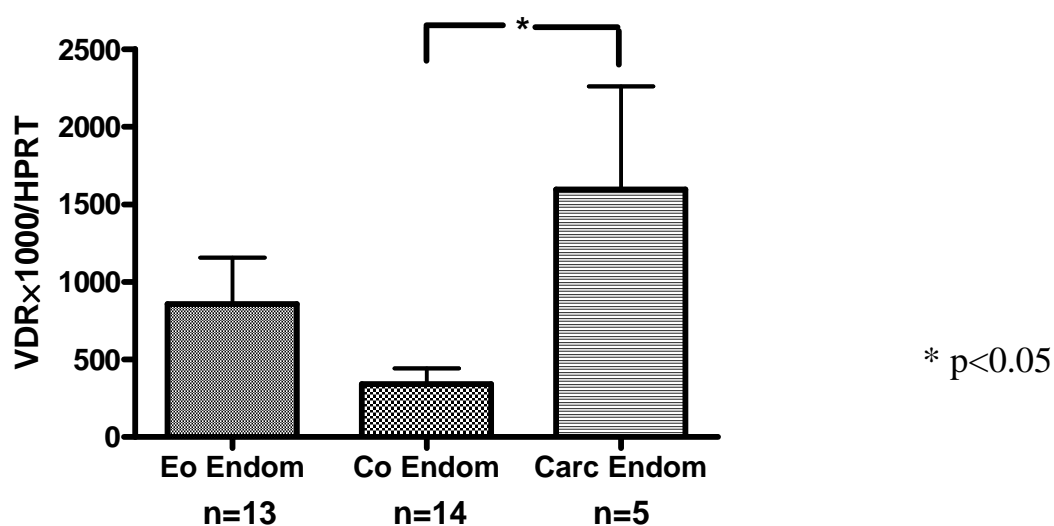

Figure 3a. *Determination of VDR mRNA expression by quantitative RT-PCR in endometrium of patients with endometriosis (Eo Endom), healthy controls (Co Endom) and patients with endometrial cancer (Carc Endom). See Table 1 for mean values $\pm$ SE.*
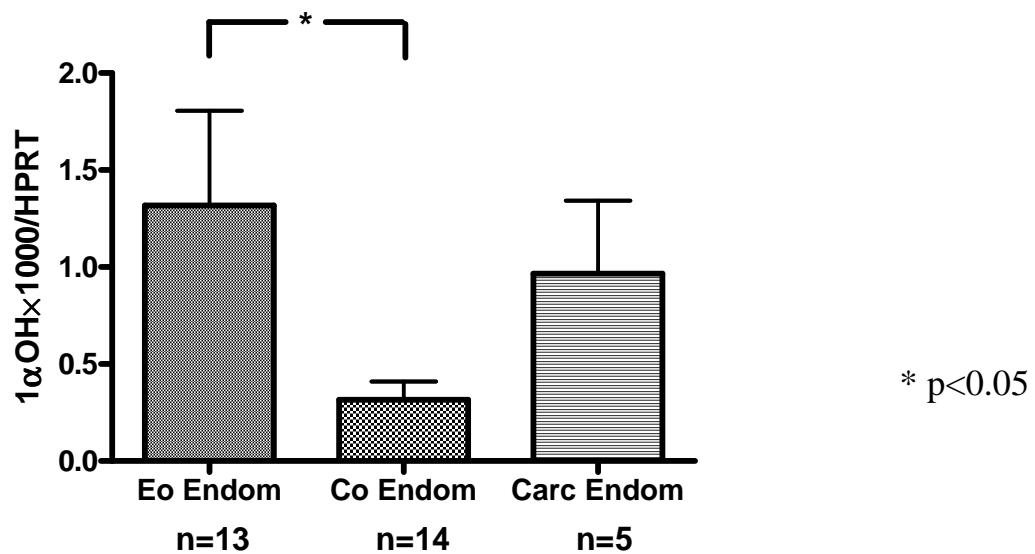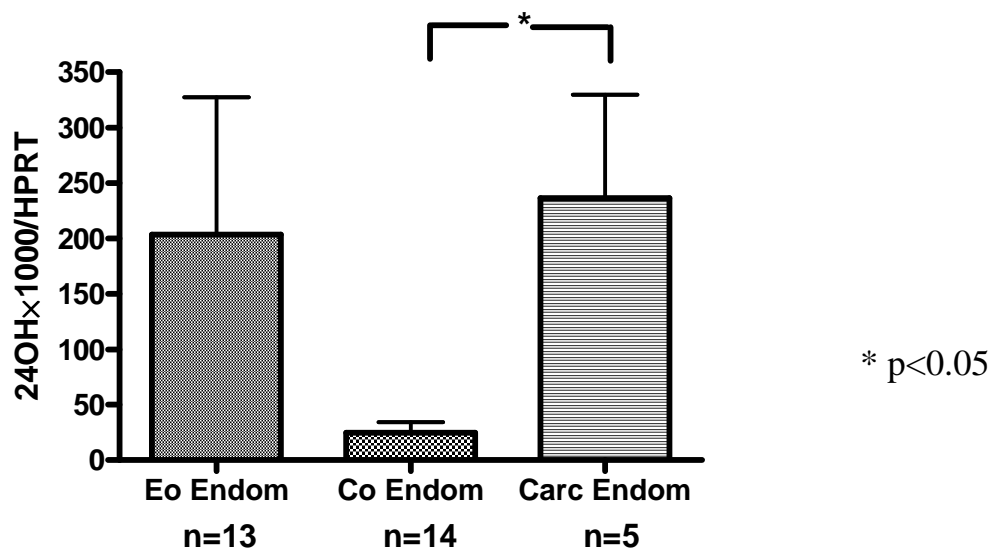
Figure 3b. Determination of 1α-hydroxylase (1α-OH) mRNA expression by quantitative RT-PCR in endometrium of patients with endometriosis (Eo Endom), healthy controls (Co Endom) and patients with endometrial cancer (Carc Endom). See Table 1 for mean values ± SE
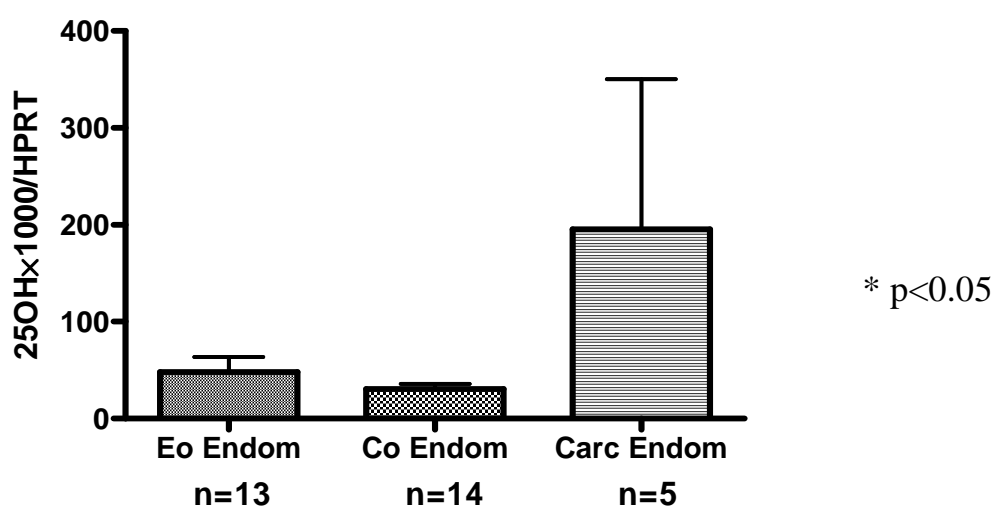

Figure 3c. Determination of 24-hydroxylase (24-OH) mRNA expression by quantitative RT-PCR in endometrium of patients with endometriosis (Eo Endom), healthy controls (Co Endom) and patients with endometrial cancer (Carc Endom). See Table 1 for mean values ± SE.

Likewise, there was no difference among the three groups (endometriosis, 48.0 ± 15.6; healthy controls, 30.4 ± 5.5; cancer, 195.5 ± 154.7) in expression of 25-hydroxylase in endometrium (p=0.55) (figure 3d).

Figure 3d. Determination of 25-hydroxylase (25-OH) mRNA expression by quantitative RT-PCR in endometrium of patients with endometriosis (Eo Endom), healthy controls (Co Endom) and patients with endometrial cancer (Carc Endom). See Table 1 for mean values $\pm$ SE.

3.3 Expression of VDR, 1α-hydroxylase, 24-hydroxylase and 25-hydroxylase in ovaries measured by realtime PCR

VDR mRNA tended to be higher in ovaries of patients with endometriosis (843.0 ± 206.6) than in controls (290.3 ± 96.1), but this did not reach a level of statistical significance (p=0.12). Nevertheless, in the ovarian cancer group (24326 ± 7997) VDR was significantly higher than in patients with endometriosis (843.0 ± 206.6) or healthy controls (290.3 ± 96.1) (p< 0.01) (figure 4a).
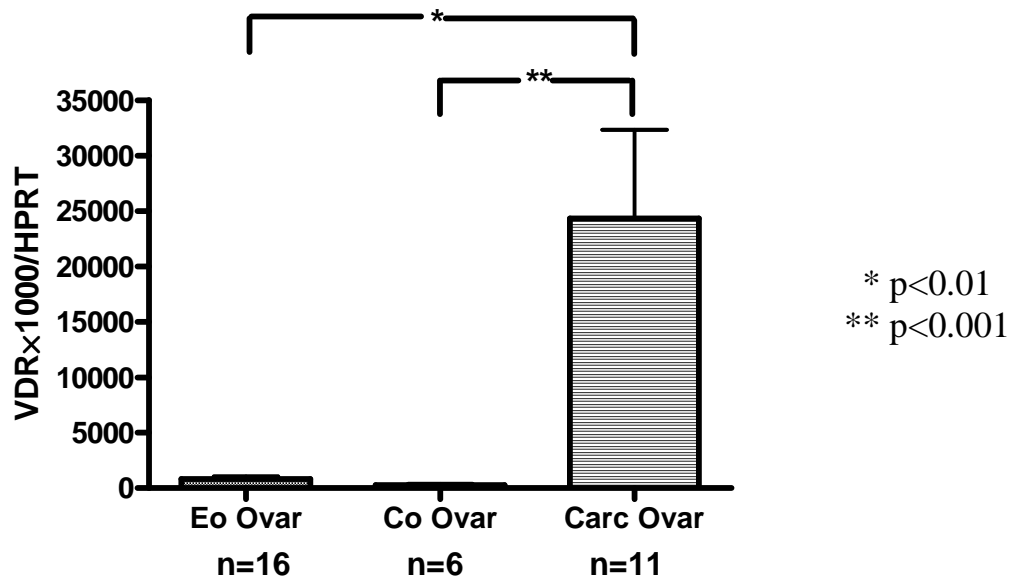
Figure 4a. Determination of VDR mRNA expression by quantitative RT-PCR in ovaries of patients with endometriosis (Eo Ovar), healthy controls (Co Ovar) and patients with ovarian cancer (Carc Ovar). See Table 1 for mean values ± SE.

There was no difference between groups in the expression of 1α-hydroxylase (endometriosis, 2.0 ± 0.3; healthy controls, 1.6 ± 0.2, cancer, 4.3 ± 1.6; p=0.3279) or 24-hydroxylase (endometriosis, 38.0 ± 17.7; healthy controls, 2.2 ± 1.1; cancer, 102.9 ± 73.4; p=0.4631) (Figure 4b and 4c).
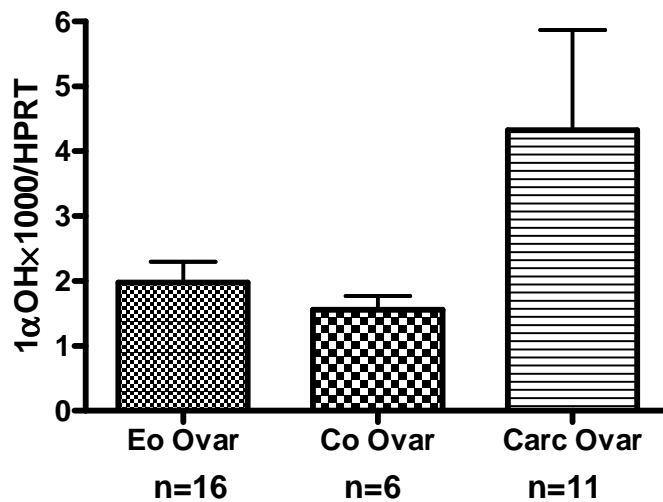

Figure 4b. Determination of 1α-hydroxylase (1α-OH) mRNA expression by quantitative RT-PCR in ovaries of patients with endometriosis (Eo Ovar), healthy controls (Co Ovar) and patients with ovarian cancer (Carc Ovar). See Table 1 for mean values ± SE.
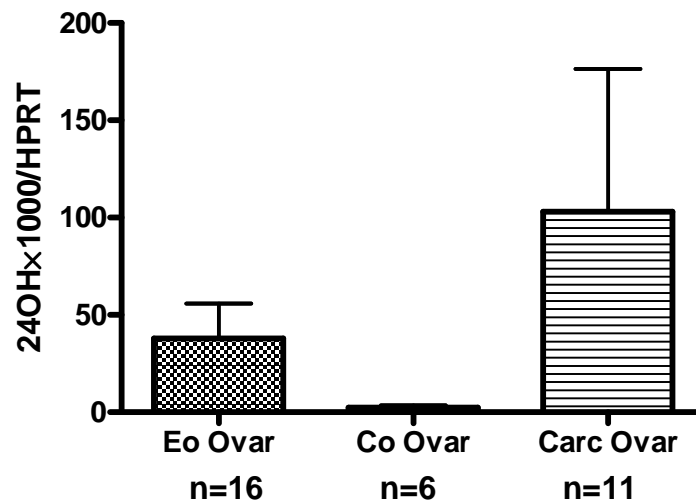
Figure 4c. *Determination of 24-hydroxylase (24-OH) mRNA expression by quantitative RT-PCR in ovaries of patients with endometriosis (Eo Ovar), healthy controls (Co Ovar) and patients with ovarian cancer (Carc Ovar). See Table 1 for mean values ± SE.*

On the contrary, expression of 25-hydroxylase was significantly higher in both endometriosis (4518.0 ± 779.7, p=0.001) and control group (7051 ± 1505, p<0.001) compared to ovarian cancer (951.8 ± 227.4) (Figure 4d).
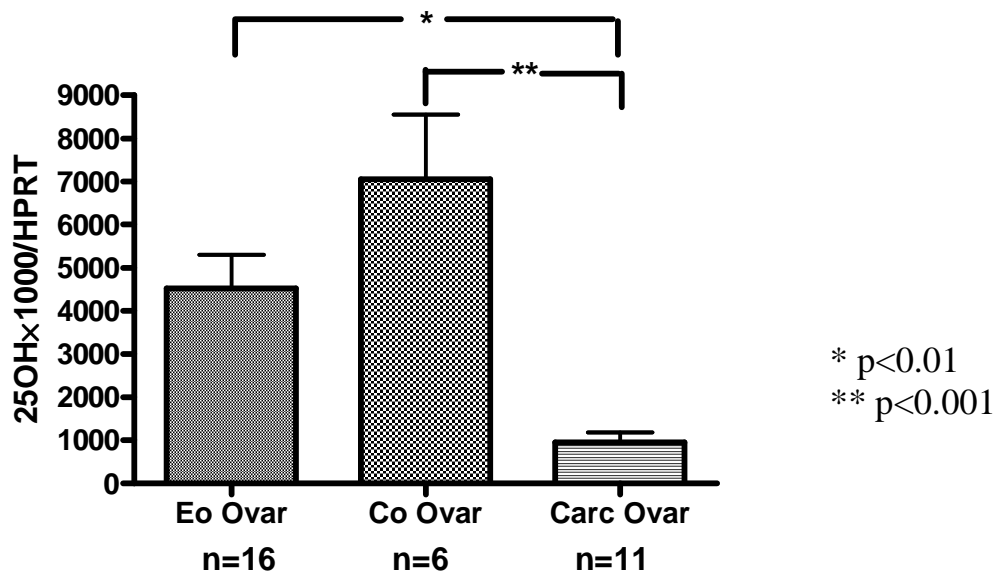

* p<0.01
** p<0.001

Figure 4d. *Determination of 25-hydroxylase (25-OH) mRNA expression by quantitative RT-PCR in ovaries of patients with endometriosis (Eo Ovar), healthy controls (Co Ovar) and patients with ovarian cancer (Carc Ovar). See Table 1 for mean values ± SE.*

3.4   VDR expression in primary endometrial cell cultures

VDR mRNA in endometrium of patients with endometriosis was significantly higher in isolated epithelial cells (71.9 ± 23.0) compared to stromal cells (31.3 ± 9.8, $p<0.01$) (figure 5a). However this trend did not reach statistical significance in the endometrium of healthy controls (figure 5b). VDR mRNA levels in epithelial cells of patients with endometriosis (71.9 ± 23.0) were greater than in healthy controls (20.8 ± 7.9, $p<0.01$) (figure 5c). Similar results were obtained comparing VDR expression in stroma of endometriosis patients (31.3 ± 9.8) and healthy controls (7.4 ± 2.6, $p<0.01$) (figure 5d).

Figure 5a. *Determination of VDR mRNA expression from primary endometrial cell culture by quantitative RT-PCR in epithelium (epi) and stroma (str) of patients with endometriosis.*
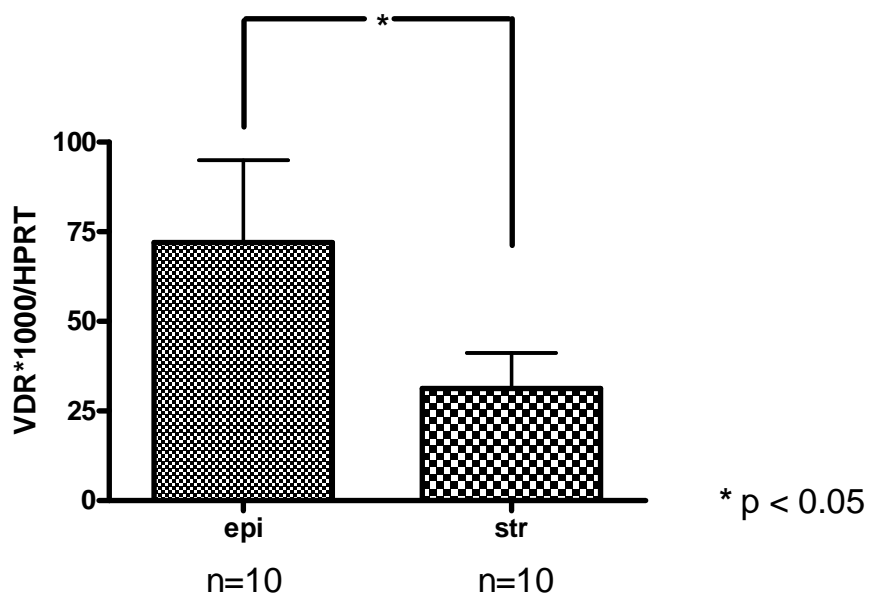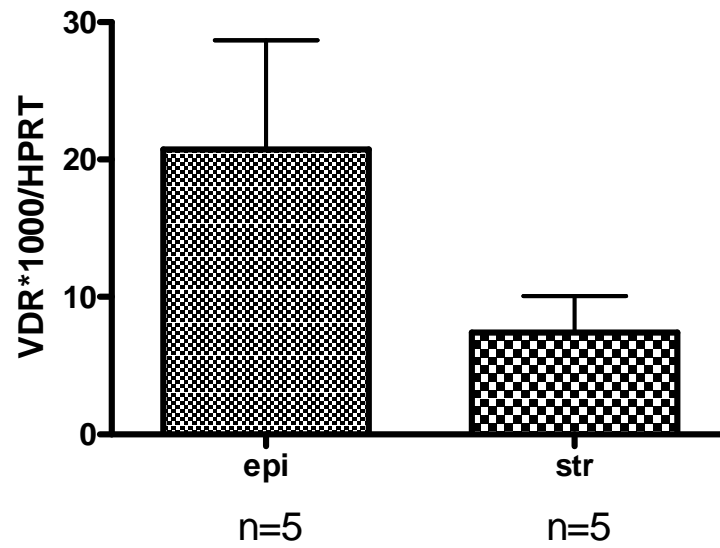
Figure 5b. Determination of VDR mRNA expression from primary endometrial cell culture by quantitative RT-PCR in epithelium (epi) and stroma (str) of healthy controls.
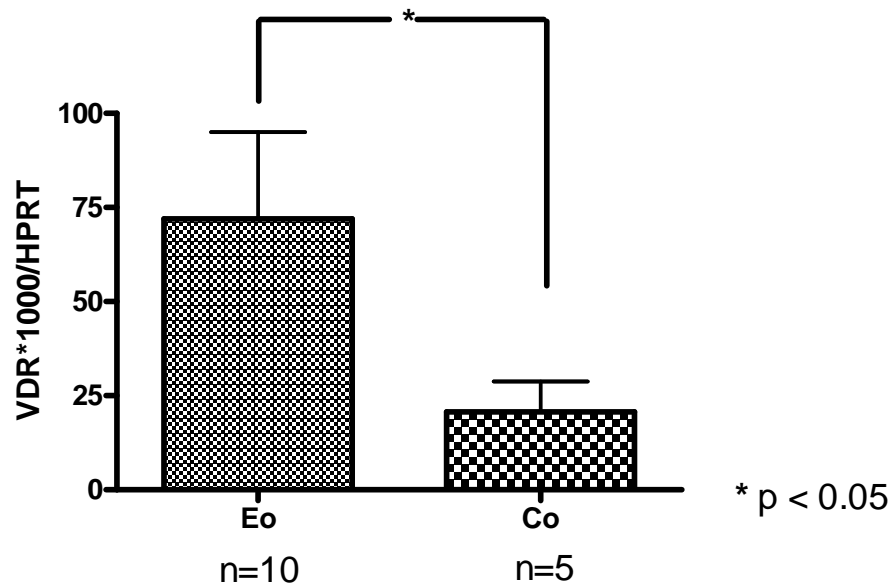

Figure 5c. Determination of VDR mRNA expression from primary endometrial cell culture by quantitative RT-PCR in epithelium of patients with endometriosis (Eo) and healthy controls (Co).
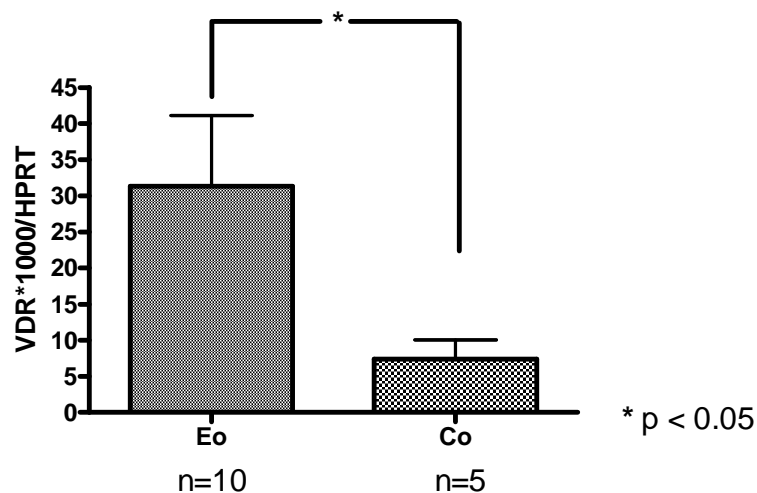
Figure 5d. Determination of VDR mRNA expression from primary endometrial cell culture by quantitative RT-PCR in stroma of patients with endometriosis (Eo) and healthy controls (Co).

3.5 Western blot for VDR, 1α-hydroxylase, 24-hydroxylase and 25-hydroxylase

Western blot analysis showed that the observed differences in VDR (Figure 6) and 1α-hydroxylase mRNA levels were maintained at the protein level. By contrast, Western blots for 24-hydroxylase and 25-hydroxylase proteins failed to corroborate significant differences in the presence of these two enzymes among the observed groups.
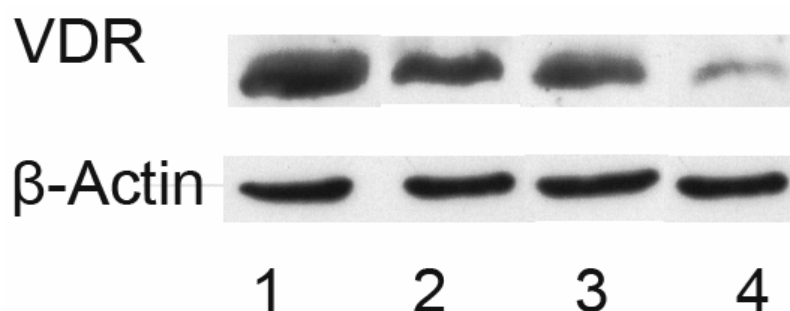

Figure 6. Expression of VDR in ovarian cancer tissue (lane 1), endometriotic tissue (lane 2), endometrium of women with endometriosis (lane 3) and endometrium of women without endometriosis (lane 4) evaluated by Western blot. Detection of β-actin was used to control for equal protein loading.

3.6   25(OH) vitamin D in peripheral blood

We found no difference in 25-OH vitamin D levels in serum between patients with endometriosis (n=46, 25.7 ± 2.1 ng/ml, p=0.3073) and healthy controls (n=33, 22.6 ± 2.0 ng/ml) (Figure 7).
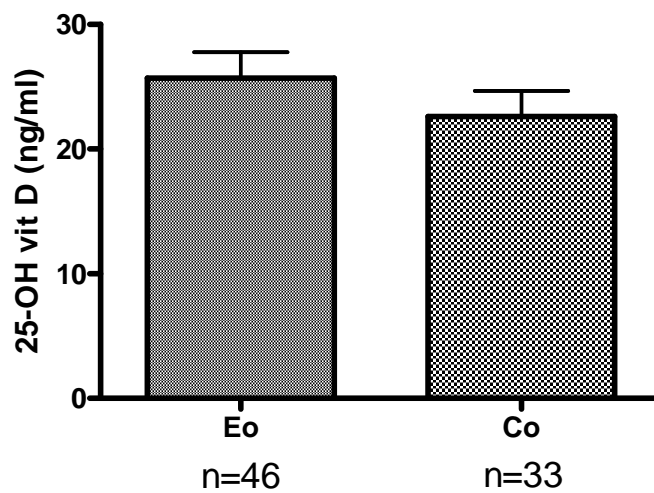

Figure 7. Serum levels of 25-OH vitamin D in patients with endometriosis (Eo) and healthy controls (Co).

4 DISCUSSION

Our study showed that there was significantly higher expression of VDR in patients with endometrial cancer compared to healthy controls, which supports the findings of Yabushita et al. (Yabushita *et al.,* 1996). While not a statistically significant difference, a tendency of elevated VDR expression in the endometrium of patients with endometriosis was noted compared to the control group, Additionally, in endometrium of patients with endometriosis, we found significantly higher VDR expression in epithelium than in stroma, which was not the case with the control group. We also found elevated expression of 1α-hydroxylase in endometrium of patients with endometriosis. 24-hydroxylase was elevated in endometrium of the endometrial cancer group and, although not statistically significant, had a tendency of higher expression in endometrium of patients with endometriosis. High expression of vitamin D key enzymes in endometrium of patients with endometriosis and patients with endometrial cancer highlights the potential for local autocrine and/or paracrine responses rather than traditional endocrine effects of vitamin D. The elevation of 1α-hydroxylase expression in patients with endometriosis suggests the increase of local production of the active form of vitamin D, 1, $25(OH)_2$ D3, which might mitigate against endometriotic cell growth. On the other hand, the elevation of 24-hydroxylase in endometriosis and endometrial cancer suggests a compensatory local deactivation of 1, $25(OH)_2$ D3. These findings suggest very intensive metabolism of vitamin D in endometrium of patients with endometriosis and endometrial cancer: both local production and deactivation of the active form of vitamin D are increased. Cross et al. reported similar relations in colon cancer (Cross *et al.,* 2005).

In the present study, VDR mRNA was found to be up-regulated in ovarian tissue of patients with endometriosis compared to normal ovarian tissue. Nevertheless, VDR mRNA up-regulation is increased further in ovarian cancer tissue. Similar findings were reported from several authors (Villena-Heinsen *et al.,* 2002, Saunders *et al.,* 1992, Friedrich *et al.,* 2003, Ahonen *et al.,* 2000, Anderson *et al.,* 2006). We found no significant differences in 1α-hydroxylase and 24-hydroxylase expression in ovaries among the observed groups. Interestingly, 25-hydroxylase was even expressed higher in ovaries of both patients with endometriosis and healthy controls, than in ovaries of patients with ovarian cancer. By contrast, Friedrich et al. reported elevated levels of 1α-hydroxylase, 24-hydroxylase and 25-hydroxylase in ovaries of patients with ovarian cancer (Friedrich *et al.,* 2003). Some of the suggested mechanisms of vitamin D inhibition of ovarian

cancer cell growth are the down-regulation of the telomerase activity and resulting ovarian cancer cell apoptosis (Feng *et al.,* 2004), p27 (tumor suppressor) stabilization and G1 arrest (Pengfei *et al.,* 2004).

It has long been recognized that people who live at higher latitudes face an increased risk of many chronic diseases such as. Crohns disease (Armitage et al., 2004), multiple sclerosis (Hernan *et al.,* 1999) and hypertension (Rostand 1979). Similar findings were reported for common solid cancers (colon, prostate, breast) (Garland *et al.,* 1989, Garland *et al.,* 1990, Ahonen *et al.,* 2000). It was suggested that this increased risk of chronic disease could be attributed to the lack of UVB from exposure to sunlight and subsequently reduced vitamin D production. Although low levels of 25-OH vitamin D are reported in some chronic diseases and cancer (Porojnicu *et al.,* 2007), we found no significant differences in the level of serum 25-OH vitamin D between patients with endometriosis and healthy controls.

To our knowledge, the present study is the first demonstration of the expression of VDR and enzymes involved in vitamin D metabolism in endometriosis. Currently available data are sparse, with a recently published study by Ferrero *et al.* reporting decreased levels of vitamin D binding protein (DBP) in the peritoneal fluid, but not in the plasma, of women with untreated endometriosis. This finding suggests that vitamin D metabolism may be relevant in the pathogenesis of this disease (Ferrero *et al.*, 2005).

Among other vitamins with possible effects on endometriosis, only vitamin E and vitamin C have been reported (Jackson *et al.,* 2005, Agarwal *et al.,* 2005). Results suggesting that oxidative stress might play a role in the development and progression of endometriosis (Jackson *et al.,* 2005) indicate that supplementation of these vitamins, with well known antioxidant action, might have potential therapeutic value. Antioxidant supplementation has been suggested as an intervention strategy to prevent preeclampsia and other female reproduction related diseases (Agarwal *et al.,* 2005).

Vitamin D has antiproliferative and antineoplastic activities which include activation of apoptosis, induction of cell cycle arrest and differentiation, inhibition of invasion and motility, and reduction of angiogenesis. These activities are exerted through both genomic and non-genomic pathways. The classic genomic response is believed to be the most responsible for the vitamin D action and it is mediated through VDR. VDR is stably expressed in normal endometrium tissue during the menstrual cycle (Vienonen *et al.,* 2004), as well as in ovarian tissue (Villena-Heinsen *et al.,* 2002) and in placenta-decidua (Evans *et al.,* 2004, Vigano *et al.,*

2006). VDR is also highly expressed in a large number of tumor tissues including ovarian, endometrial, cervix, breast and colorectal cancer (Yabushita *et al.,* 1996, Cross *et al.,* 2005, Villena-Heinsen *et al.,* 2002, Saunders *et al.,* 1992, Friedrich *et al.,* 2003, Feng *et al.,* 2004, Pengfei *et al,* 2004). As reported here, VDR is expressed in endometriosis, confirmed both by immunohistochemistry and real time PCR. Our data indicate that VDR expression in endometriosis patients, both in endometrium and ovaries, is intermediate, with levels between VDR expression of patients with cancer and healthy controls.

Several pathophysiological and clinical parallels between endometriosis and cancer have been observed, and as we have demonstrated, these extend to similarities in VDR and vitamin D related enzymes. At present, it is very difficult to know whether the increased expression of VDR and some of these enzymes is a primary event or a consequence for endometriosis. It may be more important to address whether VDR and vitamin D enzymes are constitutively expressed in endometriosis or rather a secondary response to local inflammation. Given the emerging evidence of antiproliferative and anti-inflammatory actions of vitamin D (Taverna *et al.,* 2005), we hypothesize that this vitamin might influence the activity of local immune cells and their cytokine production, which previously have been postulated as important factors for the development and the maintenance of endometriosis (Agic *et al.,* 2006). Possible benefits from the local application of exogenously supplemented vitamin D are yet to be revealed, but should be studied further to determine its potential treatment benefit in endometriosis.

5 SUMMARY

Several mechanisms of vitamin D antineoplastic activity have been suggested including induction of apoptosis, inhibition of proliferation through cell cycle arrest, induction of differentiation, inhibition of invasion and motility, and reduction of angiogenesis. Numerous in vitro and in vivo studies have shown that vitamin D potently inhibits cell proliferation in a wide range of cell types, including carcinomas of the breast, prostate, colon, skin, brain, myeloid leukemia cells, and others. Endometriosis has several pathophysiological similarities to malignant diseases such as reduced apoptosis, invasion of endometrial tissue in other organs (bowel, bladder) and increased angiogenesis. Concerning these similarities to malignant diseases, endometriosis came into our focus as a disease with possible connection to vitamin D action.

VDR distribution in endometrium and ovaries was shown by immunohistochemistry. VDR, 1α-hydroxylase, 24-hydroxylase and 25-hydroxylase mRNA in endometrium and ovaries of patients with endometriosis, control group and patients with cancer was measured by realtime PCR and Western blot. RIA was used for measuring 25(OH) vitamin D levels in peripheral blood.

Immunohistochemistry showed strong staining of VDR in endometriosis and endometrial cancer. The most intense VDR-positive staining in endometriosis and cancer is shown in epithelial cells and less in stromal cells. VDR mRNA is significantly higher expressed in patients with endometrial cancer, and higher expressed in endometriosis, compared to control group. The data showed significantly higher 1α-hydroxylase expression in patients with endometriosis compared to healthy controls. VDR mRNA in ovaries of patients with endometriosis and ovarian cancer is higher expressed than in healthy controls. On the contrary, expression of 25-hydroxylase in ovaries is significantly higher in both endometriosis and control group compared to ovarian cancer. There was no statistical difference in 25(OH) vitamin D levels in peripheral blood between endometriosis patients and healthy controls.

Realtime PCR have shown that VDR and some of the vitamin D enzymes are highly expressed in endometriosis, although not in the amount as in cancer. Western blot analysis showed that the observed differences in VDR and 1α-hydroxylase mRNA levels were maintained at the protein level. High distribution of VDR in endometriosis, especially in epithelial cells, was confirmed by immunohistochemistry. The intensity of staining is significantly higher in endometrium and endometriotic tissue of patients with endometriosis than in normal endometrium. Given its familiar antiproliferative and anti-inflammatory actions, we hypothesize that vitamin D might influence the local activity of immune cells and cytokines thought to play important pathogenic roles in the development and maintenance of endometriosis.

6   ZUSAMMENFASSUNG

Mehrere Mechanismen antineoplastischer Aktivität von Vitamin D sind vorgeschlagen worden, einschließlich Induktion der Apoptose, Proliferationshemmung durch Stillstand des Zellzyklus, Induktion der Differenzierung, Invasions- und Motilitätshemmung und Reduktion der Angiogenese. Zahlreiche *in vitro*- und *in vivo*-Studien haben gezeigt, dass Vitamin D die Proliferation in einer Reihe von Zelltypen stark hemmt, einschließlich Karzinomen der Brust, Prostata, Dickdarm, Haut, Hirn, myeloische Leukämiezellen und andere. Die Endometriose weist

mehrere pathophysiologische Ähnlichkeiten mit malignen Erkrankungen auf, wie zum Beispiel reduzierte Apoptose, Invasion von Endometriumgewebe in andere Organe (Darm, Blase) und verstärkte Angiogenese. Diese Ähnlichkeiten rückten die Endometriose in unseren Blickpunkt als eine Erkrankung, welche möglicherweise durch Vitamin D beeinflusst werden kann.

Die Verteilung des Vitamin D-Rezeptors im Endometrium und den Ovarien wurde immunhistochemisch gezeigt. Der Vitamin D-Rezeptor, die 1α-Hydroxylase, 24-Hydroxylase und 25-Hydroxylase in Endometrium bzw. Ovarien von Patientinnen mit Endometriose, mit Karzinomen sowie Kontrollpatientinnen wurden auf mRNA-Ebene mittels RT-PCR und auf Protein-Ebene mittels Westernblot analysiert. Der Gehalt an 25(OH) D_3 im peripheren Blut wurde mittels RIA bestimmt. In endometriotischem Gewebe sowie in Endometriumkarzinom-Gewebe zeigte der Vitamin D-Rezeptor eine starke immunhistochemische Färbung, wobei die Intensität in Epithelzellen stärker war als in Stromazellen. Auf mRNA-Ebene zeigten Patientinnen mit Endometriumkarzinom bzw. Endometriose eine deutlich höhere VDR-Expression im Vergleich mit der Kontrollgruppe. Die Expression von 1α-Hydroxylase war bei Endometriosepatientinnen deutlich höher als bei der Kontrollgruppe. In den Ovarien von Patientinnen mit Endometriose bzw. Ovarialkarzinom wurde die VDR-mRNA stärker exprimiert als in der Kontrollgruppe. Im Gegensatz dazu war die Expressionsrate der 25-Hydroxylase in den Ovarien von Endometriose- bzw. Kontrollpatientinnen höher als bei Patientinnen mit Ovarialkarzinom. Es gab keinen statistisch signifikanten Unterschied zwischen dem 25(OH)D3-Gehalt des peripheren Blutes von Endometriosepatientinnen und der Kontrollgruppe.

Die Realtime-PCR zeigte, dass VDR und einige der Vitamin D-Enzyme in der Endometriosegruppe höher exprimiert wurden als in der Kontrollgruppe, jedoch nicht in der Ausprägung wie bei Karzinomen. Die Westernblot-Analyse zeigte, dass die beobachteten Unterschiede auf mRNA-Ebene von VDR und 1α-Hydroxylase auch auf Proteinebene weiter bestanden. Eine hohe Konzentration von VDR in endometriotischem Gewebe, speziell in Epithelzellen, wurde durch Immunhistochemie bestätigt. Die Färbeintensität ist signifikant höher in eutopischem und ektopischem Gewebe von Patientinnen mit Endometriose als in normalem Endometrium. Im Hinblick auf seine antiproliferative und antiinflammatorische Wirkung stellen wir die These auf, dass Vitamin D die lokale Aktivität von Immunzellen und Zytokinen beeinflussen könnte, welche eine wichtige pathogenetische Rolle in der Entwicklung und dem Verlauf der Endometriose spielt.

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7   CURRICULUM VITAE

Name	**Agic**
First name	**Admir**
Gender	Male
Date of Birth	24.07.1977
Place of Birth	Priboj, Serbia
09.1984-06.1992	Primary school in Priboj/ Serbia
01.09.1992	Gymnasium in Priboj/ Serbia
25.06.1994	Language University „Kolarac", Belgrade: Englisch ("Conversation Level IV") graduation mark: excellent
03.06.1996	„Abitur" - mark: excellent
01.09.1996	Matrikulation on the Medical University in Belgrade, University in Belgrade/ Serbia
30.01.2003	Graduate and Master degree on the Medical University in Belgrade, everage mark: 9.89
01.02.2003	„Royal House of Karadjordjevic"- scholarship
03.2003-03.2004	Residency at the University Clinic „Bezanijska Kosa", Belgrade
29.03.2004	State-exam, Ministry of Health in Belgrade- medical practitionar
06.2004-06.2005	Scientific Assistent at the Medical University in Belgrade
06.2005-06.2006	Visiting scientist and Medical Doctor candidate in the Clinic for Gynecology and Obstetrics, University of Schleswig-Holstein, Campus Lübeck
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