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RESEARCH ARTICLE

Hyperandrogenemia impairs endometrial vitamin D receptor expression in polycystic ovary syndrome

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ABSTRACT

Objectives: To determine the effects of hyperandrogenemia and other phenotypic parameters on endometrial vitamin D receptor (VDR-X2 and VDR-X4) expression in women with polycystic ovary syndrome (PCOS) undergoing ovarian stimulation and total embryo freezing.

Methods: Forty-four PCOS patients were divided into four phenotypes according to the criteria for hyperandrogenemia (HA), ovulatory dysfunction (OD), and polycystic ovary morphology (PCOM): phenotype A (HA+OD+PCOM), phenotype B (HA+OD), phenotype C (HA+PCOM), and phenotype D (OD+PCOM). Endometrial VDR expression was determined by real-time PCR and immunohistochemistry. Twenty age- and body mass index (BMI)-matched couples with male infertility were included as controls.

Results: VDR-X2 and VDR-X4 expression levels were significantly lower in the PCOS group than in the control group. A significant downregulation was detected in the relative VDR-X2 and X4 expression in phenotypes A, B, and C compared to the control group. VDR-X2 and X4 expression in phenotype D was significantly higher than in phenotypes A and B. A significant negative correlation was detected among VDR-X2, VDR-X4, serum testosterone (T), androstenedione (A), DHEAS, and insulin resistance (IR). Multivariate analysis revealed that serum T, A, DHEAS, and IR levels were independently associated with both VDR-X2 and VDR X4 relative gene expression after adjusting for age and BMI. The VDR mRNA and immunoreactivity of each phenotype overlapped. The clinical pregnancy rates for each phenotype were similar.

Conclusion: VDR expression in the endometria of patients with PCOS was defective. Hyperandrogenemia and insulin resistance are the key drivers of defective VDR expression in the endometrium of patients with PCOS.

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Endometrium; phenotype; polycystic ovary syndrome; VDR

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine cause of infertility in women of reproductive ages. Although infertility in PCOS is mostly due to ovulatory dysfunction, the higher incidence of miscarriage, preeclampsia, and recurrent implantation failure in ovulatory PCOS patients suggests that the PCOS endometrium is dysfunctional [1]. Similar to human PCOS studies, different receptivity pathologies of the endometrial compartment have been reported in experimental PCOS models [2–4]. Under physiological conditions, preparation of the endometrium for implantation is provided by different steroid hormones, nuclear receptors, and coactivators [5]. Since endometrial expression of some steroid hormone receptors has long been shown to be defective in PCOS, expression of the steroid-structured vitamin D receptor (VDR) may also be defective in the endometrium [6]. Although most PCOS studies have reported low serum vitamin D (VD) levels, no studies have investigated how low VD affects endometrial VDR expression [7]. Because VDR regulates the modulation of many genes related to cell growth and immunological and inflammatory reactions in the endometrium, it may be necessary for successful implantation [8].

VD exerts its biological effects through the VDR, a member of the steroid/thyroid nuclear hormone receptor family [7,8]. The VD-VDR complex stimulates the VD response elements in DNA through the retinoid X receptor. This genomic activation mediates anti-proliferative, anti-inflammatory, pro-differentiative, and immunomodulatory effects at the feto-placental interface [7,8]. Many studies have shown that VD improves reproductive problems caused by PCOS and improves IVF outcomes [9,10]. In addition to the improvement in endometrial thickness in PCOS patients given VD replacement, a decrease in decidual VDR expression in recurrent miscarriages and preeclampsia is further evidence supporting the role of VDR in endometrial receptivity

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[11,12]. VDR is widely expressed not only in tissues responsible for calcium hemostasis but also in the endometrium [8]. Endometrial VDR expression, which is low in the proliferative phase, begins to increase in the early secretory phase and decreases again in the mid-secretory phase [9]. The endometrial VD-VDR complex may contribute to maternal tolerance of the embryo by shifting Th1/Th2 in favor of Th2, in addition to the activation of endometrial natural killer cells and dendritic cells [7,11]. In addition to VDR expression, the ability of the endometrium to produce enzymes responsible for VD production and catabolism supports the role of VDR in implantation [4]. Uterine hypoplasia, abnormal endometrial histology, defects in steroid hormone synthesis, and reduced aromatase activity observed in mice with targeted ablation of the VDR confirm the critical role of the VDR in implantation [7,11,12]. Other evidence for the role of VDR in implantation is that VD supplementation improves the expression of the receptivity gene HOXA10, uterine weight, and decidualization [9,12].

Although PCOS is a syndrome accompanied by common metabolic problems, hyperandrogenemia (HA) is mostly responsible for dysfunctional endometrium [1,7]. However, other phenotypic and metabolic parameters, such as ovulatory dysfunction (OD), polycystic ovarian morphology (PCOM), obesity, and insulin resistance can also lead to endometrial dysfunction [1,7,8]. Although many studies have reported that VD levels are low in PCOS patients and that VD replacement improves fertility outcomes [7,9,11,12], there are no studies on the VDR expression pattern in PCOS patients. The primary objective of this study was to determine the endometrial VDR expression in infertile women with PCOS who underwent ovarian stimulation and assisted conception. By classifying PCOS patients according to their phenotypes, this study aimed to analyze the effect of hyperandrogenemia and other phenotypic parameters on endometrial dysfunction and VDR expression. Specifically, this study sought to compare VDR expression patterns among phenotypes A (HA+OD+PCOM), B (HA+OD), and C (HA+PCOM), which include the hyperandrogenic component, against phenotype D (OD+PCOM), the non-hyperandrogenic form. The secondary objective of this study was to assess the influence of hyperandrogenemia and other phenotypic features on endometrial VDR expression.

Materials and methods

This cross-sectional study was conducted on women with PCOS who were treated at the IVF unit of Göztepe Medical Park Hospital and underwent controlled ovarian stimulation and total embryo freezing. After receiving ethical approval (ethics approval number: 2022/12-23, date: 20.10.2022), the study was registered at ClinicalTrials.gov (NCT05885633). Informed consent was obtained from participants who agreed to undergo total embryo freezing and endometrial sampling. In the G*Power version 3.1.9.7 (Heinrich-Heine-Uni-versität Düsseldorf, Germany) analysis, which was performed by taking into account the study of Eftekhar et al. [13], the minimum number of participants required to complete the study at a 95% confidence level ($\alpha = 0.01$) and 95% power was determined as 56 (effect size = 1.24). The study group consisted of 44 PCOS patients who did not conceive with first-line letrozole or clomiphene citrate treatment, or were referred for IVF/ICSI because of a previous failed attempt. Twenty women scheduled for IVF/ICSI due to male factor infertility, whose age and BMI were compatible with the PCOS group, were recruited as the control group (Figure 1). Women who met at least two of the criteria for hyperandrogenemia, ovulatory dysfunction, and polycystic ovarian morphology (PCOM) determined by the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) were diagnosed with PCOS [14]. Hyperandrogenism was defined either clinically based on high hirsutism scores or biochemically based on increased testosterone levels. Ovulatory dysfunction was defined as oligomenorrhoea or amenorrhea. Polycystic ovarian morphology was defined as 12 or more follicles of 2–9 mm or a volume of at least one ovary greater than 10 ml. Participants in the control group were selected from patients diagnosed with azoospermia or severe oligoasthenoteratospermia, according to the World Health Organization 2010 normative reference values. The 44 participants in the PCOS group were divided into four phenotypic groups according to the NIH consensus panel. Phenotype A: HA+OD+PCOM (classical/complete, $n = 17$); phenotype B: HA+OD (classical/non-PCOM, $n = 10$); phenotype C: HA+PCOM (ovulatory, $n = 9$); phenotype D: OD+PCOM (non-hyperandrogenic, $n = 8$).

The age, body mass index (BMI), and menstrual history of all participants were recorded. Endometrial thickness was measured using transvaginal ultrasonography on the day of the ovulation trigger. The uterus was placed in the mid-sagittal plane to ensure that the bladder was empty. The maximum echogenic length between the endometrium and myometrium was measured 1 cm from the fundal tip. Baseline hormone values and biochemical parameters were measured in the 2-5 days of progesterone withdrawal bleeding in participants with phenotypes A, B, and D, and in spontaneous menstrual bleeding in phenotype C. After at least 8-10 h of overnight fasting, blood samples were collected and stored at −20 °C. Serum 25-hydroxyvitamin D, luteinizing hormone (LH), follicular stimulating hormone (FSH), estradiol, androstenedione (A), dehydroepiandrosterone sulfate (DHEAS), insulin, and total testosterone (T) levels were analyzed using an electrochemiluminescence immunoassay method with a Cobas e601 device (Roche Diagnostics GmbH, Mannheim, Germany). Fasting blood glucose was measured using the hexokinase method with an AU 5800 device (Beckman Coulter Inc., Brea,

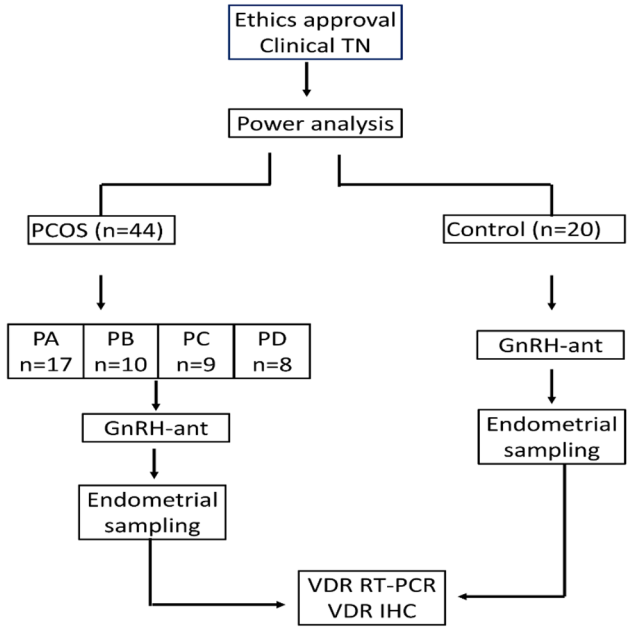

Figure 1. Flowchart of patient and control subjects selection and methodological procedures.

CA, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula: insulin (μU/mL) × glucose (mg/dL)/405). Women with congenital adrenal hyperplasia, hyperprolactinemia, Cushing's syndrome, androgen-secreting tumors, thyroid disorders, or idiopathic hirsutism were excluded from the study. Patients taking vitamin D, insulin sensitizers, lipid-lowering drugs, or other hormonal drugs in the last 3 months were excluded. Patients with a history of mechanical endometrial injury, endometrial polypectomy, hysteroscopic myomectomy, endometrioma resection, dilatation/curettage, ovarian drilling, or salpingectomy were also excluded. Participants with premalignant or malignant endometrial pathology, a history of tubal factor infertility, congenital uterine anomaly, or any systemic diseases were excluded.

Ovarian stimulation and endometrial sampling

A flexible GnRH antagonist protocol was used for ovarian stimulation. Briefly, rFSH (Gonal F, Merck, Switzerland) was started on the 2nd or 3rd day of the spontaneous or progesterone-induced cycle. The initial dose of rFSH was determined according to patient age, BMI, and AFC. For pituitary suppression, GnRH antagonist cetrotide (Cetrorelix, Merck, Switzerland) was started when the dominant follicle was ≥ 14 mm. Ovulation was triggered by a GnRH agonist when two or more follicles were ≥ 18 mm in diameter. Oocytes were collected 36 h after the ovulation trigger. Immediately after oocyte retrieval, endometrial tissue was collected using a pipelle cannula while the patient was still under anesthesia. A portion of the endometrium was placed in RNA stabilization buffer (RNA-later) for qRT-PCR and stored at −20 °C. The remaining endometrial tissue was fixed with 10% formalin, followed by paraffin blocking and preparation for immunohistochemical analysis. All metaphase II oocytes were subjected to ICSI and eligible good-quality embryos were vitrified. Frozen-thawed single good-quality blastocyst transfer was performed on artificially prepared endometrium. All the patients received luteal-phase support with intramuscular progesterone. Clinical pregnancy was defined as the presence of an intrauterine gestational sac on transvaginal ultrasonography (TVS).

Quantitative RT-PCR analysis of VDR-X2 and VDR-X4

The mRNA expression of VDR transcript 2 (VDR-X2) and VDR transcript 4 (VDR-X4) was analyzed using qRT-PCR. Three technical replicates were performed for each sample. mRNA variants occur by altering the structure of pre-mRNAs before translation *via* alternative splicing. Thus, by producing different mRNAs from a single gene, protein isoforms with different functions can be produced. As human VDR has multiple transcript variants, the receptor structure and cellular response to VD may vary [15]. Therefore, we analyzed two different transcript variants of the VDR mRNA (VDR-X2 and VDR-X4). Endometrial samples in RNAlater were homogenized using a Tissuelyser. Trizol (Dongsheng Biotech, China) was used to isolate total RNA from endometrial samples stored at −80 °C. A OneScript Plus cDNA synthesis kit (G236, Abcam, Canada) was used for complementary DNA synthesis. Sequences of all primers designed to be used as forward and reverse primers for RT-PCR were; Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH): forward 5′-GGAGCGAGATCCCTCCAAAAT-3′, reverse 5′-GGCTGTTGTCATACTTCTCATGG-3′; VDR-X2: forward 5′-ACATTGCTTTGCTTGCCTCC-3′, reverse 5′-ACGTTCCGGT CAAAGTCTCC-3′; VDR-X4: forward 5′-CTTGGCATGGAGT GGAGGAA-3′, reverse 5′-GAGGATTGAGGGAGGCAAGC-3′. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the endogenous housekeeping gene. GAPDH mRNA was used as a reference to normalize the mRNA expression of the target genes. PCR analysis was performed on an Applied Biosystems 7500 Real-Time PCR instrument using BrightGreen 2X qPCR Master Mix (Master Mix-S, abm, Canada).

Immunohistochemical analysis

Endometrial samples cut from paraffin blocks were passed through an alcohol series and citrate-buffered solution for antigen retrieval. The slides were then washed with phosphate-buffered saline (PBS; P4417, Sigma Aldrich, USA) for 3 × 5 min and incubated with hydrogen peroxide (Hydrogen Peroxide Block, TA-125-HP, LabVision Corporation, USA). Ultra V Block (TA–125-UB, LabVision Corporation, USA) solution was applied to prevent background staining. Polyclonal anti-VDR (SRB, 201r-1703; Sunred Biotechnology, Shanghai, China) was added to the sections. The sections were then incubated with a secondary antibody (biotinylated Goat Anti-Polyvalent (anti-mouse/rabbit IgG), TP-125-BN, LabVision Corporation, USA) and incubated with streptavidin-peroxidase (TS–125-HR, LabVision Corporation, USA). Subsequently, 3-amino-9- ethylcarbazole (AEC) substrate and AEC chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, LabVision Corporation, USA) were added to the sections. When the image signal was obtained counterstaining was performed with Mayer's hematoxylin (Large Volume VisionMount, TA-125-UG, LabVision Corporation, USA) and evaluated under a Leica DM500 microscope (Leica DFC295). Immunoreactivity was quantitatively evaluated using the histoscore formula (Histoscore = extent × density). For the extension (0.1: <25%, 0.4:26-50%, 0.6:51–75%, 0.9:76–100%) values were taken as the basis, while for the density (0: none, +0.5: little, + 1: low, +2: moderate, +3: severe) values were taken.

Statistical analysis

IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Continuous variables were examined for normal distribution using the Shapiro-Wilk test. Normally distributed continuous variables are shown as mean ± standard deviation; non-normally distributed parameters are shown as median (25^{th}-75^{th} percentiles); and categorical variables are shown as frequency (percentage). If the comparison between two independent groups was normally distributed, an independent samples t-test was used; if not normally distributed, the Mann-Whitney U test was used. More than two groups with a normal distribution were compared using a One-Way ANOVA test, and parameters not showing a normal distribution were compared using the Kruskal-Wallis test. The Bonferroni test was used for post-hoc paired comparison analyses. In 2 × 2 comparisons between categorical variables, if the expected values in all cells were > 25, Pearson's Chi-square; If one of the cells was less than 25 and the minimum expected value was between 5–25, Pearson's chi-square with continuity correction was used, and if the number of cells with an expected value less than 5 was more than 20% of the total number of cells, Fisher's exact test was used. For comparisons between categorical variables greater than 2 × 2, Pearson's chi-square test was used when the proportion of cells with an expected value less than 5 was less than 20%, and Fisher-Freeman-Halton test was

used when it was greater than 20%. The relationships between VDR, phenotypic determinants, and other metabolic parameters were analyzed using Spearman's non-parametric correlation test. After controlling for age and BMI, the relationship between VDR-X2 and VDR-X4 and laboratory parameters was investigated using binary logistic regression analysis. Two-tailed *p*-values of less than 0.05 were considered statistically significant. The mean cycle threshold (Ct) and ΔCt values of each sample were analyzed using DataAssist Software v3.01 (Applied Biosystems). The VDR mRNA expression of each phenotype was calculated using the ΔΔCt method after normalization to the target genes of the group. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative mRNA expression levels, which are presented as the mean ± SEM. Statistical differences in VDR mRNA expression were analyzed using One-way ANOVA and Bonferroni's multiple comparison test (GraphPad Software Inc., San Diego, CA, USA).

Table 1. Comparison of demographic and baseline hormone values between the PCOS and control groups.

Variables	PCOS ($n=44$)	Control ($n=20$)	p
Age (years)	26.77 ± 2.45	27.75 ± 3.26	0.188[a]
BMI (kg/m^2)	23.97 ± 1.87	22.83 ± 1.72	0.309
Infertility duration (yrs)	4 (3–4)	4 (3–5)	0.436[b]
Antral follicle count	21 (20–22)	12 (11–13)	<0.001[b]
Endometrial thickness (mm)	9.5 (9–10.5)	9 (8.5–10)	0.427[b]
Total gonadotropin dose (IU/L)	1846.35 ± 403.05	2376.35 ± 281.23	<0.001[a]
E2 on the day of hCG (pg/mL)	3172.34 ± 278.42	2014.22 ± 405.92	<0.001[a]
No. of oocytes retrieved	15.05 ± 2.90	9.32 ± 1.44	<0.001[a]
Vitamin D (ng/mL)	14.2 (10.6–17.9)	20.4 (19–23)	<0.001[b]
Testosterone (ng/dL)	34.24 ± 7.55	20.7 ± 4.59	<0.001[a]
Androstenedione (ng/mL)	3.6 (2.9–4.55)	2.3 (2.2–2.37)	<0.001[b]
DHEAS (μg/dL)	262 (243–294)	182.5 (166.5–205)	<0.001[b]
Estradiol (pg/mL)	45.6 (39.2–56.6)	49.9 (43.7–52.8)	0.622[b]
LH (mIU/mL)	9.46 ± 2.18	6.60 ± 1.01	<0.001[a]
FSH (mIU/mL)	5.9 (5.5–6.7)	5.7 (4.7–6.4)	0.100[b]
LH/FSH	1.61 ± 0.48	1.21 ± 0.29	<0.001[a]
Insulin (μIU/mL)	10.0 ± 2.31	6.15 ± 1.18	<0.001[a]
HOMA-IR	2.32 ± 0.53	1.31 ± 0.39	<0.001[a]
Clinical pregnancy rate (n, %)	21 (47.7%)	9 (45%)	1.000[c]

[a]Independent sample t-test.
[b]Mann-Whitney U test.
[c]Chi-Square test.

Results

The demographic and basal hormone levels of each group are presented in Table 1. The mean age, BMI, and duration of infertility were similar between the groups. The serum VD levels in the PCOS group were significantly lower than those in the control group. The total number of oocytes collected and serum testosterone, androstenedione, DHEAS, LH, LH/FSH, fasting insulin, and HOMA-IR levels were significantly higher in the PCOS group than in the control group. Significantly fewer gonadotropins were used in the PCOS group than in the controls (1846.35 ± 403.05 IU vs 2376.35 ± 281.23 IU, $p<0.001$. The peak estradiol levels were higher on the day of hCG in PCOS group compared to controls (3172.34 ± 278.42 pg/ml vs 2014.22 ± 405.92 pg/ml, $p<0.001$). Endometrial thickness on the day of oocyte retrieval did not differ between groups ($p=0.427$).

When the four phenotypic groups were evaluated, all parameters were similar, except for the serum levels of testosterone, DHEAS, androstenedione, and HOMA-IR (Table 2). The serum T levels of phenotypes with HA components (A, B, and C) were significantly higher than those of phenotype D without HA ($p<0.01$). T levels of the classical phenotypes (A and B) were similar. While the T levels of phenotype A were higher than those of phenotype C ($p<0.01$), the T levels of phenotypes B and C were similar. Serum androstenedione and DHEAS levels were significantly lower in phenotype D than in the other phenotypes ($p<0.01$ each). Serum estradiol levels on the day of hCG administration were similar across phenotypes. HOMA-IR of the three phenotypes (A, B, and C) accompanying HA was significantly higher in phenotype D ($p<0.01$). Although the HOMA-IR of phenotype A was higher than that of phenotype C ($p<0.01$), there was no significant difference between the other phenotypes.

In the comparison made by taking the VDR mRNA expression of the control group as a reference, the VDR-X2 (1.00 ± 0.01 vs. 0.34 ± 0.03, $p<0.001$) and VDR-X4 mRNA expressions of the PCOS group (1.01 ± 0.01 vs. 0.43 ± 0.03, $p<0.001$) showed significant down-regulation compared to the control (Figure 2). Compared to VDR-X2mRNA expression of control group a significant down regulation was detected in endometrial VDR-X2 mRNA expressions of phenotypes A (1.00 ± 0.01 vs. 0.21 ± 0.01; $p<0.001$), B (1.00 ± 0.01 vs.

Table 2. Phenotypic distribution of demographic and laboratory findings.

Variables	Phenotype A ($n=17$)	Phenotype B ($n=10$)	Phenotype C ($n=9$)	Phenotype D ($n=8$)	p-values
Age (yrs)	26.35 ± 2.37	27.3 ± 2.98	27.67 ± 2.69	26.0 ± 1.31	0.409[d]
BMI (kg/m^2)	24.59 ± 1.71	24.03 ± 1.91	23.42 ± 1.66	23.18 ± 2.22	0.257[d]
Infertility duration (yrs)	4 (3–4)	4 (4–5)	4 (3–4)	4 (3–4.5)	0.546[e]
Endometrial thickness (mm)	10 (9–11)	9.5 (9–10)	9 (9–10)	9.5 (9–11)	0.821[e]
Vitamin D (ng/mL)	12.4 (11.2–15.1)	14.1 (9.8–18.8)	16.9 (9.9–18.0)	15.7 (14.6–17.4)	0.764[e]
Testosterone (ng/dL)	39.65 ± 4.26*, #	35.4 ± 5.85*	32.11 ± 5.35*	23.7 ± 5.15	<0.001[d]
Estradiol (pg/mL)	48.7 (42.7–55.7)	42.6 (35.5–46.3)	49.1 (40.2–53.3)	45.7 (38.04–60.1)	0.614[e]
Androstenedione (ng/mL)	3.7 (3.5–4.4)*	3.95 (3.3–5)*	3.6 (2.9–5)*	2.32 (2.14–2.41)	<0.001[e]
DHEAS (μg/dL)	262 (252–283)*	282 (256–302)*	278 (262–302)*	211.5 (199–220.5)	<0.001[e]
LH (mIU/mL)	10.42 ± 2.72	8.5 ± 1.73	8.91 ± 1.18	9.2 ± 1.76	0.122[d]
FSH (mIU/mL)	6 (5.6–6.6)	5.9 (5.3–6.3)	5.9 (5.5–6.9)	6.1 (4.8–6.7)	0.937[e]
HOMA-IR	2.71 ± 0.33*, #	2.48 ± 0.40*	2.10 ± 0.24*	1.54 ± 0.29	<0.001[d]
E2 on the day of HCG	3145.90 ± 292.1	3253.4 ± 321.9	3160.1 ± 248.4	3141.2 ± 254.8	0.785[d]
CPR (n,%)	8 (47.1%)	5 (50%)	4 (44.4%)	4 (50%)	1.000[f]

[d]One-way Anova test.
[e]Kruskall Wallis test.
[f]Fisher-Freeman-Halton test.
*$p<.01$ vs phenotype D group.
#$p<.01$ vs phenotype C group.

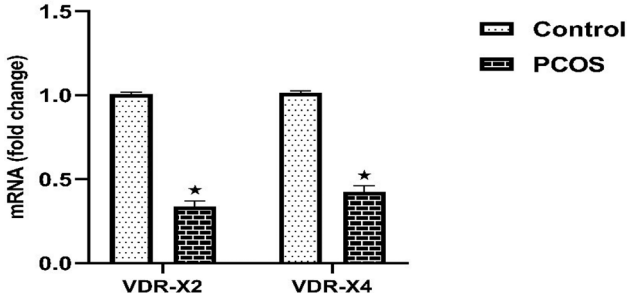

Figure 2. Graphical representation of endometrial VDR-X2 and VDR-X4 mRNA expression in the PCOS and control groups (*data are shown as mean ± SEM. *p < 0.001 vs. control*).

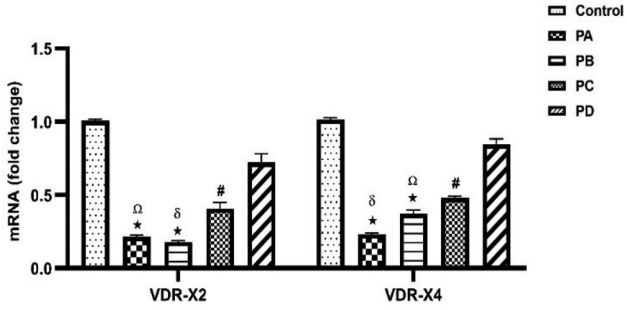

Figure 3. Graphical representation of VDR-X2 and VDR-X4 mRNA expression according to PCOS phenotypes (*data are shown as the mean ± SEM. *p < 0.001, #p < 0.05 vs. control; Ωp < 0.05, &p < 0.01 vs. phenotype D*).

Table 3. Logistic regression analysis of metabolic parameters independently associated with endometrial VDR-X2 and VDR-X4 mRNA expression.

	Unadjusted		Adjusted[a]	
	OR (95% CI)	*p*-values	OR (95% CI)	*p*-values
VDR-X2				
Testosterone (ng/dL)	0.805 (0.700–0.926)	<0.01	0.793 (0.684–0.920)	<0.01
Androstenedione(ng/mL)	0.347 (0.147–0.820)	<0.05	0.338 (0.139–0.821)	<0.05
DHEAS (µg/dL)	0.957 (0.929–0.987)	<0.01	0.948 (0.913–0.983)	<0.01
HOMA-IR	0.033 (0.002–0.482)	<0.05	0.034 (0.002–0.505)	<0.05
VDR-X4				
Testosterone (ng/dL)	0.839 (0.747–0.942)	<0.01	0.814 (0.712–0.930)	<0.01
Androstenedione(ng/mL)	0.450 (0.223–0.908)	<0.05	0.462 (0.222–0.963)	<0.05
DHEAS (µg/dL)	0.971 (0.949–0993)	<0.05	962 (0.936–0.989)	<0.01
HOMA-IR	0.054 (0.006–0.504)	<0.05	0.053 (0.005–0.560)	<0.05

[a]Adjusted with age and body mass index.
OR: Odds ratio, CI: Confidence interval.

0.18 ± 0.01; $p < 0.001$) and C (1.00 ± 0.01 vs. 0.40 ± 0.04; $p < 0.05$). VDR-X2 mRNA expression in the control group and phenotype D was similar (1.00 ± 0.01 vs. 0.72 ± 0.05; $p = 0.077$). For VDR-X2, no significant difference was detected between phenotypes A and B ($p = 0.374$) or phenotypes A and C ($p = 0.064$). VDR-X2 expression of phenotype D was significantly higher than both phenotype A (0.21 ± 0.01 vs. 0.72 ± 0.05; $p < 0.05$) and B (0.18 ± 0.01 vs. 0.72 ± 0.05; $p < 0.01$). There was no significant difference in VDR-X2 expression between phenotypes B and C ($p = 0.150$) or phenotypes C and D ($p = 0.292$) (Figure 3).

Compared to VDR-X4mRNA expression of control group a significant down regulation was detected in endometrial VDR-X4 mRNA expressions of phenotypes A (1.01 ± 0.01 vs. 0.23 ± 0.01; $p < 0.001$), B (1.01 ± 0.01 vs. 0.37 ± 0.01; $p < 0.001$) and C (1.01 ± 0.01 vs. 0.48 ± 0.01; $p < 0.05$). VDR-X4 mRNA expression of the control group and phenotype D was similar (1.01 ± 0.01 vs. 0.84 ± 0.03; $p = 0.105$). No significant difference was detected between phenotypes A and B ($p = 0.060$) or phenotypes A and C ($p = 0.059$) in terms of VDR-X4 expression. VDR-X4 expression of phenotype D was significantly higher than both phenotype A (0.23 ± 0.01 vs. 0.84 ± 0.03; $p < 0.05$) and B (0.37 ± 0.012 vs. 0.84 ± 0.03; $p < 0.05$). There was no significant difference in VDR-X4 expression between phenotypes B and C ($p = 0.403$) or phenotypes C and D ($p = 0.221$) (Figure 3).

A significant negative correlation was found between endometrial VDR-X2 mRNA levels and serum testosterone (r=-0.751, $p < 0.01$), androstenedione (r=-0.748, $p < 0.01$), and DHEAS (r=-0.752, $p < 0.01$) levels. Similarly, a negative correlation was detected between endometrial VDR-X4 mRNA and serum testosterone (r=-0.740, $p < 0.01$), androstenedione (r=-0.652, $p < 0.01$), and DHEAS (r=-0.586, $p < 0.01$). While there was a positive and significant correlation ($r = 0.611$, $p < 0.05$) between VD and VDR-X2 mRNA, no correlation was found between VDR-X4 mRNA and VD ($r = 0.322$, $p = 0.34$). There was a significant negative correlation between HOMA-IR and VDR-X2 mRNA levels (r=-0.690, $p < 0.05$). We also noted a significant negative correlation between HOMA-IR and VDR-X4 expression (r=-0.513, $p < 0.05$). Although there was a significant positive correlation between HOMA-IR and serum T ($r = 0.701$, $p < 0.01$) and A ($r = 0.654$, $p < 0.05$) levels, no correlation was observed between DHEAS and HOMA-IR. Multivariate analysis revealed that serum T, A, DHEAS, and HOMA-IR were independently associated with both VDR-X2 and VDR X4 relative gene expression after adjusting for age and BMI (Table 3).

The clinical pregnancy rates in the PCOS and control groups were similar (45% vs. 47.7%, $p = 1.000$). There was no difference in the clinical pregnancy rates calculated according to phenotype (Table 1). The VDR histoscore of the control group was significantly higher than those of each PCOS phenotype. The VDR histoscores of phenotypes A and B were similar and had the lowest value among all phenotypes. The VDR histoscores of phenotypes C and D were similar and significantly higher than those of phenotypes A and B (Figure 4).

Discussion

This study examined the effect of hyperandrogenemia and other metabolic and endocrine factors on endometrial VDR expression in women with PCOS. A total of forty-four PCOS patients, divided into four phenotypes, showed a significant decrease in VDR-X2 and VDR-X4 levels compared with controls. The decrease in VDR was most pronounced in phenotypes A, B, and C. There was a negative correlation between the VDR and serum testosterone, androstenedione, DHEAS, and insulin resistance. These findings are important, as they show for the first time that hyperandrogenemia and insulin resistance lead to defective VDR expression in the PCOS endometrium. Because studies investigating the biological functions of VDR in the endometrium are generally observational and based on animal studies, we analyzed VDR expression in the PCOS endometrium [8,16]. We showed that the PCOS endometrium is dysfunctional with respect to VDR, in addition to the defective expression of many receptivity modulators and steroid hormone receptors.

Low serum VD levels may be one of the possible causes of failed VDR expression in PCOS endometrium. The significant

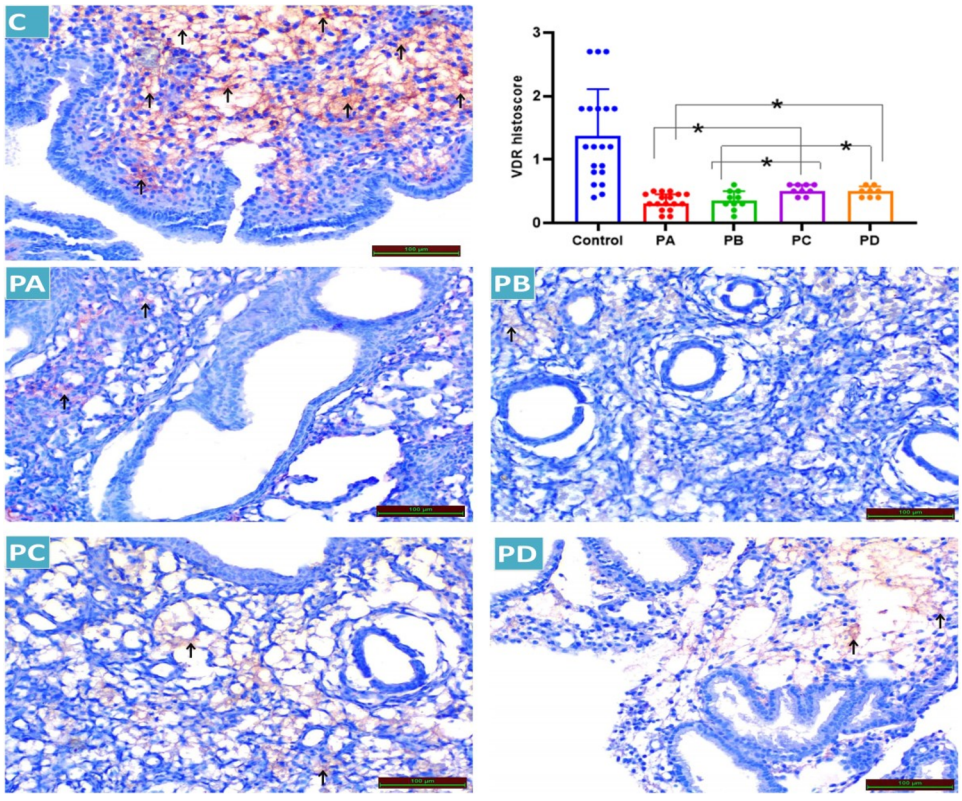

Figure 4. Representative immunostaining of endometrial VDR in each PCOS phenotype. Intense VDR immune reactivity was detected in the control group compared to all phenotypes (C). VDR immunoreactivity of phenotypes A (PA) and B (PB) was significantly lower than that of other phenotypes. The VDR immunoreactivity of phenotypes C (PC) and D (PD) were similar to and higher than those of the other two phenotypes (PA and PB). The histoscore graph in the upper right corner shows the comparison of VDR values between groups. *indicates $p<.05$ relative to the group being compared. Black arrows indicate the VDR-positive cells in each image. Magnification, X20. Scale bar, 100 µm.

positive relationship between serum VD and endometrial VDR in the PCOS group supports this idea. Although serum VD levels were higher in all phenotypes than in healthy controls, decreased VDR expression was observed only in phenotypes accompanied by hyperandrogenemia. As aromatase enzyme activity decreases in VD deficiency, the incidence of hyperandrogenemia increases [17]. The frequent prevalence of hyperandrogenemia in PCOS patients accompanied by hypovitaminosis D suggests that VD deficiency reduces VDR expression by causing hyperandrogenemia [18]. A study conducted by Jamilian et al. showed that VD supplementation led to a significant decrease in total testosterone levels [19]. The improvement in mating rates, endometrial decidualization, and uterine weight in vitamin D-deficient animals with VD replacement supports the stimulatory effect of VD on VDR [20]. The fact that VD supplementation increases Th2 levels and reduces pro-inflammatory cytokines in infertile women with vitamin D deficiency is another important piece of evidence of the positive relationship between VD and VDR [21]. However, when adjusted for confounding factors, such as age and BMI, there was no significant relationship between serum VD levels and endometrial VDR expression, suggesting that there was no clear relationship between failed VDR and serum VD levels.

Phenotypes A, B, and C had significantly lower VDR-X2 and VDR-X4 expression levels than the controls. As the common component of these three phenotypes is hyperandrogenemia, the decrease in endometrial VDR-X2 and VDR-X4 expression may be due to HA. The higher serum testosterone, androstenedione, and DHEAS levels in phenotypes A, B, and C than in the control group and phenotype D supports our hypothesis. The fact that high testosterone and androstenedione are more prominent in phenotypes A and B than in phenotype C may explain why the VDR expression defect is more prominent in these two phenotypes than in phenotype C. In light of these findings, we suggest that phenotype A, B, and C endometria express defective VDR due to their greater exposure to the adverse effects of hyperandrogenemia. In agreement with this, *in vitro* exposure to testosterone or dihydrotestosterone suppresses the expression of the key receptivity molecule, HOXA10, providing important evidence that high androgen levels negatively affect the expression of receptivity modulators [22]. The fact that VDR is better expressed in non-hyperandrogenic phenotype D than in other phenotypes is important evidence that androgen elevation can block VDR.

Under physiological conditions, androgens potentiate progesterone receptor expression and contribute to implantation [23]. HA may impair receptivity in the PCOS endometrium by inhibiting pathways related to DNA repair, cell cycle regulation, oxidative stress, insulin, and glucose metabolism [24]. High T, A, and DHT levels may impair implantation by downregulating cyclin-dependent kinase inhibitors as well as inhibiting endometrial tight junction protein expression and pinopode formation [25–27]. Moreover, high testosterone inhibits the expression of homeobox genes [22], whereas high DHEAS inhibits the expression of leukemia inhibitory factor (LIF) [2]. It is worth noting that although it is not as effective as T and A, high DHEAS 04 levels may disrupt decidualization and implantation by blocking the glucose utilization pathways of the endometrium [28]. The negative and significant correlation between VDR and serum T, A, and DHES is evidence that all types of androgens suppress transcriptional regulation of endometrial VDR. Moreover,

multivariate analysis after adjustment for age and BMI revealed a significant relationship between serum T, A, DHEAS, and endometrial VDR expression, supporting that androgens adversely affect endometrial VDR expression, regardless of their androgenic potential. However, unlike T and DHT, *in vitro* exposure to dehydroepiandrosterone or dehydroepiandrosterone sulfate has been reported to not affect receptivity gene expression, suggesting that the potency of androgens is important for preventing implantation [22]. However, the clinical pregnancy rates of the low VDR phenotypes were similar to those of the normal VDR phenotypes, suggesting that low VDR may not necessarily be associated with reduced implantation. The relatively low number of participants in each phenotype may mask the negative effect of a low VDR on clinical pregnancy rates.

Insulin resistance and compensatory hyperinsulinism may be the other causes of failed endometrial VDR expression. The negative relationship between HOMA-IR and VDR supports this hypothesis. Since the production of insulin receptor substrates and glucose transporters in the endometrium is impaired at high insulin levels, complete decidualization does not occur [29]. The increase in insulin sensitivity while decreasing androgen levels in PCOS patients administered VD supplementation is evidence of the complex relationship between VD, insulin, and androgens [17]. Hyperandrogenemia has adverse effects on the endometrium by promoting IR and impairing glucose transport [24]. The stimulation of T and A production by insulin in PCOS is evidence that insulin and androgens potentiate each other's negative effects on the endometrium [30]. HOXA10 expression was increased, while AR expression was decreased in PCOS patients treated with metformin, supporting the role of insulin in implantation [31]. When our findings and literature data are evaluated together, the positive correlation between IR and serum T and A levels strongly supports that hyperandrogenemia and IR potentiate each other's effects in impairing endometrial receptivity. The persistence of the relationship between HOMA-IR and VDR after adjustment for age and BMI suggests that IR is an independent metabolic variable that triggers defects in VDR expression.

The phenotypes of VDR expression and immunoreactivity matched perfectly. Different intensities of VDR immunoreactivity were observed for each phenotype. The high VDR histoscore in the control group indicated that VDR was suppressed in the PCOS endometrium. Weak VDR immunoreactivity in hyperandrogenic phenotypes suggests that both genomic and non-genomic activation of VDR is impaired in PCOS. Normal VDR immunoreactivity in the control group and the non-hyperandrogenemic phenotype is strong evidence of the negative effects of androgens on VDR expression.

Although this is the first study to demonstrate the underexpression of VDR in endometrial samples collected during stimulated cycles from PCOS patients experiencing infertility, the number of participants was relatively low. However, the number of participants determined by the power analysis was sufficient for statistical evaluation. Another limitation is that supraphysiological estrogen levels in stimulated cycles may adversely affect VDR expression. However, there are no data regarding whether high estradiol levels affect endometrial VDR expression. Since serum estradiol levels measured on the day of hCG administration were similar in all phenotypes, the effect of circulating estradiol levels on VDR expression can be ruled out. The fact that endometrial sampling was performed during oocyte retrieval may also be considered a limitation of this study. Since receptivity modulators peak in the mid-luteal phase, the VDR may be physiologically low in endometrial samples collected during the periovulatory phase. However, the fact that VDR expression was high in the control group despite endometrial sampling at the same time interval weakens our claim.

In conclusion, lower VDR expression in phenotypes A, B, and C than in the control group and phenotype D indicates that endometrial VDR expression varies according to PCOS phenotypes. Since no significant effect of ovarian stimulation on VD metabolism has been reported, we suggest that the VDR expression pattern we obtained is specific to PCOS and its related phenotype. Hyperandrogenemia and IR have been identified as two important metabolic components leading to inadequate endometrial VDR. Since endometrial VDR analysis is not cost-effective for the evaluation of infertile patients, VD replacement before ICSI in infertile PCOS patients with low VD may improve fertility outcomes by normalizing VDR expression. Comprehensive studies on the use of VDR in endometrial receptivity arrays are critical to elucidate the relationship between VD, VDR, and implantation.

Ethical approval

This study was approved by the Firat University Ethics Committee (approval number: 2022/12-23). Strict compliance with the standards of the Declaration of Helsinki was demonstrated throughout the study. Clinical Trial registration number: NCT05885633

Consent form

Informed consent was obtained from all the participants.

Author contributions

All the authors contributed to the conception of the work; the acquisition, analysis, and interpretation of data, drafting, and revision of the manuscript They have read and approved the final version for submission and agree to be personally accountable for their contributions and for ensuring that questions related to the accuracy or integrity of any part of this work were appropriately investigated, resolved, and documented in the literature.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data used and/or analyzed in the current study are presented within the study. There were no additional data to be requested.

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