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Article

What Unraveled Mysteries Lie in the Interplay of CD44, Vitamin D, and COX-2 in Endometriosis?

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Abstract: (1) Background: Our study aimed to examine tissue expression of CD44, vitamin D receptor (VDR), and cyclooxygenase 2 (COX-2) in endometriosis-affected women with and without progestin treatment and to inquire about their implications in the pathogenesis of endometriosis. (2) Methods: A cohort of 60 women diagnosed with endometriosis was divided into two groups, of which 26 received progestin treatment and 34 without treatment. We conducted an immunohistochemical (IHC) analysis of paraffin-embedded endometriosis tissue samples collected post endometriosis surgery. The primary goal was to elucidate VDR, COX-2, and CD44 expression patterns. (3) Results: CD44 evaluation revealed a positive score in 32 (60%) of the untreated cases, and 21 (40%) of the treated ones, but the difference didn't reach statistical significance. VDR was observed to be higher in the group without treatment, with 32 individuals (88.9%) showing positive expression, compared to 20 individuals (83.33%) in the treatment group. Endometriotic tissue consistently demonstrated uniformly strong membrane positivity for COX-2 in all of our 60 included cases. (4) Conclusions: IHC analysis of VDR, CD44, and COX-2 are supplementary tools for risk stratification and prognostic evaluation in patients diagnosed with endometriosis. Notably, this study highlights the immunological differences between untreated and treated endometriosis.

Keywords: endometriosis; immunohistochemistry; VDR; CD44; Cyclooxygenase 2; treatment; progestins

1. Introduction

Endometriosis remains an intriguing and complex gynecological condition that continues to raise significant challenges in both diagnosis and treatment. Ascertained as the presence of ectopic endometrial tissue outside the uterus, this pathology is retrieved in millions of women worldwide, leading to chronic pelvic pain, infertility, and a kaleidoscope of other clinical signs and symptoms [1]. Numerous theories attempt to explain the origins of endometriosis. However, the most widely accepted theory attributes it to retrograde menstruation [2]. While this theory is compelling and enjoys broad recognition, it falls short of elucidating why merely 3.7% of the 90% of women experiencing menstrual retrograde flow are affected by the condition. This drives us to suspect the

involvement of additional pathogenic mechanisms in the appearance of these ectopic endometrial foci. The literature describes many genetic, epigenetic, and immunological factors that interfere with the normal signaling pathways, altering the microenvironment and the immune response of the endometriosis affected individual. Researchers continue the race to uncover the molecular factors that contribute to this disease development and progression [3–6]. A combination of surgery and medical treatments are available for these women, but there is ongoing research that focuses especially on molecular targeted therapies. Despite its disputed etiopathogenesis, there seems to be a consensus about the involvement of dysfunctional cell adhesion molecules. Although the precise cause of the disease remains unclear, growing evidence indicates that dysregulated cell adhesion molecules may play a role [2].

Such an adhesion molecule, with many functions beyond the standard attributed role, is CD44. This molecule is a cell surface glycoprotein highly involved in cellular dynamics, modulating the local immune response and tissue homeostasis [7]. It has numerous isoforms resulting from its alternative splicing. These isoforms entail cell-to-cell and cell-to-matrix adhesion, maintaining tissue integrity, cell signaling; and extracellular matrix remodeling. They are also involved in processes such as embryonic development, lymphocyte homing, and wound healing [2]. CD44 is a hyaluronic acid (HA) receptor, the ligand representing an essential component for tissue organization [2]. By their interaction, CD44 and HA promote essential processes such as angiogenesis and immune evasion, emphasizing also the complexity of their functions [8]. When focusing on endometriosis, CD44 emerges as one of the key players. It is involved in the progression of this disease by governing cell adhesion, attachment, invasion, and migration of the endometrial-like cells into the ectopic sites [9,10]. The adhesion capacity explains the establishment and persistence of endometriotic lesions, furthering the understanding of endometriosis's invasive nature [11].

Only a few studies in the existing literature have explored the potential therapeutic advantages of vitamin D in addressing endometriosis. Beyond its well-known role as a secosteroid that governs calcium metabolism and bone mineralization, vitamin D and its receptor also wield significant influence over the immune system, which aids in reducing bodily inflammation. These dual functions aroused the interest of scientists, who have ventured into studying its implications in endometriosis [12,13]. Several studies within the literature have indicated that vitamin D can act as an inhibitor of cellular proliferation [14]. There is a very limited percentage of literature studies that focus on the possibility and benefits of using vitamin D supplementation for endometriosis treatment. With its immunomodulatory, anti-inflammatory, anti-proliferative, and anti-invasive properties, vitamin D is speculated to play a potentially vital role in the multifaceted pathogenesis of endometriosis [15,16]. But, this promising frontier remains relatively uncharted, with a multitude of ongoing research efforts concentrated on the attempt to elucidate the precise mechanisms of action and therapeutic implications of vitamin D in the context of endometriosis.

Due to the chronic characteristic inflammation that characterizes endometriosis, cyclooxygenase-2 (COX-2), with its inflammatory molecules synthesis, appears to play a key part in its pathogenesis [17–20]. Literature reports elevated levels of COX-2 in the ectopic endometrial-like tissue, with Bulum et al. suggesting a potential connection with pain related symptoms [21–23]. Research suggests that COX-2 contributes to various aspects of endometriosis, including implantation and growth of the ectopic tissue, angiogenesis, and modulation of the immune response [24], potentially leading to either pro-inflammatory or anti-inflammatory effects [25].
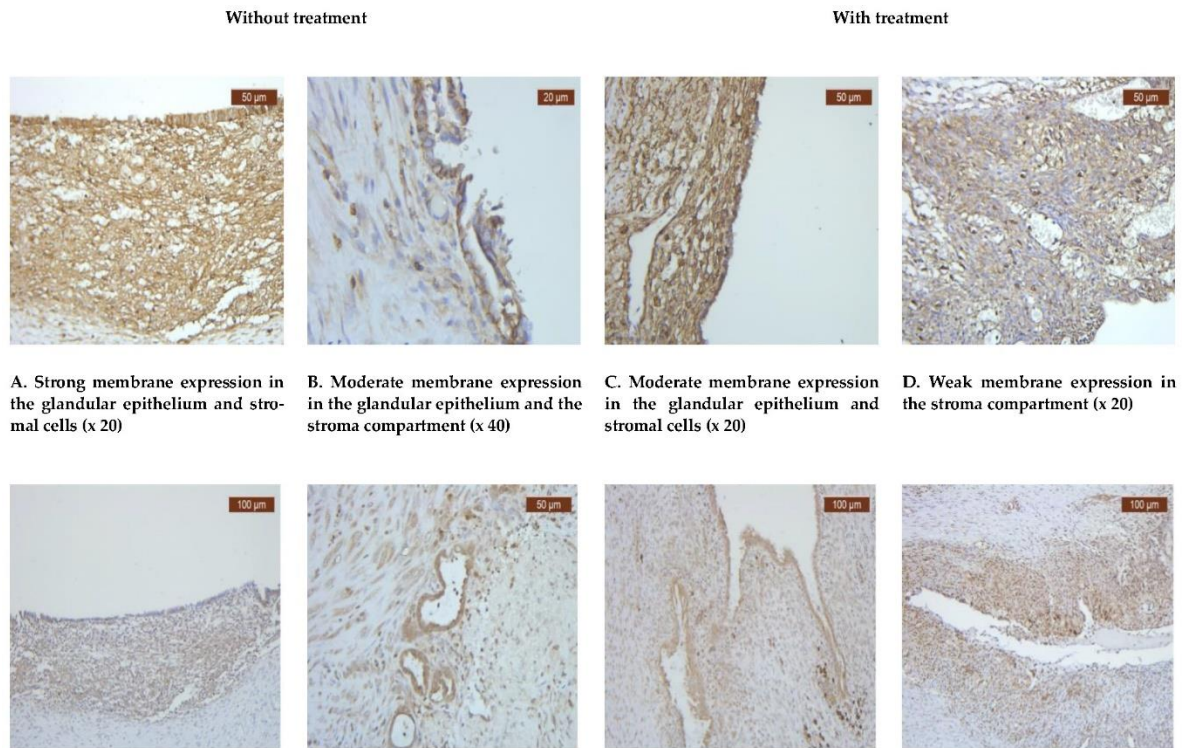
Driven by our inquisitiveness regarding this controversial pathology, our research efforts to unravel endometriosis physio-pathogenesis converged in studying these three molecule expressions (CD44, VDR, and COX-2) in the endometriotic tissue samples of progestin-treated and untreated women. The purpose of our research is to serve as a foundation for the development of new diagnostic tools and therapeutic strategies that might ease the burden for endometriosis-affected women.

2. Results

The age of our 60 included patients varied between 18 and 45 years, with a mean age of 31.92 ± 4.706 (95% CI 30.70/33.13, Std Error 0.608). Before undergoing surgery, 24 (40%) patients underwent progestin treatment with dienogest for three months, while 36 (60%) patients did not receive any treatment.

2.1. CD44

All instances of endometriosis, regardless of treatment status, exhibited a notably strong positive reaction for CD44. Despite the widespread expression of CD44 observed in all cases of endometriosis, a positive score was detected in 32 (60%) of the untreated cases and 21 (40%) of the treated ones (Table 1). Immunohistochemistry examination revealed high expression levels of CD44 in both the stroma and endometriotic cells (Figure 1A-D).

Without treatment   **With treatment**

A. Strong membrane expression in the glandular epithelium and stromal cells (x 20)

B. Moderate membrane expression in the glandular epithelium and the stroma compartment (x 40)

C. Moderate membrane expression in the glandular epithelium and stromal cells (x 20)

D. Weak membrane expression in the stroma compartment (x 20)

E. Weak nuclear expression in the stroma compartment and in a cells of glandular epithelium (x 10)

F. Weak nuclear expression in the glandular epithelium and stroma compartment (x 20)

G. Moderate nuclear expression in the glandular epithelium and stroma compartment (x 10)

H. Strong nuclear expression in the glandular epithelium and stroma compartment (x 10)

I. Strong membranar/cytoplasmatic expression in the glandular epithelium and stroma compartment (x 10)

J. Strong membranar/cytoplasmatic expression in the glandular epithelium and stroma compartment (x 20)

K. Weak membranar/cytoplasmatic expression in the glandular epithelium and stroma compartment (x 10)

L. Moderate membranar/cytoplasmatic expression in the glandular epithelium and stroma compartment (x 10)
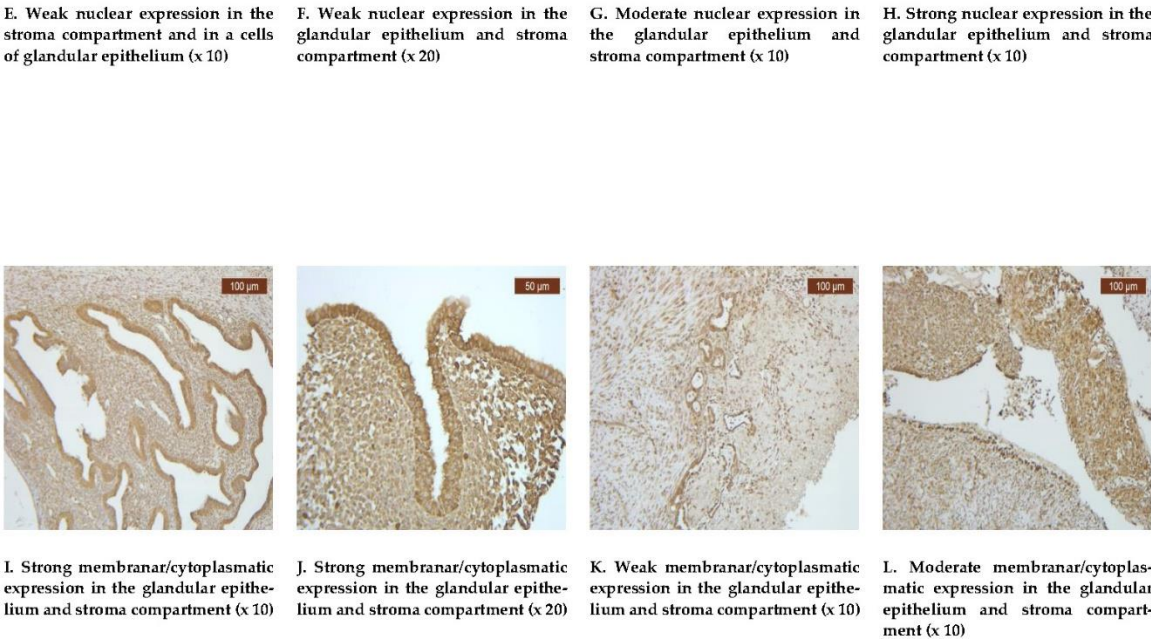
Figure 1. Immunohistochemical expression A-D of CD44; E-H of VDR; I-L of COX-2 in two groups (with and without treatment).

Table 1. Differential expression of CD44, VDR and COX 2 in endometriotic samples.

Group	N	CD44		VDR		COX 2	
		Positive	Negative	Positive	Negative	Positive	Negative
With treatment	24	21(40%)	3 (43%)	20 (83.3%)	4 (16.7%)	23	1
Without treatment	36	**32** (60%)	4 (57%)	32 (88.9%)	4 (11.1%)	36	0
p-value		0.99F		0.702 F		0.40F	

Upon scrutinizing the tissue expression of CD44, no statistically significant difference was observed between the progestin-treated and untreated groups, with a p-value of 0.99 (Table 1). When examining the CD44 expression in the epithelial and stromal compartments separately, no notable variation was detected either (Table 2).

Table 2. Differential expression of CD44, VDR, and COX-2 in endometriotic samples in the two compartments.

Group	N	CD44 EPITHELIAL Positive	Negative	CD44 STROMAL Positive	Negative	VDR EPITHELIAL Positive	Negative	VDR STROMAL Positive	Negative	COX 2 EPITHELIAL Positive	Negative	COX 2 STROMAL Positive	Negative
With treatment	24	22 (39.28%)	2 (50%)	17 (38.63%)	7 (43.75%)	20 (35.71%)	4 (100%)	16 (40%)	8 (40%)	23 (39%)	1 (100%)	23 (39%)	1 (100%)
Without treatment	36	34 (60.71%)	2 (50%)	27 (61.36%)	9 (56.25%)	36 (64.28%)	0 (0%)	24 (60%)	12(60%)	36 (61%)	0 (0%)	36 (61%)	0 (0%)
		56	4	44	16	56	4	40	20	59	1	59	1
p-value		0.00 F		0.72		0.02		1		0.40 F		0.40 F	

2.2. VDR

To elucidate the involvement of VDR in ovarian endometriotic pathology, we aimed to characterize its expression patterns and distribution. Among the 24 women included in the treatment group, 20 (83.3%) tested positive for VDR, while only 4 (16.7%) were negative. Conversely, in the

group of women with endometriosis but without any treatment, 32 (88.9%) were positive for VDR, with only 4 (11.1%) testing negative (Table 1).

The expression of VDR was observed to be higher in the group without treatment, with 32 individuals (88.9%) showing positive expression, compared to 20 individuals (83.33%) in the treatment group. Conversely, the occurrence of negative VDR expression was consistent across both groups (Table 1).

VDR exhibited a diffuse positive nuclear expression in both the epithelial and stromal compartments (Figure 1E-H). However, the analysis revealed that the expression levels of VDR in the epithelial compartment were lower in the treated group, with 20 individuals (35.71%), compared to the untreated group, with 36 individuals that proved to be positive (64.28%) (Table 2). Comparative images of the immunohistochemical staining in the two groups (with or without treatment) are illustrated in Figure 1E-H.

2.3. COX-2

Endometriotic tissue consistently demonstrated uniformly strong membrane positivity for COX-2 in all of our 60 cases, this includes epithelial cells and endometrial stroma, regardless of therapeutic status. COX-2 was uniformly positive in both our groups (24 cases with dienogest treatment and 36 cases without treatment), with a slight tendency to decrease in intensity in the treated group (Table 1).

Comparative images of the immunohistochemical staining in the two groups, with or without treatment, are depicted in Figure 1I-L.

3. Discussion

Vitamin D levels are classified as follows: deficiency (<20 ng/ml), insufficiency (20–30 ng/ml), and sufficiency (>30 ng/ml) [26]. The vitamin D nuclear receptor (VDR), which participates in the transcription of over 900 genes, facilitates the biological effects of vitamin D. This receptor is involved in various immunological processes, acting in conjunction with the active form of vitamin D, known as 1,25 (OH)2 vitamin D. Upon binding with the active vitamin D, the receptor relocates from the cytoplasm into the nucleus to initiate gene transcription. Emerging endometriosis research evidence is beginning to outline a potential therapeutic direction in the form of vitamin D, whose immunomodulatory and anti-inflammatory properties have caught considerable attention [27,28].

The literature is abundant with ongoing research into potential markers for endometriosis and treatment options, as no specific marker has yet proven highly sensitive and specific, and current treatments are still limited, especially by side effects. Owing to its incompletely understood pathophysiology, there are limited therapy options available, most of which involve progestin treatments and painkillers with less severe side effects. To treat endometriosis, progestin therapy has typically been used either alone or in combination with other drugs [11,29–32]. As an enhancement of an already complicated to manage pathology, as stated by Guidice et al., the presence of progesterone inhibitory isoform A receptor results in progesterone treatment resistant endometriotic implants [31].

The present study investigated the expression of CD44, VDR, and COX-2 in endometriotic tissues, comparing cases with and without progestin (desogestrel) treatment, with the hope of offering insights into the molecular pathophysiology of endometriosis and describing potential diagnosis and target-treatment markers.

There was no notable difference in CD44 levels between the two groups in our study. This cell-adhesion and migration molecule was expressed at similar levels in both the progestin-treated group (32 cases/60%) and the untreated ovarian cyst tissue samples (21 cases/40%). Although the difference was not statistically significant (p=0.99), further research could explore the observed trend of increased CD44 expression in progestin-treated women. In our study, we have noticed that CD44's membranar expression is typically elevated in the endometriotic tissue of women without treatment, compared to the treatment group, supporting endometriotic ectopic implant cell adhesion and lesion persistence in untreated patients. This trend is noteworthy due to CD44 implications in adhesion,

migration, and invasion, underlining its potential as a therapeutic target for future interventions [2]. We need also to emphasize that the lack of statistical significance may be attributed to the limited progestin treatment time-frame, which was restricted to just three months. Additionally, patient-specific characteristics could have influenced the outcomes as noted by Guidice et al., the presence of a specific progesterone receptor isoform can modulate an individual's response to progestin therapy [32].

Literature data about CD44 levels in endometriotic ectopic lesions is scarce and controversial [33–36], motivating our decision to further study its dynamics in progestin treated endometriosis compared to untreated ones. A 2007 research by Kim et al. detected high levels of CD44 expression in both epithelial and stroma compartments of various endometriotic lesions [37]. Conversely, a 2016 animal study by Knudtson et al. yielded intriguing results, showing that endometriotic lesions could still manifest themselves in knockout mice, even in the absence of CD44 [38]. Sancakli et al., Poncelet et al., and Nothnick et al., all found lower levels of CD44 in the tissue samples from women with endometriosis compared to controls [2,39,40], while Pazhohan et al. and Matsuzaki et al. detected exactly the opposite [10,41]. These discrepancies underline the need for expanded further research to clarify the role and implications of CD44 in endometriosis.

The biological effects of vitamin D are mediated through the vitamin D receptor (VDR), which participates in the transcription process of more than 900 genes. Acting in conjunction with the active form of vitamin D, known as 1,25 (OH) vitamin D, VDR is involved in a multitude of immunological processes, initiating gene transcription by relocating from the cytoplasm into cells nucleus. Endometriosis research results begin to underline the potential therapeutic benefits of vitamin D administration in affected women due to its immunomodulatory and anti-inflammatory properties [27,28]. Our research comes as an effort to take a further step in the direction suggested by Agic et al. 2007 that vitamin D might have more endometriosis-contributing autocrine and/or paracrine local effects [42]. Our results underline that VDR expression was detected in the majority of cases, regardless of the women's progestin treatment status (83.3% from the progestin-treated cases and 88.9% from the untreated ones). Our result also has shown weak nuclear VDR expression in untreated women, potentially contributing to the persistent inflammation associated with this condition. This ascertainment fuses with the rest of the results from literature that suggest potential VDR key roles in the modulation of the immune response and inflammation in endometriosis [1]. However, therapeutic interventions might interfere with VDR expression. This aspect comes as a result of the fact that progestin-treated women had a lower VDR expression in the epithelial compartment. Vitamin D supplementation or other treatments targeting VDR may upregulate VDR expression, leading to decreased inflammatory responses. In treated group, VDR expression often showed moderate and strong nuclear expression. However, the exact mechanism and its clinical practice implications are still to be discovered.

The well-known COX-2 pathway, which produces pro-inflammatory molecules, is considered to play a central role in endometriosis pathophysiology [3,4,43]. Our research detected strong membranous positivity for COX-2 in the epithelial cells and endometrial stroma of both treated and untreated patients. Regarding the IHC intensity, we noticed strong COX-2 expression in endometriotic lesions of women without treatment, fact that is often correlated with increased inflammatory response, while reduced COX-2 expression in treated endometriosis samples might explained its weak and moderate expression.This similitude suggests that COX-2 expression is not influenced by progestin treatment, making it a suitable diagnostic marker and a target for future therapeutic interventions. The overexpression of COX-2, regardless of progestin treatment, similar to the observations of Lai et al., implies that it may substantially contribute to the chronic inflammatory state observed in women affected by endometriosis [44].

We hope that the results of our study will contribute to the growing body of evidence regarding the molecular pathways in this remaining enigmatic disease. The patterns of our three analyzed molecular markers - VDR, CD44, and COX-2 - indicate their potential as targets for novel therapeutic strategies. Specifically, VDR may provide a new direction in managing the immune response in endometriosis by modulating it, while inhibitors targeting CD44 and COX-2 could be exploited

therapeutically due to their roles in reducing invasiveness and inflammation. A more comprehensive understanding of endometriosis pathophysiology might be achieved by exploring the interplay of these markers.

The impact of our research limitations must be carefully considered for an accurate interpretation of our results. The first limitation resides in our small sample size and in the short 3 months progestin treatment time-frame. To validate our observations, we need to expand our research to a broader population and extend the progestin treatment time-frame. The second limitation arises from our group selection criteria, as we included only women with endometriotic ovarian cysts (stages III or IV of the disease) from a diverse phenotypic pool. To be able to generalize our findings, we need to validate them in a population that includes all stages of endometriosis. Despite these obvious limitations, we are confident that our research will contribute to advancing the understanding of this complex disease.

4. Materials and Methods

4.1. Patients and Tissue Samples

The study period was from January 2021 to January 2022. Prior to the enrollment in the study all of the patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr. T. Popa" from Iasi (No. 10/28.09.2020) and the University of Medicine and Pharmacy "Carol Davila" from Bucharest (No. 6/21.12.2021).

Tissue samples were collected during ovarian cyst laparoscopic interventions from 60 women diagnosed with endometriosis. These women underwent surgery at the Obstetrics and Gynecology Hospital "Cuza-Voda" in Iasi, and the Obstetrics and Gynecology Hospital "Panait Sirbu" in Bucharest. We have divided them into two groups. The first group of women diagnosed with endometriosis decided to undergo progestin (dienogest) treatment for three months before the surgical interventions. The second group of women chose not to undergo dienogest treatment before surgery.

Inclusion criteria:

We included only Caucasian women with the histological examination confirming the suspected endometriosis diagnosis in every case we included. All of the enrolled women were diagnosed during the surgical procedure with endometriosis classified as stages III or IV, according to the American Society for Reproductive Medicine (ASRM) criteria [29,30]. Tissue samples were collected from all of the included patients. The women who underwent hormone therapy had 2 mg of dienogest administered daily for three months prior to surgery. The selection of untreated endometriosis patients was done by matching their age and body mass index (BMI) with those receiving dienogest therapy. All the tissue samples were obtained during the proliferative phase of the menstrual cycle.

Exclusion criteria:

To mitigate potential confounding variables, we excluded individuals with a body mass index (BMI) exceeding 30, as well as those diagnosed with malignancy or other tumoral lesions, diabetes, depression, genetic syndromes, any infectious or autoimmune diseases, smokers, pregnant women, individuals receiving hormonal therapy other than dienogest, or any other treatment known to interfere with bone and mineral metabolism.

4.2. Immunohistochemistry

Routinely prepared hematoxylin and eosin (H&E) sections have been examined, and IHC has been independently evaluated by two pathologists to confirm the diagnosis.

Monoclonal antibodies targeting CD44 were employed for analysis. Prior to analysis, the samples underwent fixation with 10% neutral formalin, followed by embedding in paraffin and sectioning to achieve a thickness of 4-5 micrometers. IHC was utilized to assess the expressions of

CD44, employing specific (1:250) dilutions provided by Abcam Company. For CD44 the membrane immunostaining pattern was considered positive (Table 3).

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, and immunohistochemical staining was performed using protocols optimized for COX-2 antibody. The antibody clone name, source, dilution, and pattern of expression are listed in Table 3. The membrane pattern of COX-2 immunostaining was considered positive, however, if a cytoplasmic and/or membranous pattern was present, it was considered positive. The expression levels of CD44, VDR, and COX-2 were quantified by assessing the percentage of positively stained cells and the staining intensity in each section. For overall positivity, immunostaining in >5% of cells were considered positive, and < 5% positive cells was considered negative. The expression of intensity was evaluated as weak, moderate, and strong both at the epithelial and stromal compartments.

Table 3. Immunohistochemical panel of antibodies used in the study.

Antibody	Clone, manufacturer	Dilution	Expression
Anti - CD44	rabbit polyclonal IgG isotype, Abcam (ab157107)	1:250	membranar
Anti - VDR	rabbit polyclonal IgG isotype, Abcam (ab3508)	1:3000	nuclear
Anti- Cox-2	rabbit polyclonal Ig G isotype, Abcam (ab15191)	1:100	membranar

VRD: vitamin D receptor; IgG: Immunoglobulin G; COX-2:.

Vitamin D anti-receptor dilution 1:3000 (Abcam, Cambridge, UK) was incubated for an overnight period at 4°C. The sections were washed, exposed to the secondary antibody for 45 minutes at 37 degrees, and then thoroughly cleaned with *phosphate-buffered saline (PBS)*. Hematoxylin was used as a counterstain in the standard avidin-biotin-peroxidase technique, which used a liquid DAB (diaminobenzidine) substrate and chromogen system for viewing. As a negative control, primary antibodies were left out. For VDR the nuclear immunostaining pattern was considered positive. Human jejunum was utilized as a positive reference for vitamin D receptor (Table 3).

4.3. Statistical Analysis

Medical data were imported and verified in Microsoft Excel and then analyzed in SPSS 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). The information was in the form of measurable numerical values (age), respectively categorical variables. Within the descriptive statistics, we calculated the values of the following statistical measures: sample size (N), mean, standard deviation, standard error, and 95% confidence interval for mean, min, max, and absolute and relative frequencies, respectively. Statistical hypothesis tests were done by Chi-square or Fisher exact tests (for categorical type). The standard cut-off of 5% or 0.05 significance was used to decide on the conclusion of the hypothesis.

5. Conclusions

The current study concludes by highlighting the importance of VDR, CD44, and COX-2 as major participants in endometriosis pathogenesis. The tissue expression of CD44 reavealed no statistically significant difference between the progestin-treated and untreated groups, with a p-value of 0.99. Our results indicated a lower expression of VDR in the epithelial compartment of the treated cases, compared to untreated ones, with 40% of treated and 60% of untreated cases showing positive expression. COX-2 was highly expressed across all endometriotic tissue samples, irrespective of treatment status. Even though the precise mechanisms are still not fully understood, our findings provide a basis for further investigation and possible clinical uses targeted at enhancing endometriosis treatment.

Author Contributions: Conceptualization, D.R.M. and L.L.; methodology, C.E.M. and M.O.; software, I.E.B and L.V.B.; validation, I.E.B., and M.O.; formal analysis, L.L. and M.G.; investigation, D.R.M.; resources, M.G. and A.U.; data curation, A.U.; writing—original draft preparation, D.R.M.; writing—review and editing, A.U.;

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