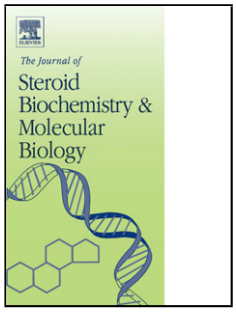

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Expression and shedding of CD44 in the endometrium of women with endometriosis and modulating effects of vitamin D: a randomized exploratory trial

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Highlights

- The endometrium of endometriosis patients expresses higher levels of CD44s, CD44V, and CD44v6.
- The endometrial fluid and serum concentration of sCD44 is higher in endometriosis patients.
- Vitamin D down regulates the expression of CD44s, CD44V, and CD44v6 in the endometriosis patients.
- Vitamin D regulates the shedding of sCD44 in the endometrium of endometriosis patients.

Abstract

Endometriosis is an estrogen-dependent disease. The impaired estrogen and progesterone signaling over-activates the Wnt/β-catenin pathway in endometriosis patients, which can explain the increased invasion potency of endometrial cells derived from the endometrium of women with endometriosis. The regulatory effects of vitamin D on Wnt/β-catenin pathway were demonstrated by previous studies. According to gene prioritization method, among Wnt target genes, *CD44* was in high ranking in relation to endometriosis. The aim of this study is to investigate the expression of CD44 in the endometrium of women with endometriosis and to study the effects of vitamin D on its expression. This prospective study was performed, during a 12 months period from December 2015 to November 2016, on healthy women as the control group (n=14) and endometriosis patients (n=34). The endometriosis patients randomly divided

into two groups: One group treated according to the routine protocol and the other group, alongside the routine protocol, took 50000 IU vitamin D weekly for 12-14 weeks. Blood, endometrial fluid, and endometrial tissue samples were obtained from the control group and endometriosis groups before and after the intervention. We used *in silico* gene prioritization to study the relevance of *CD44*. The expression of CD44 was evaluated using the techniques of Western blot, real-time polymerase chain reaction, and ELISA. The eutopic endometrium of women with endometriosis in mid-secretory phase expressed significantly higher levels of CD44s, CD44V, and CD44v6. The concentration of soluble CD44 in the serum and endometrial fluid of endometriosis patients was higher than of healthy women. The expression level of CD44s, CD44V, and CD44v6 in the eutopic endometrium as well as the concentration of soluble CD44 in the endometrial fluid were decreased after modification of the circulating levels of 25(OH)D. It seems that the increased expression and extensive shedding of CD44 in eutopic endometrium play a role in the pathogenesis of endometriosis. Vitamin D can control and modify this process at least in part. We suggest more in vivo investigations on the therapeutic potency of vitamin D in endometriosis.

Keywords: endometriosis; endometrium; CD44; vitamin D

1. Introduction

In women with endometriosis, endometrial cells from eutopic endometrium may be more aggressive and susceptible to implant in ectopic sites, which is facilitated by altered microenvironment of the peritoneal cavity [1]. A previous in vitro study showed that endometrial fragments can easily attach to intact peritoneal mesothelial cell (PMC) layer and the invasion begins rapidly [2]. It was also demonstrated that the significant variability in adherence of both

endometrial epithelial and stromal cells to peritoneum depends on the source of these cells rather than PMCs [3]. In a study by Griffith et al., the menstrual endometrial epithelial and stromal cells from women with endometriosis expressed a higher rate of some splice variants of *CD44* including *CD44v6*, which probably can explain the increased binding capacity of these cells to PMC monolayers [4]. CD44 has the key role in the attachment of ovarian [5] and gastric cancer cells [6] to the peritoneum. The results of a recent study, by using *CD44* knockout model, supported the basic in vivo role of CD44 in developing of the endometriotic lesion [7].

CD44 belongs to the family of transmembrane glycoproteins and is the key receptor of hyaluronan as an essential element of the extracellular matrix (ECM). It plays several roles in cell functions such as adhesion, signaling as well as migration [8]. *CD44*, a Wnt target gene [9], is encoded by a single gene, and alternative splicing generates up to ten different *CD44* variant isoforms. The expression of *CD44v6* has been linked to the invasive potency of some cancer cells [10].

CD44 standard (CD44s) is the smallest isoform, lacks variant exons and its related protein contains three functional domains including an intracellular domain, a transmembrane domain, and an extracellular domain [11]. In addition to hyaluronic acid, the extracellular domain interacts with several ECM components including collagens, fibronectin, cytokines and chemokines, growth factors, and metalloproteinases [12]. The cytoplasmic tail of CD44 connects to the actin-cytoskeleton via binding to ERM (ezrin, radixin, and moesin), takes part in the remodeling of the cytoskeleton and involves in the processes such as the regulation of the epithelial-mesenchymal transition, cell adhesion, motility, and invasion [13]. An essential process in the regulation of CD44-dependent functions is the shedding of CD44 from the cell membrane. Cleavage of CD44 is firstly induced by some metalloproteases such as MT1-MMP

[14], ADAM10, and ADAM17 within the ectodomain, which forms soluble CD44 (sCD44) and promotes cell detachment from its surrounding matrix. In the second step, presenilin-dependent γ-secretase induces the cleavage of its intracellular domain, which generates an intracellular fragment [15]. The last fragment enters the nucleus and stimulates the transcription of *CD44* and its target genes. The newly synthesized CD44 promotes CD44-mediated cell-surface interactions including cell adhesion to its surrounding matrix [15]. Excessive turnover and shedding of CD44 lead to increased cell motility in highly aggressive melanoma cells [16].

Human endometrium is a dynamic tissue, undergoes cyclical remodeling controlled by sex hormones secreted by ovaries [17]. It is demonstrated that the estrogen and progesterone exert their function on endometrium via modulating the Wnt/β-catenin signaling [18]. It is noteworthy that Wnt/β-catenin target genes have an essential role in cell migration, survival, adhesion, and invasion [19]; all are fundamental processes in the establishment of endometriosis [20]. Recent studies showed that the expression of some target genes of Wnt/β-catenin such as *Cyclin D1* [21], *SOX9* [22], some matrix metalloproteinases (*MMP-9* and *MMP-2*) [23], and *vascular endothelial cell growth factor* [24] are dysregulated in the endometrium of women with endometriosis. However, the role of most Wnt target genes and their contribution to the endometriosis onset and progression are not yet completely understood.

The expression level of vitamin D receptor (VDR) in the endometrium of women with endometriosis is similar to healthy endometrium while the higher expression of enzymes, involved in the production and deactivation of vitamin D, indicates its elevated metabolism in the endometrium of same patients [25]. A similar pattern of expression was reported in colon cancer [26]. A large prospective study showed that the women with higher predicted serum levels of 25(OH)D are less likely to suffer from endometriosis. Also, vitamin D intake from

foods was negatively associated with endometriosis [27]. In colon cancer cells, vitamin D inhibits the expression of *CD44* and some other target genes of β-catenin/TCF pathway, completely dependent on VDR expression [28-30]. Also, analogs of vitamin D down-regulate the expression of CD44 in breast cancer cells in a VDR-dependent manner [31].

This study proposed to investigate the role of the WNT-target gene, CD44, in the pathogenesis of endometriosis using bioinformatics and experimental approaches. The aim of the study was to assess the expression of CD44 and CD44v6 in the endometrium of women with endometriosis as well as the in vivo effects of vitamin D on the modulating of CD44 in mRNA and protein levels in the same patients.

2. Materials and Methods

2.1. The bioinformatics study

2.1.1 Prioritization of Wnt target genes contribution in endometriosis

The list of Wnt target genes was obtained from the Wnt homepage database (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). Servers such as Disease-Connect (http://disease-connect.org/), MalaCards (http://www.malacards.org/), and SNPs3D (http://www.snps3d.org/) were used to get the list of candidate genes for the disease. But, considering the importance of training gene set size, only the SNPs3D reported gene set was used in subsequent analysis. Overall, seven training gene sets were applied. After that, prioritizing servers including ToppGene (https://toppgene.cchmc.org/prioritization.jsp) that ranks and prioritizes candidate genes according to functional similarity to training gene set, ToppNet (https://toppgene.cchmc.org/network.jsp) which prioritizes and ranks candidate genes on the basis of topological features in the network of protein-protein interaction, and Endeavour

(https://endeavour.esat.kuleuven.be/) that works based on similarity of a candidate gene with a profile derived from genes already known to be involved in the disease, were used for finding the *CD44* rank among Wnt signaling target genes in relation to endometriosis. All the prioritization procedure designed and performed as described by Moreau and Tranchevent [32].

2.2. The experimental study

This study was carried out from December 2015 to November 2016 at Alzahra hospital, connected to Tabriz University of Medical Science, Iran. A case-control study was carried out to assess the expression of CD44 and CD44v6 in the endometrium of endometriosis patients compared to the healthy endometrium. Simultaneously, a clinical trial was conducted to evaluate the effects of vitamin D on the modulating of CD44 and CD44v6 in mRNA and protein levels in the same patients.

2.2.1. The case-control study

The initial inclusion criteria were: age between 22 to 37 years, having a normal body mass index (18.5<BMI<30) and regular menstrual cycles, and no usage of any supplements, hormones or intrauterine devices for at least 6 months prior to laparoscopy. The exclusion criteria were: a history of diabetes mellitus, chronic infections or immune system diseases.

The control group consisted of 14 healthy fertile women who underwent laparoscopy for reversal of tubal ligation with no findings of macroscopic pathology in the pelvic cavity. Also, a total of 46 infertile women of reproductive age who met our initial inclusion criteria and planned to undergo diagnostic laparoscopy, accepted to participate in the study.

The laparoscopy was set at the mid-secretory phase of the menstrual cycle in all women according to their last menstrual period and urinary LH surge monitoring (Donacheck ovulacion; Novalab Iberica, Spain). The mid-secretory phase (LH+6 to LH+10) was confirmed using the histological assessment according to the Noyes' criteria [33] in all included endometrial samples by an independent specialist gynecologist histopathologist. All samples collection, storage, and processing were done according to the recommendations of World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) [34, 35]. In the first step, prior to the induction of anesthesia for laparoscopy, 10 mL venous blood was collected into a sterile tube. Blood samples were centrifuged at 2,500 x g for 10 minutes at a temperature of 4° C; serum was separated and stored in aliquots at -80 °C until further assessment. For obtaining the endometrial fluid (EF), we used the uterine aspiration method described by Boomsma et al. [36]. In summary, after insertion of a speculum, we cleansed the cervix with a swab and then gently introduced Frydman embryo transfer catheter (1306045, CCD Laboratories, Paris, France) into the uterine cavity. The uterine secretion gradually aspirated by a syringe connected to the catheter. Before removing the catheter and to prevent suction of cervical mucus, it was clamped distal to external os of the cervical channel. The content of catheter was snap frozen in liquid nitrogen and stored at -80 °C. The aspirates were viscose and their volumes were varied between 5 to 10 µl.

The endometrial tissue samples were collected with Pipelle curette (Pipelle de Cornier Mark II, Paris, France). The obtained endometrial tissues were divided into three sections, the first part immersed in RNAlater (76104, Qiagen, Hilden, Germany) and stored at -80°C for RNA extraction, the second part was washed with sterile cold PBS (phosphate-buffered saline) and

stored in liquid nitrogen for western blot analysis, and the last part was fixed in formalin for endometrial dating.

In 39 out of 46 patients, endometriosis was diagnosed by laparoscopy and pathology. Endometriosis severity was staged according to the revised American Society for Reproductive Medicine classification system [37]; all visible endometriotic lesions were completely removed at the same laparoscopy. Only 34 endometriosis stage III-IV patients were included in the study as endometriosis group and the samples of the others were excluded from the study.

2.2.2. The clinical trial study

According to the routine protocol for treatment of infertility in women with endometriosis in our hospital, after operative laparoscopy, the women are given a chance to get pregnant spontaneously for 3 months. After this period, in the lack of pregnancy, the patients are candidates for treatment with in vitro fertilization, usually with GnRH antagonist gonadotropin stimulation protocol. Before antagonist stimulation, oral contraceptives (OCPs) are prescribed for 14 to 28 days for scheduling of menstrual cycle and coordinating follicular development [38].

After an endometriosis diagnosis, the patient was randomized into ESD group or ES group. The ESD group (n=17) took 50000 IU vitamin D (D-VIGEL 50000, Daana Pharma Co.) orally once a week for 12-14 weeks along with OCP in the last three weeks. Taking vitamin D and its possible side effects were followed-up by weekly phone calls and monthly visits. One woman of this group spontaneously conceived in the second cycle, so she was excluded from the study [n=16 (stage III, n=9; stage IV, n=7)]. The treatment of ES group [n=17 (stage III, n=9; stage IV, n=8)] arranged according to the routine protocol as described above without any intervention. After 12-

14 weeks, the second blood, EF, and endometrial tissue sampling were done in the mid-secretory phase of menstrual cycle which OCP was taken. OCP use was monitored with daily phone calls. All participants were compliant with OCP according to their self-reported diary records.

To randomized allocation of patients into ESD or ES groups, an independent hospital staff elicited uniform, opaque, traceless, intact, and sealed envelopes. The principal investigators (A.P. and A.S.) were blinded to group allocation for the duration of study as well as the data assessment period. Distribution of the participants in the trial study is shown in the CONSORT (Consolidated Standards of Reporting Trials) flow diagram Figure 1.

The study was approved by Tehran Medical University Research Ethical Committee (IR.TUMS.REC.1394.576). Participation in the study was voluntary and after being informed that they could leave the study at any stage, the participants gave their informed written consent.

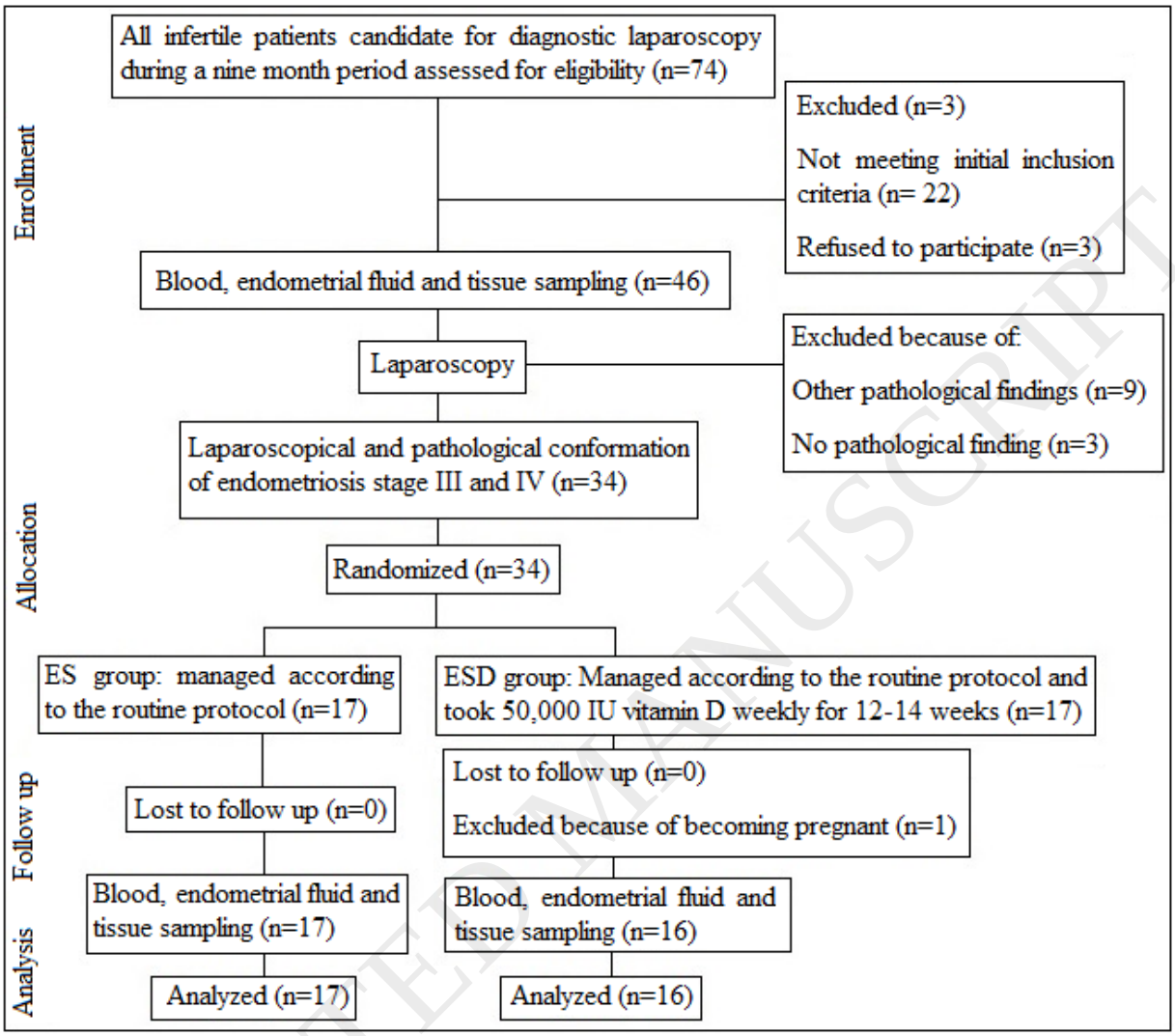

Figure 1. CONSORT flow chart of patients.

2.3. Real-time quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR method was used to quantify the transcripts of *CD44* and *CD44v6*. Thirty milligrams of the frozen endometrial tissues were individually homogenized and total RNA was extracted using the RNeasy Mini Kit (74104, Qiagen, Hilden, Germany). The RNA samples incubated with DNase I (RNase-free) (EN0521, Fermentas, Opelstrasse, Germany) to eliminate

the remaining genomic DNA. The concentration of the RNA was assayed by using Nanodrop 2000 spectrophotometer (Thermo Scientific, Copenhagen, Denmark). For confirmation of the integrity of mRNA, agarose gel (1%) electrophoresis and visualizing the proper ribosomal bands via an ultraviolet transilluminator were used. An equal amount of total RNA was reverse transcribed into cDNA by using RevertAid First Strand cDNA Synthesis Kit (K1622, Fermantas, Opelstrasse, Germany). We used Oligo (dT)-18 primer to broaden all mRNA. We utilized the PRIMERBLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) for designing the primers sequences. The used primer sequences were as follows: *CD44*: forward, 5′-TGGCGCAGATCGATTTGAATATAAC-3′, reverse, 5′-CACCACGTGCCCTTCTATGA-3′; *CD44v6*: forward, 5′-AGTCACAGACCTGCCCAATG-3′, reverse, 5′-ATCATTCCTATTGGTAGCAGGGA-3′ and *GAPDH*: forward, 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse, 5′- GAAGATGGTGATGGGATTTC-3′.

All transcripts were quantified by using Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) apparatus. The total reaction volume was 20µl containing 12 µl RealQ Plus 2x Master Mix Green - Ampliqon, 0.25 mM of each primer and diluted cDNA 1:100. Amplification was carried out at 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 40 seconds, and 72°C for 40 seconds. All samples were analyzed in triplicate. The PCR products were run on agarose gels (3%) and single bands with proper size were acquired for *CD44*, *CD44v6,* and for *GAPDH* as control housekeeping gene. Also, the melting curves analysis confirmed the specificity of PCR reactions. Livak method ($2^{-\Delta\Delta Ct}$) [39] was utilized to quantify the mRNA expression of CD44 and CD44v6 in each sample relative to calibrator sample and $2^{-\Delta\Delta Ct}$ reported as mRNA fold change.

2.4. Measurement of sCD44

Commercially available enzyme-linked immunosorbent assay (ELISA) kit (BMS209/2, Bender Med Systems, Vienna, Austria) was used to measure the concentrations of sCD44, based on the manufacturer's directions, in all serum and EF samples. According to the kit instruction 10 µl of serum and 10 µl of diluted EF (2 µl EF + 8 µl cold sterile PBS) were used for determination of sCD44 in the samples. The range of quantitation and the limit of detection of sCD44 were 0.07-8 ng/mL and 0.02 ng/mL, respectively. The inter- and intra-assay coefficients of variation were 4.1% and 4.8%, respectively.

2.5. Measurement of 25(OH)D

Serum concentrations of 25(OH)D was measured by using radioimmunoassay (RIA) method (DiaSorin, Saluggia, Italy; the lower limit and reference range for the assay were 5 ng/mL and 9-47 ng/mL, respectivily) with a coefficient of variation <5% and reported in ng/mL. Based on the manufacture instruction 50 µl of serum were used for measuring of 25(OH)D consentration in the samples.

2.6. Western blot

The protein content of endometrial tissues was extracted by using RIPA lysis buffer containing protease inhibitors (sc-24948, Santa Cruz Biotechnology, Heidelberg, Germany). The cell lysates were centrifuged at 13,000×g for15 minutes at 4°C. The protein concentration of supernatants was measured by using Bradford assay (23236, Pierce, IL, USA). Samples were electrophoresed on SDS–polyacrylamide gel (20 µg of protein per lane) and proteins were shifted to a nitrocellulose membrane (1620115, Bio-Rad, CA, USA). The membranes were blocked with 5%

non-fat dry milk in 0.1% TBS-T (Tris-buffered saline/ Tween-20). Then, the membranes were incubated with mouse monoclonal anti-CD44 antibody (sc-7297, 1:200; Santa Cruz Biotechnology, Heidelberg, Germany, #D0115) for 4h at room temperature which detects both standard (CD44s) and variant (CD44V) forms. The membranes were washed with TBS-T, incubated with HRP-labeled secondary antibodies against mouse (sc-2005, 1:5000; Santa Cruz Biotechnology, Heidelberg, Germany, #B1616) for 1h at room temperature and washed again with TBS-T. Immunodetection was achieved by using ECL detection kit (34080, Thermo Scientific, IL, USA) and chemiluminescence was captured using Western Blot Imaging System (Sabz Biomedical, Tehran, Iran). The membranes were stripped and again were blocked and used for detection of CD44v6 (by using mouse monoclonal anti-CD44v6 antibody, ab78960, 1:500; Abcam, MA, USA, # GR295277) and GAPDH as an internal control (by using rabbit polyclonal anti-GAPDH antibody, sc-25778, 1:200; Santa Cruz Biotechnology, Heidelberg, Germany, #I3015). The human colon adenocarcinoma HT29 and the mouse embryonic fibroblast NIH3T3 cell lysates were utilized as the positive and negative controls, respectively. Also, for validation of detection, the primary antibodies were omitted. The signal intensity of CD44s, CD44V, and CD44v6 relative to the density of GAPDH band was computed using a densitometric analysis software (ImageJ version 1.50i).

2.7. Statistical Analyses

The normality of distribution of data was checked by Kolmogorov test and it was negative in all variables except age, BMI, and serum concentration of 25(OH)D. Data were described as median and range in parentheses or mean and SD. Statistical analyses were performed by the non-

parametric Wilcoxon and Mann-Whitney tests or independent sample *t* test, as appropriate, using SPSS version 22 software. Significance was set at p value less than 0.05.

3. Results

3.1. Gene prioritization

The hypothesis of CD44 contribution in endometriosis was tested, at first, using gene prioritization bioinformatics approach. This approach infers disease genes through a set of training genes and scores and ranks test genes based on their average similarity to the training set using statistical methods. Because of the role of Wnt signaling in endometriosis, in this study, the Wnt signaling target genes were used as test genes set to find *CD44* priority among them in relation to endometriosis. Also, multiple training gene sets established searching experimental reports and using disease gene prediction servers. As listed in Supplementary Table 1, Kao et al. [40] identified 60 up- and 85 down-regulated genes in endometriosis. Also, Meola et al. [41] reported 191 and 102 respectively up- and down-regulated genes for endometriosis. Up and down-regulated genes in separate groups and also in a combined list were used as experimentally reported training gene set of endometriosis. The Wnt homepage database reports Wnt target genes in different species and its last update (September 2016) has listed 53 reported genes as Wnt target genes in human tissues and cell lines (Supplementary Table 1). Disease-Connect server identified 27 genes in relation with endometriosis and MalaCards reported 31 genes related to this disease. However, unlike the two previous servers, SNPs3D introduced 216 possible endometriosis-related genes. Due to the importance of the size of training genes to perform a correct prioritization, the SNPs3D reported list was used and following results

obtained. Because of different approaches applied by ToppGene, ToppNet, and Endeavour to compare training and test gene sets, and the importance of prioritizing candidate genes in relation to a disease, the order of candidate genes in the output of these servers is different (Supplementary Table 2). However, among prioritization results, *CD44* got high ranks and listed in top ten genes among Wnt target genes in relation to endometriosis (Table 1). Endeavour provides a diagram for priority p values of test genes in numerous databases; however, the server stated that this is not a real statistical p-value. But, it could be used as a comparative item to showing the place of a special gene among candidates list. The CD44 Endeavour p values among Wnt target genes depicted in Supplementary Figure 1. Overall, this part of the study revealed that among Wnt target genes in human, the CD44 is a promising candidate for studying and analyzing in endometriosis.

Table 1. *CD44* ranking in prioritization results of Wnt target genes vs. endometriosis-related genes.

Training gene set		*CD44* ranking among test genes		
		ToppNet	ToppGene	Endeavour
Kao et al., 2003	Up-regulated genes	9	7	4
	Down-regulated genes	5	1	2
	All genes with altered expression	4	1	2
Meola et al., 2010	Up-regulated genes	10	8	5
	Down-regulated genes	8	3	3
	All genes with altered expression	7	1	6
SNP3 predicted list		5	1	3

3.2. The results of case-control study

3.2.1. Participant's characteristics

All participants easily tolerated sampling process without any complication. There were no statistical differences in mean (±SD) age (31.28±1.68 vs. 30.15±2.38) and BMI (28.49±1.25 vs. 27.81±1.05) between control and endometriosis groups (Table 2).

Table 2. The baseline characteristics of participants

	Control (n=14)	Endometriosis (n=34)	P value
Age (years)	31.28±1.68	30.15±2.38	0.71
BMI (Kg/m^2)	28.49±1.25	27.81±1.05	0.86
Serum 25(OH)D (ng/mL)	14.42±2.02	13.21±1.81	0.066

BMI, body mass index; 25(OH)D, 25 hydroxyvitamin D
Data are expressed as mean ± SD

The mean serum concentration of 25(OH)D in endometriosis group (13.21±1.81 ng/mL) was lower than in control group (14.42±2.02 ng/mL), although the difference did not reach a significant level (p=0.066).

3.2.2. The expression of CD44, CD44V, and CD44v6 in the eutopic endometrium of women with endometriosis

The mRNA expression of *CD44* and *CD44v6* in all endometrial samples was assessed using real-time PCR technique. It was found that mRNA expression of both *CD44* ($p<0.001$) and *CD44v6* ($p<0.001$) in endometriosis group is significantly higher than their expression in controls (Figure 2A).

The expression levels of CD44s, CD44V and CD44v6 proteins in all endometrial samples were evaluated using western blot technique. Our results showed that the eutopic endometrium of healthy women in mid-secretory phase expresses CD44s, CD44V as well as CD44v6; but their related bands were significantly intensive in women with endometriosis (p=0.038 for CD44s, p=0.005 for CD44V, and $p<0.001$ for CD44v6) (Figures 2B and 2C).

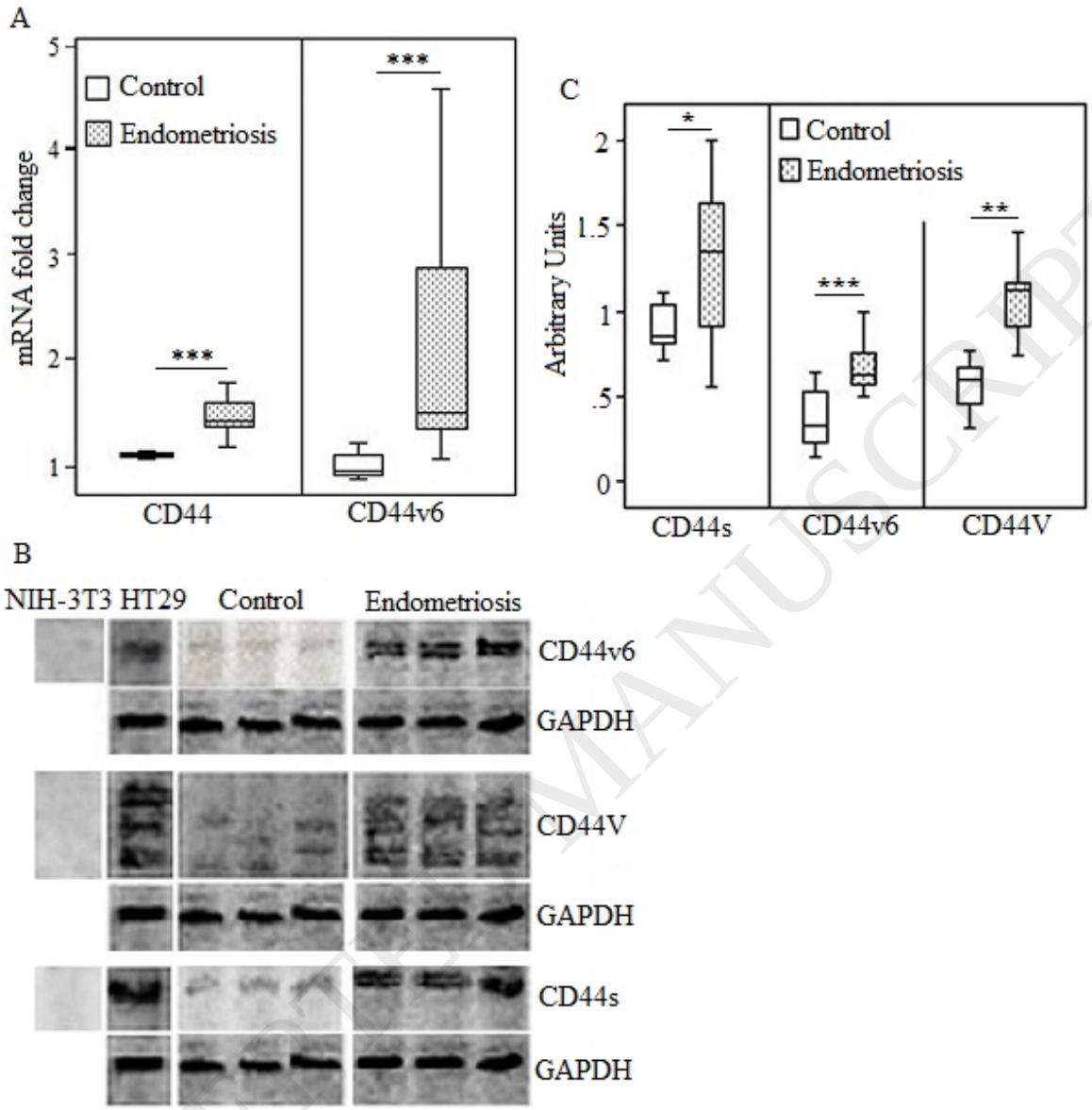

Figure 2. The expression of CD44, CD44V and CD44v6 in the endometrium of endometriosis and healthy control groups. Endometrial cells of endometriosis group (n=34) expressed a significantly higher level of both CD44 and *CD44v6* mRNAs as well as CD44, CD44V, and CD44v6 proteins compared to those of control group (n=14). *: P<.05; **: P<.01, and ***: P<.001.

3.2.3. The serum and endometrial fluid concentration of sCD44 in women with endometriosis

We used ELISA method to measure the concentration of sCD44 in serum and EF of all participants. The concentration of sCD44 in serum [542 (455-631) vs. 254.5 (197-313) ng/mL, $p<0.001$] and EF [522 (392-616) vs. 227.5 (183-308) ng/mL, $p< 0.001$] of endometriosis group was significantly greater than those of control group.

3.3. The results of clinical trial study

The endometriosis patients were randomly divided into two groups; one group [ES group, n=17 (stage III, n=9; stage IV, n=8)] was treated according to the hospital routine protocol and the other group [ESD group, n=16 (stage III, n=9; stage IV, n=7)], alongside the routine treatment, took 50000 IU vitamin D weekly for 12-14 weeks. Second blood, EF, and endometrial tissue samples were obtained from both ES and ESD groups at the end of intervention period. The participants reported no side effect of vitamin D. As expected, the circulating level of 25(OH)D in ESD group in the second sampling increased significantly after intervention (35.19±3.35 vs. 13.12±1.98 ng/mL, $p<0.001$) while it didn't change significantly in ES group (13.26±1.49 vs. 13.29±1.69 ng/mL, $p=0.827$).

3.3.1. The modulating effects of vitamin D on expression of CD44, CD44V, and CD44v6

There was no significant difference between ESD and ES groups in the expression of *CD44* and *CD44v6* mRNAs as well as CD44s, CD44V and CD44v6 proteins before the intervention. The significantly regulatory effects of vitamin D on mRNA expression of *CD44* and *CD44v6* were shown in Figure 3A. In ESD group, the mRNA expression of *CD44* ($p<0.001$) and *CD44v6*

(P=0.017) were significantly decreased when compared to ES group after the intervention period. Also, at the end of the intervention period, compared with before intervention, the expression of CD44 ($p<0.001$) and CD44v6 ($p<0.001$) mRNAs were significantly decreased within ESD group.

After the intervention, the expression levels of CD44s, CD44V and CD44v6 proteins were significantly decreased in ESD group when compared with before intervention ($p<0.001$ for CD44s, $p=0.002$ for CD44v6, and $p=0.015$ for CD44V) as well as with ES group ($p=0.001$ for CD44s, $p=0.031$ for CD44v6, and $p=0.005$ for CD44V) (Figure 3C).

3.3.2. The effects of vitamin D on serum and endometrial fluid concentration of sCD44

At the end of intervention period, compared to before intervention, the serum levels of sCD44 lowered significantly within both ES [493 (455-512) vs. 548 (455-617) ng/mL, $p=0.007$] and ESD [488 (419-528) vs. 536 (475-631) ng/mL, $p=0.002$] groups while the differences between two groups were not significant [493 (455-512) vs. 488 (419-528) ng/mL]. After the intervention period, the EF levels of sCD44 in ESD group were significantly lower than in ES group [297 (209-379) vs. 517 (388-599) ng/mL]. Also, its level was statistically changed within ESD group after the intervention ($p<0.001$), indicating that vitamin D down-regulated the shedding of CD44 into the endometrium cavity (Table 3). The concentration of sCD44 was not significantly changed within ES group at the end of the intervention period ($p=0.309$).

Table 3. Serum and EF concentration of sCD44 in ES and ESD groups

Concentration of sCD44 (ng/mL)	ES group (n=17)	ESD group (n=16)	P value
Serum, before intervention	548 (455-617)	536 (475-631)	0.845
Serum, after intervention	493 (455-512)	488 (419-528)	0.511
EF, before intervention	543 (442-616)	510 (392-591)	0.094
EF, after intervention	517 (388-599)	297 (209-379)	0.000

EF, endometrial fluid; sCD44, soluble CD44; ES group, endometriosis patients managed with the routine protocol; ESD group, endometriosis patients managed with the routine protocol and took 50000 IU vitamin D weekly for 12-14 weeks.
Data are expressed as median (range)

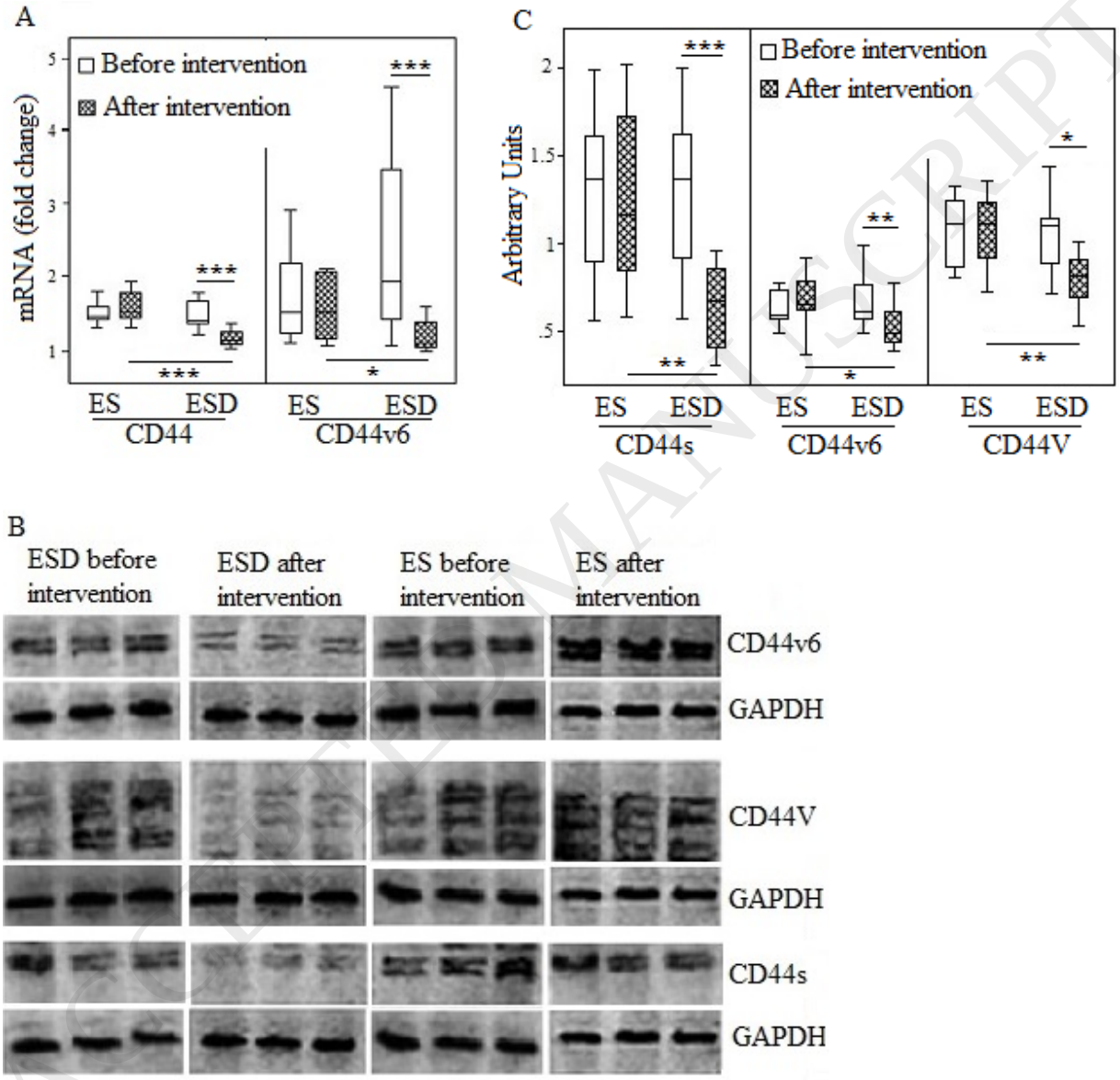

Figure 3. The expression of CD44, CD44V and CD44v6 in the endometrium of ES and ESD groups. At the end of intervention period, the expression level of *CD44* and *CD44v6* mRNAs as well as the expression level of CD44s, CD44V and CD44v6 proteins were significantly

decreased in ESD group in comparison with ES group. ES group: endometriosis patients (n=17) managed with the routine protocol and ESD group: endometriosis patients (n=16) managed with the routine protocol and took 50000 IU vitamin D weekly for 12-14 weeks. *: P<.05; **: P<.01, and ***: P<.001.

4. Discussion

As it was predicted in our gene prioritization bioinformatics analysis, the experimental findings of this study revealed that CD44 regulation is in significant relation with endometriosis. Our study showed an up-regulated turnover of CD44 in eutopic endometrium of women with endometriosis. This finding supports the hypothesis that the endometrial cells of these patients have more capacity to invasion. In healthy endometrium, both epithelial and stromal cells express CD44 depending on the phase of the menstrual cycle with the maximum expression in late secretory phase [42]. Delbandi et al. showed that endometrial stromal cells from women with endometriosis have higher attachment capacity to ECM [43]. In a worthy study, Griffith et al. demonstrated that the menstrual endometrial cells from women with endometriosis were more likely to firmly adhere to peritoneal mesothelial cells, which the authors tried to explain it with the higher expression of some variants of CD44 including v6, v7, v8, and v9 in these cells [4]. Burkle et al. demonstrated the over-expression of CD44v6 in endometriosis with lymphatic spreading, suggesting its role in the establishment of new lesions within pelvic lymph nodes [44]. In the present study, we showed that alongside the higher mRNA and protein expression of CD44s, its ectodomain cleavage is an active process in the endometrium of women with endometriosis. The concentration of sCD44 in ovarian endometriotic cysts is higher than its

concentration in other ovarian cysts [45]. Also, the levels of sCD44 in peritoneal fluid and serum of women with endometriosis are higher than in peritoneal fluid and serum of healthy women [46]. Our findings confirmed the higher concentration of sCD44 in the serum of women with endometriosis than in controls. Shedding of CD44 in the peritoneal cavity [46] and in the endometrial fluid of women with endometriosis are significantly more than in those of control subjects. Although the source of sCD44 in peritoneal fluid is unknown, it may be released from the surface of newly entered endometrial cells to peritoneal cavity via retrograde menstruation, expressing and shedding a great level of CD44.

The extensive cleavage of CD44 can be explained by over-expression of MT1-MMP [47] and MMP-9 [48] in the eutopic endometrium of women with endometriosis. It was demonstrated that MT1-MMP [14] and MMP-9 cleave the ectodomain of membrane-bound CD44 [49]. Extensive shedding of CD44 from the cell membrane accompanied by the elevated protein synthesis of CD44 may show, at least in part, the invasive properties of eutopic endometrial cells of women with endometriosis. This phenomenon was previously described in aggressive melanoma cell lines, too [16]. It is notable that the circulating level of sCD44 is increased in some human tumors [50] and is associated with the potency of some cancers to metastasis [15].

Endometriosis mimics some properties of malignant diseases such as high recurrence rate, elevated local angiogenesis, invasion and decreased apoptosis [51]. Also, it can be considered as an inflammatory disease or immune-related disorder because of local rising in some pro-inflammatory cytokines [52] and reduced cytotoxicity of natural killer cells [53].

The relationship between vitamin D deficiency and some cancers such as breast [54] and colorectal cancer [55] as well as the immunomodulatory and anti-inflammatory effects of

vitamin D is well documented. 1α-hydroxylase, 24-hydroxylase, and VDR are highly expressed in the endometrium of women with endometriosis [25] indicating a dysregulation in function of vitamin D in the endometrium of these women.

Recently, in a prospective study, the inverse relationship between the predicted serum levels of 25(OH)D and endometriosis was reported by Harris et al [27]. In our study all women with endometriosis (n=34) were vitamin D deficient, although the mean concentration of 25(OH)D in endometriosis group (13.21±1.81 ng/mL) was lower than in the control group (14.42±2.02 ng/mL) but their difference didn't reach the significant level (p=0.066) which can be explained by the small size of our groups. Based on Endocrine Society Clinical Practice Guideline, we defined vitamin D deficiency as a serum concentration of 25(OH)D below 20 ng/mL and vitamin D sufficiency as a 25(OH)D level of 30-50 ng/mL [56].

The present study showed that the correction of vitamin D status from the deficiency to the sufficiency level can change the turnover of CD44 toward its normal levels in the endometrium of women with endometriosis. The significant reduction occurred in protein expression of CD44s, CD44V, and CD44v6 in the endometrium of endometriosis patients following 3 months of weekly intake of 50000 IU vitamin D.

It was previously demonstrated that vitamin D and its analogs decrease the expression of CD44s and CD44V in breast cancer stem cells in a VDR dependent manner and repress the growth of xenograft mammary tumors [31]. Also, in the other study, treating the colorectal adenoma cells with vitamin D suppressed the expression of CD44 [57]. Elocalcitol, a VDR agonist, diminished the attachment capacity of endometrial cells to collagen and reduced the levels of some inflammatory cytokines [58]. In an in vitro study, vitamin D decreased the invasive properties of endometrial cells derived from the endometrium of women with endometriosis [59]. In an

animal model of endometriosis, the size of endometriotic lesions decreased by about 50% following three weeks of daily intake of vitamin D [60]. Although the exact mechanism of vitamin D in modulating of some cell properties is unknown, it can be probably related to its regulatory effects on Wnt/β-catenin pathway [28, 61]. Over-expression of the total and active forms of β-catenin was shown in the mid-secretory phase of the eutopic endometrium of women with endometriosis [62] indicating a nuclear accumulation of β-catenin leading to the excessive activation of this signaling pathway [63]. Over-activated Wnt/β-catenin pathway up-regulates several target genes such as *CD44*, *cyclin D1*, c-*MYC* and others, all involved in cell proliferation, invasion, survival, and migration [64].

While the regulatory mechanism of vitamin D on the expression of CD44 in endometriosis needs more investigations but different mechanisms in other pathologic processes, depending on cell and microenvironment type, have been suggested in the literature. It was demonstrated that the complex of VDR-vitamin D can directly attach to the promoter region of *CD44* and inhibits its transcription [31]. Vitamin D stimulates the relocation of β-catenin from the nucleus to the cytoplasm, also it enhances the binding of VDR to β-catenin which inhibits the transcriptional activity of β-catenin [28] suppressing the expression of Wnt/β-catenin target genes such as *CD44* [28]. As the other possible mechanism of vitamin D in decreasing the expression of *CD44*, vitamin D induces the expression of DKK-1, an inhibitor of Wnt/β-catenin pathway, dependently to the presence of VDR [61]. Finally, IL-1β, secreted by macrophages, prevents GSK3β activity in phosphorylation and deactivation of β-catenin so that enhances the transcriptional activity of β-catenin. Vitamin D disrupts this pathway by inhibiting production of IL-1β [65]. It is worth noticing that the local production of IL-1β increases in endometriosis [66].

Interestingly, the serum levels of sCD44 reduced following the surgical resection of endometriotic lesions and vitamin D had no more effect on its serum concentration. Vitamin D significantly reduced the levels of sCD44 in the EF. Recently, it was demonstrated that vitamin D suppresses the expression of MMP-9 in the eutopic endometrium of women with endometriosis [67] as well as MT1-MMP in human uterine fibroid cells [68], both involved in the cleavage and shedding of CD44. However, it needs more investigations to clarify whether the reduction in sCD44 in EF is only a reflection of its effects on reducing CD44 expression or it is secondary to effects of vitamin D on CD44 sheddases or both.

The use of standard sampling, processing, and storage methods were the strengths of this study. These standard methods optimize the sample quality and reduce the variability [35]. Also, endometriosis (in endometriosis group) or absence of any pelvic diseases (in control group) in the recruited women was definitely diagnosed by laparoscopy and histo-pathologic examinations, immediately before sampling, which enhances the validity of our findings.

In this study, all of the endometriosis patients were stage III-IV and infertile, which may limit the generalizability of the findings to all endometriosis patients.

5. Conclusion

We demonstrated that the expression of CD44s, CD44V, and CD44v6 in the eutopic endometrium of women with endometriosis in mid-secretory phase is higher than in the endometrium of healthy women. Also, the serum and EF concentration of sCD44 was higher in endometriosis patients. Vitamin D supplementation improved the serum concentration of 25(OH)D, as well as the expression of CD44s, CD44V, and CD44v6 in the endometrium of patients. Additionally, we showed that the surgical removing of endometriotic lesions decreased

the serum levels of sCD44. Also, vitamin D decreased the EF concentration of sCD44 in endometriosis patients. More investigations will reveal if vitamin D can exert positive effects on treatment of endometriosis patients.

Conflict of interest: The authors have no conflict of interest to declare.

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