Glycosaminoglycan Adenogenesis Factors: Immunohistochemical Expression in Endometriosis Tissues Compared with the Endometrium

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ABSTRACT: Endometriosis is a chronic inflammatory pathology estrogen-dependent. It is a condition affecting 5%–10% of women of reproductive age worldwide. Recent evidence indicating an embryological origin of endometriosis has provided new insights into its pathogenesis and potential therapeutic approaches. In this study, we compared the immunohistochemical expression of extracellular matrix molecules involved in the interaction between epithelium and stroma in endometriotic lesions and normal endometrial tissue. A total of 41 cases were analyzed. We examined the immunohistochemical expression of chondroitin sulfate proteoglycan 4 (CSPG4), keratan sulfate, chondroitin sulfate (CS-56), hyaluronic acid, and heparan sulfate (HEP). Our results showed higher expression of CSPG4 and CS-56 in epithelial endometriosis samples compared with normal endometrial tissue, while HEP, keratan sulfate, and hyaluronic acid showed decreased expression in epithelial endometriosis samples relative to normal endometrial tissue. Additionally, endometriotic stroma exhibited more frequent low intensity of hyaluronic acid and HEP compared with normal endometrial stroma. Investigating the levels of these molecules in eutopic and ectopic endometrial tissues enables the identification of potential therapeutic targets, and the development of novel treatments aimed at disrupting the adhesive and invasive properties of endometriotic lesions.

KEY WORDS: endometriosis, immunohistochemistry, extracellular matrix, chondroitin sulfate proteoglycan 4, keratan sulfate, chondroitin sulfate, hyaluronic acid, heparan sulfate, glycosaminoglycans, infertility

I. INTRODUCTION

The various embryological and postnatal development stages of the uterus in mammals are well recognized.[1] In detail, organogenesis of the uterus together with cervix, oviducts and anterior vagina is a sophisticated and strictly regulated spatio-temporal mechanism of differentiation of the Mülleran ducts.[2] Indeed, a complicated genetic network still not completely described, is responsible for the correct progression of the organogenesis.[3] As a logical consequence, defects in this so complicated mechanism of organogenesis, the professed anomalies of the Müllerian ducts, are quite frequent,[4] with an estimated prevalence ranging from 0.1 to 7% of the female population.[5] This incidence increases radically in patients with fertility and miscarriage problems.[6] Interestingly, these percentages of incidence are very similar to that documented for endometriosis.[7,8] Peculiar characteristic of this pathological condition is the presence of endometrial glandular and stromal structures exterior to the uterine cavity.[9] When endometriotic lesions are localized below the peritoneum, the term deep endometriosis is used.[10] To date, this is a very frequent estrogen-dependent inflammatory disease pathology, that causes various symptoms such as chronic pelvic pain, dysmenorrhea, dyspareunia and it is recognized as the main cause of infertility.[11,12] Concerning the anatomical location of endometriosis lesions, the highest prevalence is found in the deep peritoneum and in the pouch of Douglas, while the superficial peritoneum exhibited a very low prevalence.[11,12]

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Despite its high incidence and significant clinical impact, the pathogenesis of endometriosis is still not fully defined, the diagnosis is habitually late and there is no efficacious therapy for its eradication.[13] Among the various pathogenetic theories, we can cite the one of the retrograde menstruations suggested by Sampson about a century ago, the theory of the metaplasia of coelomic tissue or that of activation of stem cells.[14] Among the others, the embryogenetic theory, propose endometriosis because of defects in the mechanisms of organogenesis of the uterus, has recently gained interest, thanks to the demonstration of the presence of ectopic endometrium in fetal age in the identical anatomical compartments where endometriosis is normally described in adult patients.[15–17] Consequently, it has been proposed to include endometriosis in the multifaceted network of anomalies of the Müllerian ducts.[18] Among all the molecular mechanisms responsible for the fine regulation of uterus organogenesis, the ones involved in epithelial-mesenchymal interactions are indeed fundamental for a correct uterine development.[1] Indeed, these factors control several phenomena, such as cell movement and adhesion, cell differentiation and cell proliferation, that are responsible for local control and coordination of morphogenetic vital cell actions in uterine morphogenesis.[1] In this article we have compared, by means of immunohistochemistry, the expression in deep endometriotic lesions and in normal endometrial tissue, of some glycosaminoglycans (GAGs), members of the family of extracellular matrix, known to be involved in the interplay between the epithelium and stroma, that is fundamental for the correct uterine gland morphogenesis. The correlation data produced can provide clues on some of the molecular mechanisms in control of the adenogenesis and survival of endometriosis structures outside of the uterus.

II. MATERIALS AND METHODS

A. Patients and Tissue Samples

All cases were collected from patients who underwent surgery for infertility, pelvic pain symptoms or adnexal masses between 2000 and 2010 at the "Centro Italiano Endometriosi." Cases were selected based on the presence at histological examination of endometriotic glands. A total of 41 cases were entitled for the analysis, as summarized in Table 1. The median age of the women was 34 years old. All patients provided written informed consent before enrollment in the study to permit the use of the data generated in retrospective analyses. The Human Normal Endometrium Tissue MicroArray CYN1 (SUPER BIO CHIPS, Seoul, Korea) including 59 normal endometrial samples both in the proliferative and secretory phase, was used to analyze the expression of each protein selected for the study in the normal endometrium. Main patients' characteristics are summarized in Table 1. The study was approved by the Scientific Committee of Fondazione Italiana Endometriosi. The experimentation was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000 and 2008 and in agreement with Italian law (Italian DLgs No. 196/03, Codex on Privacy, as modified by the UE 2016/679 law of the European Parliament and Commission), that requires at the time of surgery, for studies based only on retrospective analyses on routine archival

TABLE 1: Characteristics of the 41 patients included in the study

	N	%
Age (years) AFS stage[a]	41	
I	0	0
II	1	3
III	3	7
IV	37	90

[a]American Fertility Society stage, based on the American Fertility Society, 1985. Mean age = 35 ± 5 years.

FFPE-tissue, a written informed consent from the living patients.

B. Immunohistochemistry

Immunohistochemistry was performed as previously described.[19,20] Slides were incubated at room temperature at 1:100 dilutions with the following primary antibodies: chondroitin sulfate proteoglycan antibody (CSPG4) from Abnova (Catalog Number PAB30332), keratan sulfate antibody from Biorbyt (catalog #orb157743), chondroitin sulfate antibody (CS-56) form Abcam (catalog #ab 11570), hyaluronic acid antibody from GeneTex (catalog #GTX17370), heparane sulfate antibody from ThermoFisher (catalog #MA5-46186). After three washes in PBS to eliminate the surplus of antiserum, the slides were incubated with UltraTek HRP secondary antibody (ScyTek Laboratories, Logan, UT) for 1 hour. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (ScyTek Laboratories, Logan, UT) was used as the final chromogen and hematoxylin was used as the nuclear counterstain. Negative controls for each tissue section were prepared by leaving out the primary antiserum. The intensity of the different antigen expression was described as: score 1 (absent to very low); score 2 (low); score 3 (moderate), score 4 (intense). An average of 22 fields was observed for each specimen. The level of concordance, expressed as the percentage of agreement between the observers (A.B. and M.M.), was 92%. In the remaining specimens, the score was obtained after collegial revision and agreement.

C. Statistical Analyses

Continuous variables were reported as either the means and standard deviation or median and interquartile ranges (IQRs) according to their distribution, as assessed by the Shapiro-Wilk normality test. Categorical variables were reported as percentages. Differences in clinical characteristics between eutopic (i.e., control group) and ectopic endometrium (i.e., endometriosis group) patients' groups were tested by *t*-test or Wilcoxon test (according to their distribution) and Pearson's chi-squared test or Fisher's exact test for continuous and categorical variables, respectively. To measure the linear association between continuous variables Pearson correlation coefficient was used if variables had a normally distribution, otherwise Spearman's correlation coefficient was calculated. To assess stromal and epithelial expression degrees, a proportional odd linear regression (POLR) model with three-levels (i.e., none, low, medium, and high) endpoint was used. The POLR model is a class of generalized linear models used for modeling the dependence of an ordinal response on discrete or continuous covariates. In particular, ten different univariate POLR models were performed using as response variables CSPG4, CS-56, HEP, keratan, and hyaluronic acid for epithelium and stroma, respectively. As covariate eutopic and ectopic endometrium groups-variable was used. Moreover, these models were adjusted by patients' age. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to measure the effects of endometriosis group on high molecular expression in the epithelium and stromal tissues. Statistical tests with $P < 0.05$ were considered statistically significant. All the statistical analyses were performed with the R Studio Statistical software, version 4.1.3.

III. RESULTS

Stromal and epithelial endometriosis samples (i.e., Endometriosis group) and stromal and epithelial normal endometrium samples (i.e., Control groups) were examined indifferently in the proliferative and secretory phases. All the cases of endometriosis were located under the peritoneum, mostly in the recto-vaginal septum, therefore belonging to deep endometriosis. The glands exhibited an endometrioid aspect, while the endometriotic stroma was comparable to eutopic endometrial stroma. Moreover, frequently there was an infiltrate made up of inflammatory cells, lipofuscin and hemosiderin pigment, conceivably produced by hemorrhage and menstrual changes in endometriosis lesions. Lastly, the occurrence of a network of small arterioles, nervous fibers and nodular holes of smooth muscle was usually detected in the stroma.

We analyzed by immunohistochemistry the expression of chondroitin sulfate proteoglycan (CSPG4), chondroitin sulfate (CS-56), heparane sulfate (HEP), keratan sulfate and hyaluronic acid.

We found that CSPG4 in the epithelium was expressed with low intensity in 4/41 cases (9.8%), medium intensity in 21/41 cases (51.2%) and high intensity in 16/41 cases (39%), no one had absent expression; in the normal endometrial samples the expression intensity was absent in 2/59 cases (3.4%), low in 26/59 cases (44.1%), medium in 17/59 cases (28.8%) and high in 14/59 cases (23.7%). For what concern the expression of CSPG4 in the stroma of the endometriosis patients, our result was an absent or very low intensity in 17/41 cases (41.5%), low intensity in 22/41 cases (53.7%), medium intensity in 2/41 cases (4.9%), no one had high intensity expression; in the normal endometrial samples the expression intensity in the stroma was absent in 22/59 cases (37.3%), low in 29/59 cases (49.2%), medium in 8/59 cases (13.6%) and no one had high intensity expression. Therefore, as shown in Table 2 and Figs. 1 to 5, we can say that the expression of CSPG4 in the epithelial endometriosis samples

TABLE 2: Clinical characteristics of patients by diagnosis groups

	Group		
Characteristic	**Control (*n* = 59)**[a]	**Endometriosis (*n* = 41)**[a]	***P* value**[b]
Age	39.00 (6.50)	33.00 (3.00)	< 0.01
CSPG4 epithelium			< 0.01
None	2.0 (3.4%)	0.0 (0.0%)	
Low	26.0 (44.1%)	4.0 (9.8%)	
Medium	17.0 (28.8%)	21.0 (51.2%)	
High	14.0 (23.7%)	16.0 (39.0%)	
CSPG4 stroma			0.37
None	22.0 (37.3%)	17.0 (41.5%)	
Low	29.0 (49.2%)	22.0 (53.7%)	
Medium	8.0 (13.6%)	2.0 (4.9%)	
High	0.0 (0.0%)	0.0 (0.0%)	
CS-56 epithelium			< 0.01
None	48.0 (81.4%)	14.0 (34.1%)	
Low	11.0 (18.6%)	21.0 (51.2%)	
Medium	0.0 (0.0%)	6.0 (14.6%)	
High	0.0 (0.0%)	0.0 (0.0%)	
CS-56 stroma			0.07
None	0.0 (0.0%)	0.0 (0.0%)	
Low	0.0 (0.0%)	0.0 (0.0%)	
Medium	4.0 (6.8%)	8.0 (19.5%)	
High	55.0 (93.2%)	33.0 (80.5%)	
HEP epithelium			< 0.01
None	0.0 (0.0%)	0.0 (0.0%)	
Low	9.0 (15.3%)	0.0 (0.0%)	
Medium	38.0 (64.4%)	7.0 (17.1%)	
High	12.0 (20.3%)	34.0 (82.9%)	

TABLE 2: (*continued*)

HEP stroma			0.15
None	0.0 (0.0%)	0.0 (0.0%)	
Low	2.0 (3.4%)	6.0 (14.6%)	
Medium	30.0 (50.8%)	18.0 (43.9%)	
High	27.0 (45.8%)	17.0 (41.5%)	
Keratan epithelium			< 0.01
None	18.0 (30.5%)	31.0 (75.6%)	
Low	31.0 (52.5%)	10.0 (24.4%)	
Medium	10.0 (16.9%)	0.0 (0.0%)	
High	0.0 (0.0%)	0.0 (0.0%)	
Keratan stroma			< 0.01
None	0.0 (0.0%)	0.0 (0.0%)	
Low	11.0 (18.6%)	38.0 (92.7%)	
Medium	25.0 (42.4%)	3.0 (7.3%)	
High	23.0 (39.0%)	0.0 (0.0%)	
Hyaluronic acid epithelium			< 0.01
None	1.0 (1.7%)	0.0 (0.0%)	
Low	10.0 (16.9%)	15.0 (36.6%)	
Medium	26.0 (44.1%)	26.0 (63.4%)	
High	22.0 (37.3%)	0.0 (0.0%)	
Hyaluronic acid stroma			< 0.01
None	2.0 (3.4%)	15.0 (36.6%)	
Low	21.0 (35.6%)	22.0 (53.7%)	
Medium	32.0 (54.2%)	4.0 (9.8%)	
High	4.0 (6.8%)	0.0 (0.0%)	

[a]Median (IQR) or frequency (%). [b]Wilcoxon rank sum test or Fisher's exact test.

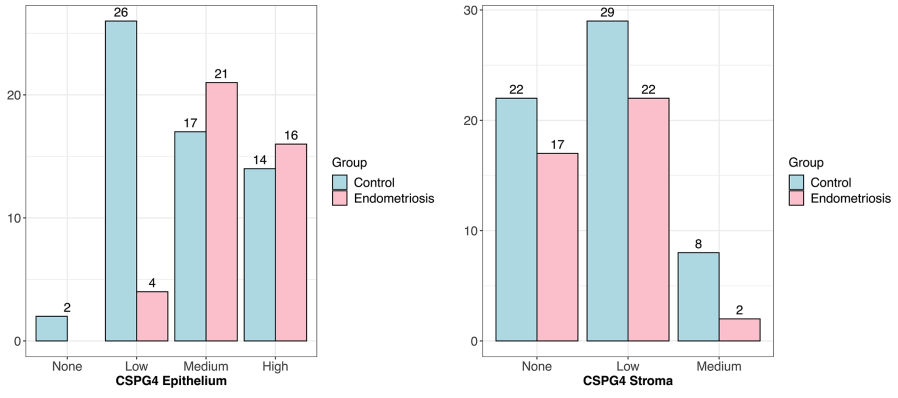

FIG. 1: Degree of expression of CSPG4 in epithelium (left) and stroma (right) composition for endometriosis affected and unaffected groups

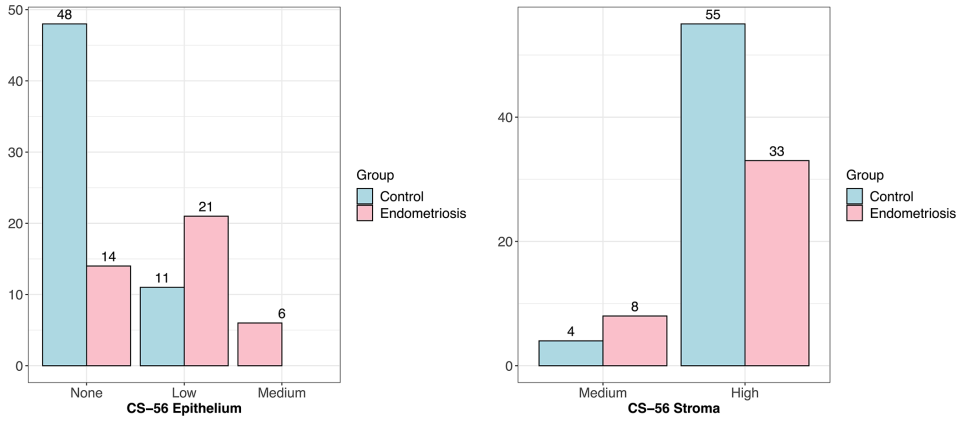

FIG. 2: Degree of expression of CS-56 in epithelium (left) and stroma (right) composition for endometriosis affected and unaffected groups

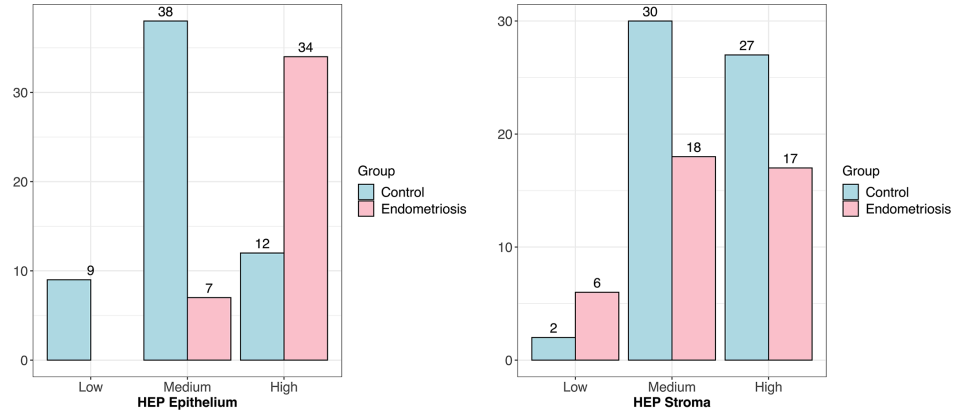

FIG. 3: Degree of expression of HEP in epithelium (left) and stroma (right) composition for endometriosis affected and unaffected groups

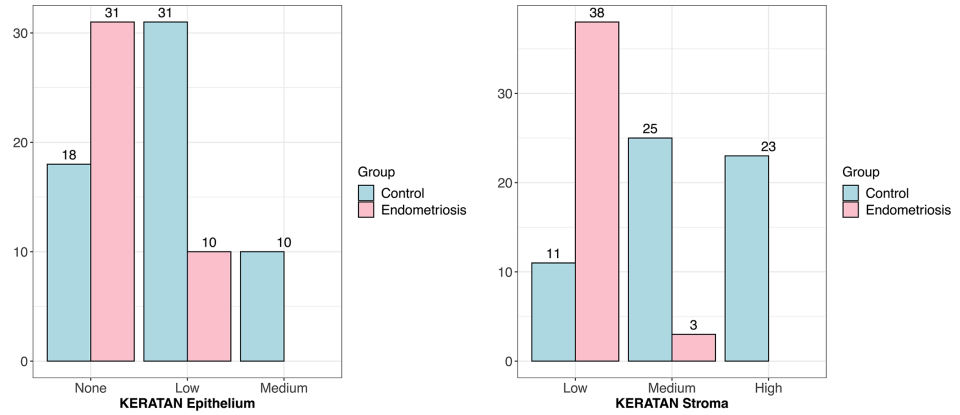

FIG. 4: Degree of expression of keratan in epithelium (left) and stroma (right) composition for endometriosis affected and unaffected groups

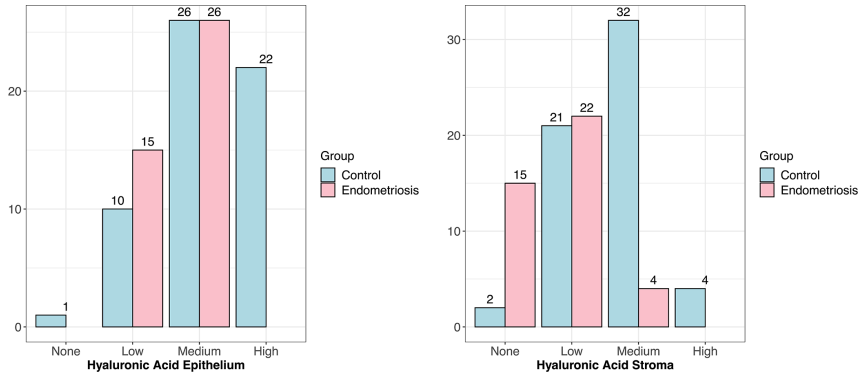

FIG. 5: Degree of expression of hyaluronic acid epithelium in epithelium (left) and stroma (right-plot) composition for endometriosis affected and unaffected groups

has an increased intensity compared with the normal endometrial expression ($P < 0.01$); then in the endometriosis stroma and in the normal endometrial stroma the expression no difference was detected ($P = 0.37$).

The results of the evaluation of CS-56 expression showed in the endometriotic epithelium an absent or low intensity in 14/41 cases (34.1%), low intensity in 21/41 cases (51.2%), medium intensity in 6/41 cases (14.6%) and no one had high expression; in the normal endometrial samples the expression intensity was absent in 48/59 cases (81.4%), low in 11/59 cases (18.6%) and no one showed medium or high expression. For what concern the expression of CS-56 in the stroma of the endometriosis patients, our result was a medium intensity in 8/41 cases (19.5%), high intensity in 33/41 cases (80.5%) and no one with absent or low intensity expression; in the normal endometrial samples the expression intensity in the stroma was medium in 4/59 cases (6.8%), high in 55/59 cases (93.2%) and no one had absent or low intensity expression.

Consequently, we can say that the expression of CS-56 in the epithelial endometriosis samples has an increased intensity compared with the normal endometrial expression ($P < 0.01$); then in the endometriosis stroma and in the normal endometrial stroma the expression no difference was detected ($P = 0.07$). The immunohistochemical expression of HEP showed in the epithelium a medium intensity in 7/41 cases (17.1%), high intensity in 34/41 cases (82.9%) and no one had absent or low expression; in the normal endometrial samples the expression intensity was low in 9/59 cases (15.3%), medium in 38/59 cases (64.4%), high intensity in 12/59 cases (20.3%) and no one showed absent expression. The expression of HEP in the stroma of the endometriosis patients resulted with low intensity in 6/41 cases (14.6%), medium intensity in 18/41 cases (43.9%), high intensity in 17/41 cases (41.5%) and no one with absent or very low intensity expression; in the normal endometrial stroma the expression intensity was medium in 2/59 cases (3.4%), medium in 30/59 (50.8%), high in 27/59 cases (45.8%) and no one had absent or low intensity expression. So, we can say that the endometrial expression of HEP in the epithelial endometriosis samples has a decreased intensity compared with the normal endometrial expression ($P < 0.01$); then in the endometriosis stroma and in the normal endometrial stroma the expression did not show a significant difference ($P = 0.15$). We found that the keratan sulfate in the epithelium was expressed with absent or very low intensity in 31/41 cases (75.6%), low intensity in 10/41 cases (24.4%), and no one had medium or high expression; in the normal endometrial samples the expression intensity was absent ore very low in 18/59 cases (30.5%), low in 31/59 cases (52.5%), medium intensity in 10/59 cases (16.9%) and no one had high intensity expression. Regarding the expression of keratan sulfate in the stroma of the endometriosis patients, our result was a low intensity in 38/41 cases (92.7%), medium intensity in 3/41 cases (7.3%), and no one had absent or high intensity expression; in the normal

endometrial samples the expression intensity in the stroma was low in 11/59 cases (18.6%), medium intensity in 25/59 cases (42.4%), high in 23/59 cases (39%) and no one had absent intensity expression. What we can learn from these results is that keratan sulfate in the endometriotic epithelium is increased the absence of expression as compared with the normal epithelium ($P < 0.01$) and in the endometriotic stroma is more present a low intensity rather than the medium/high of the normal endometrial stroma ($P < 0.01$).

The analysis on hyaluronic acid expression showed in the epithelium of our endometriosis cases displayed a low intensity in 15/41 cases (36.6%), and medium intensity in 26/41 cases (63.4%), while no one had absent or high expression; in the normal endometrial samples the expression intensity was absent in 1/59 cases (1.7%), low in 10/59 cases (16.9%), medium intensity in 26/59 cases (44.1%), high intensity in 22/59 cases (37.3%). The expression of hyaluronic acid in the stroma of the endometriosis patients, our results show absent or low intensity in 15/41 cases (36.6%), low intensity in 22/41 cases (53.7%), medium intensity in 4/41 cases (9.8%), and no one with high intensity expression; in the normal endometrial samples the expression intensity in the stroma was absent in 2/59 cases (3.4%), low intensity in 21/59 cases (35.6%), medium in 32/59 cases (54.2%), high intensity expression in 4/59 cases (6.8%). Thus, we can say that the expression of hyaluronic acid in the epithelial endometriosis samples compared with the normal endometrial is less intense ($P < 0.01$); then in the endometriosis stroma the expression intensity is lower than the normal endometrial stroma ($P < 0.01$).

The results obtained by POLR models agreed with the results shown above. In particular, Table 3 shows that for the variable "CSPG4 epithelium," the OR was 3.467 (95% CI: 1.511–8.210), with $P = 0.004$. This suggests that the probability of belonging to the endometriosis group significantly increased when high expression of CSPG4 was present in the epithelium compared with the control group; for the variable "CSPG4 stroma," the OR was 0.815 (95% CI: 0.347–1.905), with a P of 0.636. This indicates that the presence of CSPG4 in the stromal tissue did not have a significant impact on the probability of belonging to the endometriosis group or the control group; for the variable "CS-56 epithelium," the OR was 7.918 (95% CI: 3.080–21.746), with a p-value less than 0.01. This suggests that the probability of belonging to endometriosis group significantly increased when high expression of CS-56 was present in the epithelium compared with the control group; for the variable "CS-56 stroma," the OR was 0.451 (95% CI: 0.101–1.856), with $P = 0.274$. This indicates that the presence of CS-56 in the stromal tissue did not have a significant impact on the probability of belonging to the endometriosis group or the control

TABLE 3: Estimated odds ratios of endometriosis affected group on expression degrees in epithelium (left-plot) and stroma (right-plot) composition adjusted for age. Estimates obtained by univariate proportional odds-models

Response variable	OR	Lower 95%	Upper 95%	*P* value
CSPG4 epithelium	3.467	1.511	8.210	0.004
CSPG4 stroma	0.815	0.347	1.905	0.636
CS-56 epithelium	7.918	3.080	21.746	< 0.01
CS-56 stroma	0.451	0.101	1.856	0.274
HEP epithelium	22.033	7.600	74.469	< 0.01
HEP stroma	0.690	0.291	1.622	0.395
Keratan epithelium	0.111	0.038	0.290	< 0.01
Keratan stroma	0.012	0.002	0.047	< 0.01
Hyaluronic acid epithelium	0.190	0.073	0.465	< 0.01
Hyaluronic acid stroma	0.065	0.020	0.179	< 0.01

group; for the variable "HEP epithelium," the OR was 22.033 (95% CI: 7.600–74.469), with a p-value less than 0.01. This suggests that the probability of belonging to endometriosis group significantly increased when high expression of HEP was present in the epithelium compared with the control group; for the variable "HEP stroma," the OR was 0.690 (95% CI: 0.291–1.622), with a P of 0.395. This indicates that the presence of HEP in the stromal tissue did not have a significant impact on the probability of belonging to the endometriosis group or the control group; for the variable "keratan epithelium," the OR was 0.111 (95% CI: 0.038–0.290), with $P < 0.01$. This suggests that the probability of belonging to endometriosis group significantly reduced when high expression of keratan was present in the epithelium compared with the control group; for the variable "keratan stroma," the OR was 0.012 (95% CI: 0.002–0.047), with $P < 0.01$. This indicates that the presence of high expression of keratan in the stromal tissue significantly reduced the probability of belonging to the endometriosis group compared with the control group; for the variable "hyaluronic acid epithelium," the OR was 0.190 (95% CI: 0.073–0.465), with $P < 0.01$. This suggests that the probability of belonging to the endometriosis group significantly reduced when high expression of hyaluronic acid was present in the epithelium compared with the control group; for the variable "hyaluronic acid stroma," the OR was 0.065 (95% CI: 0.020–0.179), with $P < 0.01$. This indicates that the presence of high expression of hyaluronic acid in the stromal tissue significantly reduced the probability of belonging to the endometriosis group compared with the control group.

In Fig. 6, representative immunostainings for HEP and keratan in endometriosis tissue and normal endometrium are depicted.

IV. DISCUSSION

Endometriosis is a prevalent condition affecting 5%–10% of women of reproductive age worldwide.[21] The primary theory regarding its origin is Sampson's theory of reflux menstruation.[22]

Since Sampson's postulation of the retrograde menstruation theory, numerous studies have been conducted to elucidate the pathogenic mechanisms, which contribute to the development of the disease and to identify novel therapeutic targets. In particular, the recent demonstration of an embryological origin of endometriosis, shed new light on the pathogenesis and, consequently, on the possible therapeutic strategies for endometriosis.[18]

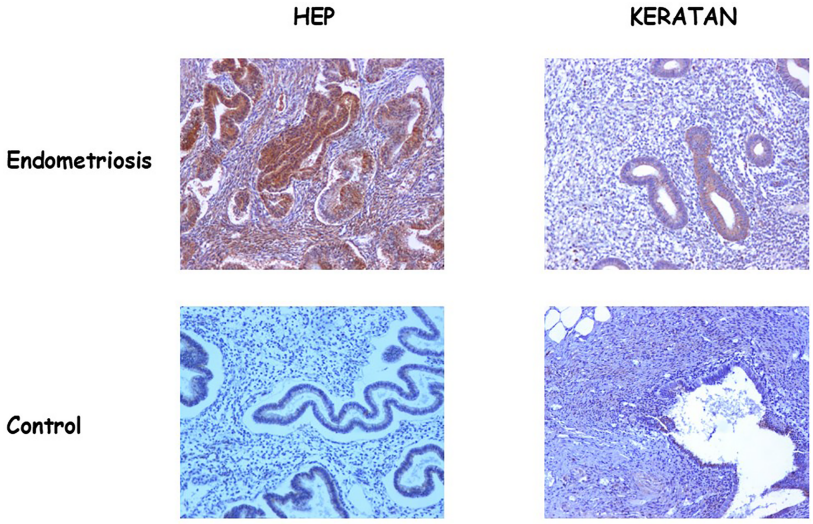

FIG. 6: Representative immunostainings for HEP and keratan in endometriosis tissue and normal endometrium (controls); original magnification 20×

Currently, conventional pharmacological treatment of endometriosis focuses on the generation of a hypoestrogenic environment, achieving it by modulation of steroid hormones through oral contraceptives, progestins and GnRH analogs. The rationale behind this is that the onset and growth of endometriotic lesion is crucially dependent on estrogen stimulation. In numerous prior studies, both ourselves and others have highlighted several discrepancies between eutopic and ectopic endometrium, one possible explanation for the failure of many medical therapies.[23] We posit that these variances are crucial for comprehending the mechanisms driving endometriosis development, particularly in adenogenesis and epithelial-mesenchymal interaction processes.[1] Indeed, both uterine development and endometriosis are partially dependent on epithelial–mesenchymal interactions, chiefly regulated by stromal-derived growth factors. These interactions play pivotal roles in epithelial proliferation, differentiation, and proliferation.

A. Chondroitin Sulfate Proteoglycan (CSPG4)

The CSPG4 is a cell surface proteoglycan that serves various functions in the tissues where it is present, as maintaining the structure and elasticity, regulating cellular activity, and modulating inflammatory response, it is overexpressed in a huge range of human neoplastic lesions by tumor cells, tumor microenvironment and cancer initiating cells.[24] Also known as NG2 (neuron-glial antigen 2), it is crucially involved in cell survival, migration and angiogenesis. Moreover, this factor underlies the inflammation regulation and hypoxia and is mediated by methyltransferases, transcription factors and miRNAs.[25] Assuming this, our study demonstrates that the expression of CSPG4 in the epithelial endometriosis has an increased epithelial intensity although in the endometriotic stroma is not significantly changed although the normal expression.

B. Chondroitin Sulfate (CS-56)

The chondroitin sulfate proteoglycans contribute to the regulation of inflammatory response in the endometrium, the modulation of cell adhesion, and intercellular communication. As revealed in the study of Nasciutti, by the biochemistry analysis, they demonstrate that CS is very abundant in human endometrium, with CS deposition in the epithelial basal membrane regions, suggesting its participation in basal membrane composition and function.[26] It is also reported in the literature that CS, due to its important role in EMT, could impair endometrial cancer progression by reducing cancer cell proliferation, migration.[27] Our immunohistochemical evaluation indicates an increased intensity in the endometrial epithelium of patients with endometriosis, but not in the stroma.

C. Heparan Sufate

HS proteoglycans regulate cell signaling pathways by acting as receptors/coreceptors of a ligand, modulating receptor trafficking and secretion, and/or establishing signaling gradients via regulating extracellular matrix structures.[28] Heparanase-1 is an endoglycosidase that specifically degrades heparan sulfate proteoglycans.[29] stimulating angiogenesis by releasing growth factors such as fibroblast growth factors and vascular endothelial cell growth factors stored in the extracellular matrix and inducing the expression of endothelial cell growth factors.[30] At present, very few studies have evaluated the expression of HS in eutopic versus ectopic endometrium, despite its proven importance in epithelial-mesenchymal interaction. A recent study conducted by Timofeeva Y.S. and colleagues[31] analyzed the content of heparan sulfate proteoglycans (HSPGs) in eutopic and heterotopic endometrium in women with ovarian endometriosis, using real-time semi-quantitative PCR and IHC. They found that real-time PCR analysis of the relative expression of core proteins of HSPGs in endometrial heterotopias revealed an increase in transcriptional activity of the gene HSPG2 and HPSE compared with eutopic endometrium with a concomitant hyper-expression of these proteins at IHC analysis. The IHC analysis in our study revealed a higher expression of HSPGs at the epithelial level in endometriosis samples compared with controls, while there was no statistically significant difference in stromal expression of

HSPGs between cases and controls. It has also been reported that in a population of women with polycystic ovary syndrome, higher level of endometrial HS was recorded compared with healthy patients, and this data was related to higher infertility rate.[32] This finding suggests the importance of the link between HSPGs and ectopic endometrial cells, even under conditions of normal stromal expression of HS, for the development and sustenance of endometriosis pathology.

D. Keratan Sulfate

It is widely acknowledged that endometrial secretions are rich in highly sulfated lactosaminoglycan chains, to regulate the sensitivity of the cells to hormonal signalling.[33] Keratan sulphate (KS) is present in association with glandular epithelium throughout the normal cycle, its production is hormonally regulated, as indicated by the significant increase observed in the secretory phase of the cycle.[34] The distribution of KS in normal endometrium has been studied by immunohistochemistry using monoclonal antibody 5D4 (Graham). As for previous markers, in the current study we have registered a higher intensity of expression of this marker in eutopic endometrial mucosa (controls), particularly in endometrial stromal cells, compared with endometriosis samples.

The presence of these glycoproteins, which are more prominently evident in eutopic endometrium, strengthens the hypothesis of a different phenotypic nature of ectopic endometrium, which also manifests as distinct epithelial-mesenchymal interactions and consequently, different sensitivity to sex hormones.

E. Hyaluronic Acid

HA, a large glycosaminoglycan protein found in the extracellular matrix, has function in clinical use as an anti-inflammatory, reducing proinflammatory cytokines.[35] In recent years, evidence has accumulated that HA plays a key role in the pathogenesis of endometriosis.

Thus, the role of HA in the literature is still conflicting, and further studies need to be conducted to shed light on the issue.

Previous studies[36,37] reported that use of hyaluronic acid reagent significantly reduced endometriosis lesion amount in mice model, by preventing the interaction of CD44-positive endometrial cells with the peritoneum.

Instead, according to a study conducted by Yu and colleagues,[38] no significant difference in HA concentration among early, late endometriosis patients, and normal controls was evident.

Finally, in the present study, expression of HA was lower in the endometriosis samples (both epithelial and stromal compounds) compared with control samples that showed medium-intense expression at immunohistochemistry.

The results of the statistical analyses highlight the importance of the expression of CSPG4, CS-56, HEP, keratan, and hyaluronic acid in the uterine epithelium as potential indicators of endometriosis. The presence of these molecules in the epithelium appears to be significantly associated with an increased probability of belonging to the endometriosis group, suggesting their potential role in the pathogenesis or progression of the condition. Conversely, the lack of significance in the effect of these molecules in stromal tissue suggests that the role of stromal tissue may be less central in determining the endometriosis status. This distinction between epithelium and stromal tissue may have diagnostic and therapeutic implications in endometriosis management, paving the way for the development of new targeted diagnostic and therapeutic strategies.

V. CONCLUSIONS

Analyzing the expression of CSPG4, CS-56, HEP, keratan sulfate, and hyaluronic acid in the study of endometriosis has been crucial for several reasons. Firstly, these molecules are integral components of the extracellular matrix and play significant roles in cellular adhesion, migration, and tissue remodeling. Understanding their expression patterns can provide insights into the pathophysiology of endometriosis, including the mechanisms underlying implantation, invasion, and proliferation of ectopic endometrial tissue.

Secondly, alterations in the expression of these molecules may contribute to the establishment and

maintenance of endometriotic lesions. Investigating their levels in eutopic and ectopic endometrial tissues allows for the identification of potential therapeutic targets and the development of novel treatment strategies aimed at disrupting the adhesive and invasive properties of endometriotic lesions.

Furthermore, studying the expression of these molecules can aid in the diagnosis and prognosis of endometriosis. By characterizing specific molecular signatures associated with the disease, clinicians can improve the accuracy of diagnostic tests and better predict disease progression and response to treatment.

Overall, the comprehensive analysis of CSPG4, CS-56, HEP, keratan sulfate, and hyaluronic acid in endometriosis research enhances our understanding of the disease's pathogenesis and offers promising avenues for therapeutic intervention and clinical management.

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