The Influence of Progesterone on Immunohystochemical Markers in Endometriosis

CLAUDIA MEHEDINTU[1], MARINA ANTONOVICI[1], LACRAMIOARA BRINDUSE[1], ELVIRA BRATILA[1], RUXANDRA STANCULESCU[1], COSTIN BERCEANU[2]*, OVIDIU BRATU[1], SILVIU PITURU[1], MIRCEA ONOFRIESCU[3], DANIELA ROXANA MATASARIU[3]

[1] Carol Davila University of Medicine and Pharmacy, 8th Eroii Sanitari Blvd., 050474, Bucharest, Romania

[2] University of Medicine and Pharmacy, 2nd Petru Rares Str., 200349, Craiova, Romania

[3] Grigore T. Popa University of Medicine and Pharmacy, 16th University Str., 70115, Iasi, Romania

Endometriosis, one of the most common gynecologic pathologies, is defined as an inflammatory, estrogen-dependent disease characterized by the growth of endometrial stroma and glands outside the uterine cavity. It is a multifactorial disease, conditioned by genetic and immune factors and triggered by hormonal and environmental factors. Estrogen receptors (ER) and progesterone receptors (PR) expression is significantly modified in endometriotic tissue, compared to normal endometrium. We performed a prospective study that included 16 patients with endometriosis: 9 patients that underwent progesterone treatment with 0.075 mg desogestrel, daily for 24 weeks prior to the surgical procedure, and 7 patients that did not follow any kind of treatment. The purpose of the study was to evaluate the changes that occurred in the expression of ER, PR, B-cell lymphoma 2 (Bcl-2) and Ki-67 from the endometriotic tissue. Oral 0.075 mg desogestrel administration proved its benefits in the management of endometriomas.

Keywords: endometriosis, progesterone receptor, estrogen receptor, Bcl-2, Ki-67

Endometriosis is an enigmatic and complex inflammatory disease characterized by the presence of endo-metrial glands and stroma outside of the uterine cavity, affecting with predilection the ovaries and the peritoneum [1,2]. It is considered to be a multifactorial disease, conditioned by genetic and immune factors and triggered by hormonal and environmental factors [3,4].

Within the endometriotic tissue, large quantities of estrogen and progesterone are produced, due to the abnormal activity in the steroidogenesis pathway [5,6]. The resulting estrogen has been shown to have a major role in the development of endometriosis. The dissemination, alongside the underlying inflammation can be considered responsible for the main clinical manifestations of endometriosis: chronic pelvic pain and infertility [6-8]. While the estrogens are playing an important role in the dissemination and proliferation of ectopic endometrial cells, the prostaglandins together with cytokines promote the inflammatory reaction that leads to pain and infertility. The presence of ER within the edometriotic implants, prove the high receptivity of this tissue to estrogen's action [6,9,10].

An ideal treatment for endometriosis should include a series of objectives, among which, two ought to be considered primarily: pelvic pain relief and infertility management. These facts can be achieved by preventing the endometriosis' rebound, using both medical and surgical therapeutic means [3,11,12].

The main objective of the present research was to see the potential of progesterone treatment for patients with endometriosis, by observing the changes occurring in the expression of ER, PR, Bcl-2 and Ki-67 in the endometrial glands and stroma of the endometriotic tissue.

Experimental part

This was a prospective study that included 16 patients, who were given an ultrasound examination and had the presumptive diagnosis of endometriosis. The patients were further investigated using laparoscopy and biopsy, and the final diagnosis of endometriosis was based on the histopathological findings. Our research took place during the years 2016-2017. All the patients enrolled in the study signed a written informed consent, which was previously approved by the Ethical Medical Committee of the University of Medicine and Pharmacy Gr. T. Popa, Iasi and the University of Medicine and Pharmacy Carol Davila, Bucharest.

The exclusion criteria were the following: BMI ≥ 30 kg/m^2, autoimmune, genetic, infectious conditions, diabetes mellitus, neoplasia, ongoing pregnancy, chronic anti-inflammatory or hormonal treatment for other conditions and depression or treatment for depressive conditions.

The patients enrolled in the study were divided in two groups: the first group included 9 patients that underwent progesterone treatment with 0.075 mg desogestrel, daily (Cerazette) for 24 weeks prior to the surgical procedure, and the second group included 7 patients that did not follow any kind of treatment. The biopsy samples were analyzed using immunohistochemistry (IHC). The aim was to highlight the ER, PR, Bcl-2 and Ki-67 expression.

The primary antibodies used are shown in table 1.

Table 1
THE MAIN CHARACTERISTICS OF THE ANTIBODIES

ANTIBODY	CLONE, SOURCE	DILUTION	EXPRESSION
ER	Clone SP1, EDTA*, IgG isotype, Biocare	1:1000	Nuclear
PR	Clone PGR 16, EDTA*, IgG1 isotype,	1:1000	Nuclear
Bcl-2	Clone 100/D5, EDTA*, IgG1/kappa, Biocare	1:100	Cytoplasmatic
Ki-67	Clone MM1, EDTA*, IgG1 isotype, Biocare	1:250	Nuclear

*Ethylenediaminetetraacetic acid

* email: dr_berceanu@yahoo.com; Phone: (+)4 0722728180 All authors have contributed equally to this paper.

The IHC reaction for ER and PR analyzed in the mesenchymal tissue (stroma) and the glandular epithelium was evaluated using a semi-quantitative score [13], based on the percent of positive cells and the intensity of the color reaction, the cases being classified as follows: 0 = absent immune reaction, 1 =≤ 1% positive cells, 2 = 1 – 10%, 3 = 10 – 33%, 4 = 33-66%, 5 = 66-100%, with the associated intensity noted as follows: 0 = absent, 1 = weak (+), 2 = moderate (++) and 3 = strong (+++). The cases with an absent IHC reaction (with a positive control) were considered negative. The final score was obtained by summing up the two scores – positivity and intensity. A final score that ranged between 0 and 2 was considered negative, and the scores from 3 to 8 were considered positive.

Regarding the IHC reaction for Bcl-2, the semi-quantitative evaluation was made using a scoring system based exclusively on the percent of positive cells [14], as follows: 0 = < 1% positive cells, 1 = 1 - 25%, 2 = 26- 50%, 3 = 51-75%, 4 = > 75%, without any remarks regarding the intensity of the reaction. A zero score was considered negative, while scores between 1 and 4 were considered positive.

For assessing the positive reaction for Ki-67 proliferation marker, we used a score (adapted Allred score), based on the analysis of the positive cells, associated with the intensity of the final reaction product. The identifying of Ki-67 positive nuclei within the decidual stromal and epithelial cells was objectified by counting the positive cells in 10 fields of view (x20), their sum being considered the final result. In positive cases, the score ranged between 3 and 8. The evaluation score of the Ki-67 expression was realized according to the intensity of coloration: weak = +1, moderate = +2, intense = +3; and the incidence: 0 = absent, 1 = ≤ 1%, 2 = 1-10%, 3 = 10 -33%, 4 = 33 - 66%, 5 = 66-100%.

Statistical analysis

The results are presented as ranges, averages and standard deviations for the quantitative variables and as absolute frequencies for the qualitative variables. Comparison of averages for the continuous quantitative variables was performed using the nonparametric Mann Whitney U test and comparison of frequencies was performed using Fisher's exact test. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using the software SPSS 23.0.

Results and discussions

The study group included patients aged between 22 and 39 years old (with an average age of 32.12±4.74), with confirmed endometriosis.

Stromal ER varied between 20 and 70% in patients with treatment and from 0% up to 100% in patients without treatment (fig. 1). The average level of ER in the stroma was slightly lower in patients that underwent treatment ($p = 0.933$, table 2).

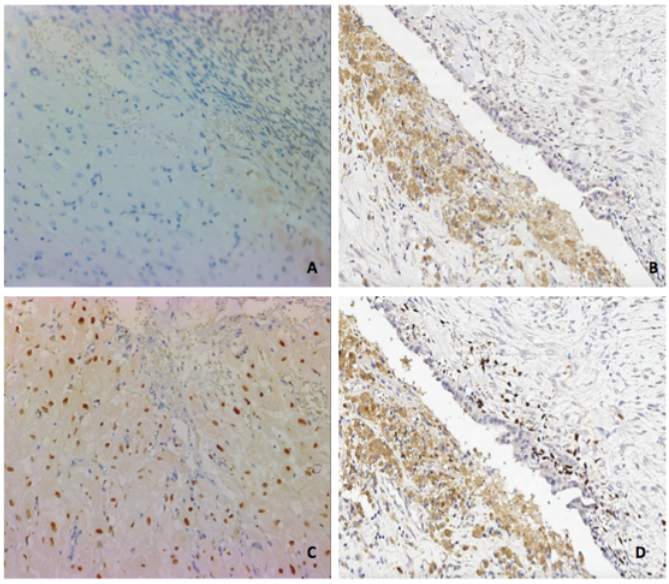

Fig. 1. ER expression in the decidualized stroma A. Without treatment; B. With Tratment; PR expression in the decidualized stroma: C. Without treatment; D. With Tratment

Epithelial ER varied between 0 and 70% in patients that followed treatment and between 0 and 90% in patients that did not underwent any kind of treatment. The average level of epithelial ER was not significantly different between the study groups ($p = 0.885$, table 3).

Epithelial PR ranged between 0% and 100% in both study groups. The average level of epithelial PR was significantly lower in patients that followed treatment ($p = 0.02$).

Stromal PR varied from 70% up to 100% in patients that followed treatment and from 5 to 100% in patients that did not underwent any kind of treatment (fig. 1). The average level of stromal PR was significantly higher in patients that underwent treatment ($p = 0.025$, table 2).

Stromal Bcl-2 varied between 40% and 90% in patients that underwent treatment and between 0% and 90% within patients without treatment (fig. 2). The average level of stromal Bcl-2 was significantly higher in patients that underwent treatment ($p = 0.012$). The frequency of positive patients in treatment group was significantly higher ($p = 0.019$, table 3).

Epithelial Bcl-2 ranged between 0% and 90% both in treated and without treatment patients, but the average level of epithelial Bcl-2 was significantly higher in patients that received treatment ($p = 0.050$, table 3).

Stromal Ki-67 ranged between 0% and 2% in patients with treatment and between 0 and 90% in patients without treatment (fig. 2). The average level of Ki-67 in the stroma was significantly lower in patients that underwent treatment ($p = 0.001$).

Epithelial Ki-67 varied from 0% to 5% within the group of patients that underwent treatment and from 0 to 35% within

		Treatment* (+/-)	% positive cells (M±SD)	p-value	Positive/negative (N)	p-value
ER	epithelial	+	23.33±24.49	0.885	6/3	0.315
		–	25.86±29.99		2/5	
	stromal	+	44.44±18.10	0.933	9/0	0.175
		–	45.86±38.12		5/2	
PR	epithelial	+	25.00±40.31	0.020	5/4	0.308
		–	53.57±39.19		6/1	
	stromal	+	90.00±12.24	0.025	9/0	-
		–	71.43±31.00		7/0	

Table 2
COMPARISON OF THE EXPRESSION OF ER AND PR IN THE STROMA AND EPITHELIUM BETWEEN PATIENTS WITH AND WITHOUT TREATMENT

**0.075 mg desogestrel for 24 weeks*

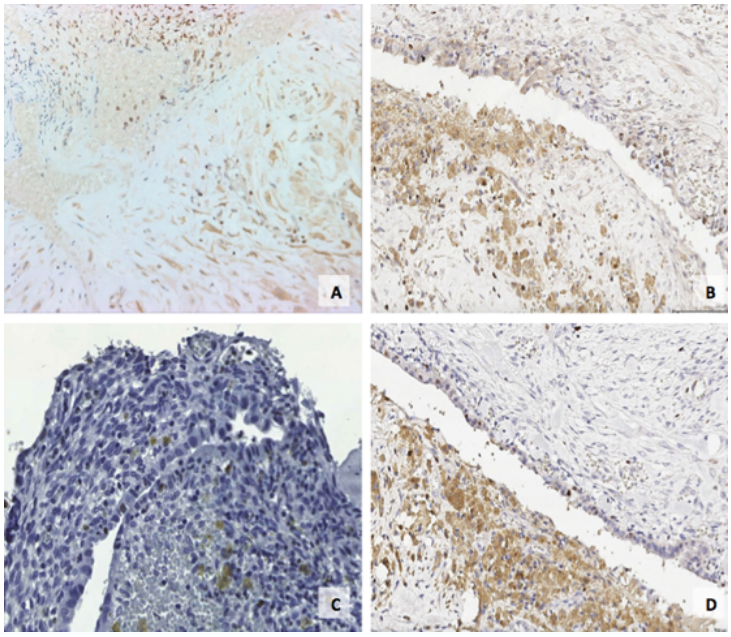

Fig. 2. Bcl-2 expression in the decidualized stroma A. Without treatment; B. With Tratment; Ki-67 expression in the decidualized stroma: C. Without treatment; D. With Tratment

		Treatment* (+/-)	% positive cells M±SD	p-value	Positive/negative N	p-value
Bcl-2	epithelial	+	51.11±40.14	0.050	6/3	0.615
		–	24.43±30.22		3/4	
Bcl-2	stromal	+	70.00±17.32	0.012	9/0	0.019
		–	27.29±29.18		3/4	
Ki-67	epithelial	+	1.22±2.17	0.028	2/7	0.126
		–	8.00±11.82		5/2	
Ki-67	stromal	+	0.67±0.86	0.001	2/7	0.126
		–	20.00±34.15		5/2	

Table 3
COMPARISON OF THE EXPRESSION OF BCL-2 AND KI-67 IN THE STROMA AND EPITHELIUM BETWEEN PATIENTS WITH AND WITHOUT TREATMENT

**0.075 mg desogestrel for 24 weeks*

the patients without treatment. The average level of epithelial Ki-67 was significantly lower within the group that received treatment (p = 0.028, table 3).

Our study showed that in the case of patients that followed treatment with desogestrel, the PR expression was significantly increased in the stroma, compared with those that did not follow medical treatment (p=0.025). This finding is highly suggestive for an increase of the progesterone sensibility in the stroma of treated patients. Un the other hand we registered a decrease in the epithelial PR expression in the same group of patients. The present research states that oral progesterone has a limited effect on the ER expression both in the stroma and the epithelium. In our research Bcl-2 seems to be under the control of progesterone. To support this affirmation, we note that after treatment, Bcl-2 expression increases exponentially especially in the stroma but also in the epithelium.

The present study also shows that an oral dose of 0.075 mg desogestrel, given daily for 24 weeks, decreases the Ki-67 expression both in the endometrial glands and especially in the stroma of the endometriotic cyst (from an average of 20% of positive cells, to 0.66% positive cells in the stroma).

Our study has as major limitation the low number of patients included in the study and this may impact the statistical power of our analysis.

The main strength of our study relates to the prospective recording of data and the use of IHC carried out by a single histo-pathologist with extensive experience in endometriosis.

Brandenberger et al. in 1999 and Bratila et al. in 2015 concluded that ER and PR expression is significantly modified in endometriotic tissue, compared to normal endometrium [6,15].

It is known that endometriosis is partially caused by the progesterone resistance and by the loss of progesterone signaling in the endometrial tissue [16,17].

Our results are concordant with the research of Hayashi et al. who reported that dienogest ameliorates the progesterone resistance in endometriotic tissue, by increasing PR expression and by decreasing ER expression [18,19].

Exogenous progesterone has a strong inhibitory action on cellular proliferation. This inhibitory action on the stroma is clinically seen as a stagnation or even decrease in cyst's dimensions and also as an improvement of the intraoperative conditions, enabling a better dissection of the cyst's wall due to the increased laxity of the tissues, and a diminished bleeding in the remaining ovary.

Our findings were consistent with the research of Nguyen et al. who showed in 2016 that the percentage of Ki-67 positive cells was significantly lower both in the epithelial and stromal cells of the cysts, in women that followed treatment with dienogest [19].

Streuli et al. in 2013 and Aznaurova et al. in 2014 were stating that hormonal treatment has no effect on the adhesion of endometriotic cells and cannot improve fertility [20,21]. These statements are contradicted by our results, oral treatment with 0.075 mg desogestrel being useful not only for improving intraoperative conditions but also for

better conserving the ovarian reserve, which may increase the chances of pregnancy.

Conclusions

We managed not only to emphasize the influence of this treatment on the specific receptors, but also on Bcl-2 and Ki-67 markers of cell apoptosis and proliferation, facts that we consider to be of major interest in nowadays research of endometriosis. The results of this study are new evidences regarding the utility of progesterone in the treatment of endometriosis and are an impulse to continue the research in this field, for a more precise identification of the underlying mechanisms that characterize this condition.

Oral treatment with 0.075 mg desogestrel proved its benefits on endometriomas, by acting at molecular level and increasing the expression of PR and decreasing Ki-67 expression, effects that are seen in clinical practice as an improvement of the symptoms, a decrease in the dimensions of the cyst and an improvement of the intraoperative conditions.

References

1.BULUN, S.E. N. Engl. J. Med., **360**, 2009, p. 268.

2.GIUDICE, L.C. N. Engl. J. Med., **362**, no. 25, 2010; p. 2389.

3.CROSIGNANI, P., OLIVE, D., BERGQVIST, A., LUCIANO, A. Hum. Reprod. Update, **12**, no. 2, 2006, p. 179.

4.CHO, S., AHN, Y.S., CHOI, Y.S., SEO, S.K., NAM, A., KIM, H.Y., KIM, J.H., PARK, K.H., CHO, D.J., LEE, B.S., Am J Reprod Immunol., **61**, 2009, p. 286.

5.BULUN, S.E, LIN, Z, IMIR, G AMIN, S., DEMURA, M., YILMAZ, B., MARTIN, R., UTSUNOMIYA, H., THUNG, S., GURATES, B., TAMURA, M., LANGOI, D., DEB, S., Pharmacol Rev., **57**, no. 3, 2005, p. 359.

6.CRISTESCU, C., VELISCU, A., MARINESCU, B., PATRASCU, A., TRASCA, E.T., POP, O.T., Rom J Morphol Embryol., **54**, no. 1, 2013, p. 91.

7.DRĂGHICI, I.M., DRAGHICI, L., COJOCARU, M., GORGAN, C.L., VRABIE, C.D., Rom J Morphol Embryol., **56**, no. 1, 2015, p. 133.

8.BRATILA, E., BRATILA, C.P., COMANDASU, D.E., BAUSIC, V., VLĂDESCU, C.T., MEHEDINU, C., BERCEANU, C., CIRSTOIU, M.M., MITROI, G., STANCULESCU, R., Rom J Morphol Embryol., **56**, no. 4, 2015, p. 1301.

9.NOBLE, L.S., TAKAYAMA, K., ZEITOUN, K.M., PUTMAN, J.M., JOHNS, D.A., HINSHELWOOD, M.M., AGARWAL, V.R., ZHAO, Y., CARR., B.R., BULUN, S.E., J Clin Endocrinol Metab., **82**, no. 2, 1997, p. 600.

10.NNOAHAM, K.E., HUMMELSHOJ, L., WEBSTER, P., ET. AL., Fertil Steril., **96**, no. 2, 2011, p. 366.

11.RICE, V.M. Ann N Y Acad Sci., **955**, 2002, p. 343.

12.VALLE, R.F., SCIARRA, J.J. Ann N Y Acad Sci., **997**, 2003, p. 229.

13.ALLRED, D.C., HARVEY, J.M., BERARDO, M., CLARK, G.M. Mod Pathol., **11**, no. 2, 1998, p. 155-68.

14.SUZUKY, Y., HONMA, T., HAYASHI, S., AJIOKA, Y., ASAKURA, H., J Clin Pathol., **55**, no. 3, 2002, p. 212.

15.BRANDENBERGER, A.W., LEBOVIC, D.I., TEE, M.K., RYAN, I.P., TSENG, J.F., JAFFE, R.B., TAYLOR, R.N., Mol Hum Reprod., **5**, no. 7, 1999, p. 651.

16.AL-SABBAGH, M., LAM, E.W., BROSENS, J.J. Mol Cell Endocrinol., **358**, 2012, p. 208.

17.ALBU (MATASARIU), D.R., MIHALCEANU, E., PANGAL, A., VULPOI, C., ONOFRIESCU, M., NITOI, L., MIHAILA, A., COSTACHESCU,G., CONSTANTINESCU, D., DUMITRASCU, I., Rev. Chim. (Bucharest), **68**, no. 9, 2017, p.2132.

18.HAYASHI, A., TANABE, A., KAWABE, S, HAYASHI, M., YUGUCHI, H., YAMASHITA, Y., OKUDA, K., OHMICHI, M., J Ovarian Res., **5**, no. 1, 2012, p. 31.

19.NGUYEN, T.T., HACHISUGA, T., URABE, R., UEDA, T., KURITA, T., KAGAMI, S., KAWAGOE, T., HISAOKA, M., J Uoeh., **38,** no. 4, 2016, p. 271.

20.STREULI, I., ZIEGLER, D., SANTULLI, P., MARCELLIN, L., BORGHESE, B., BATTEUX, F., CHAPRON, C., Expert Opin Pharmacother., **14**, no. 3,2013, p. 291.

21.AZNAUROVA, Y.B., ZHUMATAEV, M.B., ROBERTS, T.K., ALIPER, A.M., ZHAVORONKOV, A.A., Reprod Biol Endocrin., **12**, 2014, p. 50.

Manuscript received: 21.12.2017