The assessment of immunohistochemical profile of endometriosis implants, a practical method to appreciate the aggressiveness and recurrence risk of endometriosis

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Abstract
Endometriosis represents a chronic female genital tract disease characterized by implants outside the endometrial cavity, leading to alteration of pelvic anatomy and having as result chronic pelvic pain and infertility. Aim: From the molecular perspective, the aim of studying endometriosis is identifying a cause and a consequence, that lead to the appearance and perpetual arising of new implants. The description of the immunohistochemical (IHC) profile of ectopic endometrium could represent a new element in the pathogenesis of endometriosis and also a practical method to appreciate the aggressiveness and possibility of recurrence of the disease. The study consisting of histopathological and immunohistochemical (IHC) analysis of the tissues excised included 14 patients, operated from June to December 2014, to which was confirmed the presumptive diagnosis of endometriosis, based on anamnesis, clinical examination and ultrasound appearance. We identified the expression of estrogen and progesterone receptors, whose presence in the ectopic endometrium guides the medical hormone postoperative treatment. We also identified the expression of a cellular proliferation marker – Ki-67, and inhibition marker of cellular apoptosis – Bcl-2, in order to characterize the aggressiveness of endometriosis implantations and a stromal marker CD10. Although there are plenty of medical and surgical therapeutic methods available, the treatment of endometriosis must be individualized for every patient taking into consideration the IHC analysis. Consolidation of surgical treatment by prescription of a medical long-term treatment is indispensable, because endometriosis is a chronic relapsing disease.

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

Introduction
Endometriosis represents a chronic disease of the female genital tract characterized by the appearance of endometrium implants outside the endometrial cavity (most common in the abdomen and pelvis), causing alteration of the pelvic anatomy and having as consequence chronic pelvic pain and infertility in women between aged 15–49-year-old [1–3]. At present, there is no consensus regarding the pathogenic mechanisms and the best treatment of endometriosis [4–6].

Implantation and survival of ectopic endometrium cells as well as clinical signs of endometriosis are probably the final part of a complex of abnormal biological processes. From the molecular point of view, the aim of studying endometriosis as disease is identifying of a cause and of a result, which lead to constituting and permanent reappearance of implantations of endometrium, a subject more and more frequently approached in fundamental research. The description of immunohistochemical (IHC) profile of ectopic endometrium may represent a new element of study in the pathogenesis of endometriosis as well as a practical method of evaluating the aggressiveness and reappearance probability of the disease [7–10].

The final result is the introduction of a medium- or long-term more efficacious hormone treatment to the patients depending on IHC profile of endometriotic implants, treatment that will be taken until the women decide to procreate.

The paper aims to discover a new approach of examination and therapeutic intervention in endometriosis, based on molecular markers identified in the endometriosis tissue using IHC techniques.
Materials and Methods

The study consisted of histopathological examination and IHC analysis of pelvic implants from 14 patients operated between June–December 2014, in which there was confirmed the diagnosis of endometriosis based on histopathological exam.

The main characteristics of the study group were represented of average age of patients 30 years (24–39 years), nulliparous, nine of them presenting primary infertility. The suspicion diagnosis of endometriosis was established by clinical and laboratory findings (transvaginal echography, gel ultrasonovaginography). In order to standardize the preoperative ultrasonography examination, an ultrasound score was formulated that describes the localization of endometriosis lesions on the pelvic organs and confers a score to every localization, in order to easily calculate a final score for every patient that describes the most faithfully the possible localization and number of endometriosis implants. The ultrasound score aims the appearance of endometriosis implants from all the pelvic organs.

Ectopic endometrial tissue was obtained by laparoscopy; laparoscopic operation was done for diagnosis and therapeutic purpose. After establishing the endometriosis diagnosis based on histopathological analyses of the tissues extracted, the fragments were IHC analyzed in order to realize the cellular markers profile. Within the endometriosis implants, we identified the expression of estrogen receptors (ER) (clone SP1, EDTA – Ethylenediaminetetraacetic acid, IgG isotype, Biocare) and progesterone receptors (PR) (clone PGR 16, EDTA, IgG1 isotype, Biocare), receptors whose presence in the ectopic endometrium guides the medical treatment applied to the patients suffering of endometriosis. We also identified the expression of a marker of cellular proliferation, Ki-67 (clone MM1, EDTA, IgG1 isotype, Biocare) and of a marker of inhibition of cellular apoptosis, Bcl-2 (clone 100/D5, EDTA, IgG1/kappa, Biocare) in order to characterize the aggressiveness of endometriosis implantations. In order to mark the endometriosis areas in endometriosis implants, we used a stromal marker, CD10 (clone 56C6, EDTA, pH 9, 1/100 dilution, Dako).

Results

Within this study group has been noticed a correlation among clinical examination, ultrasound preoperative score and intraoperative appearances of implantations. Intraoperative evaluation allowed staging of endometriosis (according to staging score system of American Society of Fertility and Sterility), the group being characterizes as it follows: four cases stage II, mild form, score 6–15; six cases stage III, moderate form, score 16–40; and four cases stage IV, severe form, score >40.

For the four patients diagnosed with endometriosis stage II intraoperative, it was noticed an ovarian endometriosis cyst without other peritoneal implantations. In the isles of endometriosis from the capsule of the cyst, the marker CD10, with cytoplasm expression, allowed the pointing out of cytogenic chorion elements, thus permitting marking and extension of endometriosis areas (Figure 1). The IHC profile of preparations analyzed for this stage of endometriosis was extremely fluctuating for all the markers analyzed. The identification of estrogen receptors (ER) in the four cases of mild endometriosis pointed out a low nuclear index at the epithelial element – 35% and a variable nuclear index at stromal element 10–65% (Figure 2). The expression of progesterone receptors (PR) was pointed out in a low ratio in the epithelial element (10–20%) but in stromal element, the expression was noteworthy 45–65% (Figure 3). The expression of cytoplasm Bcl-2 marker in three cases had a low value at the glandular cells of 8% (Figure 4) and in another case, it was expressed having a ratio of 20%. The index of cells proliferation Ki-67 pointed out the same variable expression at the glandular epithelium and in stromal cells, in three cases being 10% expressed (Figure 5) and in one case, the expression had the value of 35%.

Most of the patients diagnosed with endometriosis stage III (six patients out of 14) had ovarian cysts of 6–9 cm, accompanied by minimal lesions of peritoneal endometriosis. The ER analysis pointed out a variable nuclear index with values of 25–45% at the epithelial component and an index of less than 10% at the stromal component. Progesterone receptors in the fragments of cyst walls pointed out a marked expression at the epithelial and stromal component of 60% (Figure 6) in four cases, while in two cases the expression PR was low, respectively 30% in the stromal and epithelial component. The expression of cytoplasm protein Bcl-2 in the cytogenic chorion and in the glandular epithelium had values of 10–20% (Figure 7). In three cases, the expression of cellular proliferation marker was differently expressed in the same lesion because in the same section the stromal component was identified under the influence of estrogen hormone and of progesterone influence. In stromal component, being under estrogen influence the marker Ki-67 was expressed in a value of 20% while under progesterone influence the expression of Ki-67 was under 10% in the stromal component and in the epithelial component the expression was accentuated – 35%.

Patients diagnosed with severe endometriosis, stage IV (four patients), had an intraoperative appearance of bilateral endometriosis cysts, multiple peritoneal lesions, recto-vaginal endometriosis or bladder endometriosis nodule. The histopathological exam of the extracted implants pointed out the typical appearance of endometriosis – glands, endometriosis stroma and bleeding isles. The IHC analysis of ER pointed out a variable nuclear index of 30–60% in tissue glands and in endometrium stromal cells. PR analysis in endometriosis tissue also expressed variable index in endometriosis stroma less than 10% (two cases) and 65% in other two cases. In all cases of severe endometriosis, the expression Bcl-2 and Ki-67 was pointed out.

In a case of severe endometriosis, the histopathological exam pointed out at glandular tissue with focal areas of glandular hyperplasia with atypia. Atypia elements were suggested by the appearance of the nucleus that showed polarization – round nucleus with emphasizing nucleolus. The analysis of ER expression pointed out in this case a nucleus index of 80% in hyperplasic nucleus and a stromal index of 60% (Figure 8). The PR expression was pointed out in a percent of 90% in glandular tissue and of 70%...
in stromal cells (Figure 9). The expression of cytoplasm marker Bcl-2 had a high level in the epithelium, fact suggested also by the morphological appearance of glandular structure, the disposition of pseudo-stratification of epithelium cells with a plan of nucleus polarization (Figure 10). The expression of the Ki-67 marker was intense with an index of 30% in the hyperplastic glandular epithelium with atypia (Figure 11).

Figure 1 – Stage II endometriosis. Expression of CD10 marker. Anti-CD10 immunostaining, ×100.

Figure 2 – Stage II endometriosis. Expression of ER at the level of stromal (65%) and epithelial (35%) component. Anti-ER immunostaining, ×400.

Figure 3 – Stage II endometriosis. Expression of PR at the level of stromal (65%) and epithelial (10%) component. Anti-PR immunostaining, x400.

Figure 4 – Stage II endometriosis. Expression of Bcl-2 at the level of glandular cells (8%). Anti-Bcl-2 immunostaining, ×100.

Figure 5 – Stage II endometriosis. Expression of Ki-67 at the level of glandular epithelium (10%). Anti-Ki-67 immunostaining, ×100.

Figure 6 – Stage III endometriosis. Expression of PR at the level of stromal and epithelial component (60%). Anti-PR immunostaining, ×200.
Figure 7 – Stage III endometriosis. Expression of Bcl-2 at the level of stromal cells (20%). Anti-Bcl-2 immunostaining, ×400.

Figure 8 – Stage IV endometriosis. Nuclear expression of ER at the level of glandular and stromal epithelium. Anti-ER immunostaining, ×100.

Figure 9 – Stage IV endometriosis. PR expression at the level of epithelial and stromal cells. Anti-PR immunostaining, ×200.

Figure 10 – Stage IV endometriosis. Bcl-2 expression at the level of epithelial cells, expression correlated with the proliferative level of the endometrium. Anti-Bcl-2 immunostaining, ×400.

Figure 11 – Stage IV endometriosis. Ki-67 expression at the level of glandular and stromal epithelium. Anti-Ki-67 immunostaining, ×400.

Discussion

Endometriosis is an estrogen-dependent inflammatory disease, which affects women at fertile age. The clinical signs of endometriosis suggest the negative effect of ovulation cycles in developing and maintaining the endometriosis [11, 12], its symptoms appearing usually after the first period and disappearing after menopause [13]. Exceptionally, an endometriosis nodule may be symptomatic after the installing of menopause, suggesting the independence of endometriosis from the ovarian secretion of estrogen [14]. The interrupting of ovulation by using gonadotropine releasing hormone (GnRH) analogues, oral contraceptives, progestogen or anti-progesterone diminish the pain associated with endometriosis [15, 16]. At the same time, aromatase inhibitors, which stop the producing of estrogen, have favorable effects in patients suffering from endometriosis [17]. The levels of nuclear receptors for estrogen and progesterone are considerable modified in ectopic endometrial tissue as compared to normal endometrium. The level of β-receptors of estrogen in endometriosis tissue is 142 times bigger than normal in endometrium and the level of α-receptors of estrogen is nine times bigger in endometriosis tissue as compared to the normal one [18]. The increased expression of α- and β-estrogen receptors in stromal endometriosis cells leads to binding the precursors of the progesterone receptors to the β-estrogen receptors generating low levels of
progesterone receptors [19, 20]. Biologically important quantities of estrogen and progesterone are produced in the endometriosis tissue, due to an abnormal activity of steroidogenesis cascade [21]. The quantity of estrogen produced plays a decisive role in endometriosis. The estrogen in endometriosis implants leads to the dissemination of endometriosis cells. The cells dissemination and the inflammation are responsible for the two main clinic manifestations of endometriosis: the chronic pelvic pain and infertility [22–25]. The estrogen sustains the survival and dissemination of endometriosis tissue, while prosta
glandins and cytokines promote inflammation, pain and infertility. The presence of estrogen receptors in endome
triosis implants reveals the responsiveness of this tissue to estrogen’s action [26, 27]. The expression of ER in the examined cases in this study was of 25–80%, with a marked expression in glandular epithelium cells. In the stromal cells, the estrogen receptors were expressed by a nuclear index of 10–60% and their presence at both stromal and epithelium compounds prove the sensitiveness of endometriosis tissue at estrogen hormone stimulus. Generally, the level of ER expression was lower than the PR expression in our group, unlike in the studies conducted by Nnoaham et al. [26] and Noble et al. [27], which showed a very low PR expression with a moderate ER expression.

In endometriosis tissues, the level of PR-B receptors is undetectable while the level of PR-A receptors is con
siderably diminished [19]. In eight (66%) of the studied cases, from the IHC point of view, progesterone receptors were identified in an important percent, over 60% both in epithelium and stromal components. Unlike literature data communicated so far, however, the IHC analysis of the implants extracted in the presented cases highlights the PR presence at a high level, especially in medium and severe forms of endometriosis, fact that explains the clinical response of this disease at progesterone treatment. Despite the results published by Attia et al., in 2000 [19], we identified the PR at a relatively high level in all endome
triosis lesions studied (at a range of 35–38% with extreme values between 14% and 50%), especially the ones in advanced stages. The progesterone resistance in some endometriosis cases is explained by the extremely low levels of progesterone receptors in endometriosis tissue.

The interrupting of normal process of apoptosis leads to a large variety of affections [28]. The lack of balance between pro- and anti-apoptosis proteins leads to abnormal cells dissemination and to the impossibility of a correct answer at apoptotic stimulus and a low answer at therapies based on inducing the cells apoptosis [29]. There have been identified more than 25 members of Bcl-2 protein family (B-cell lymphoma 2), responsible of adjusting cell apoptosis, encrypted by Bcl-2 gene, which is situated on chromosome 18 [30].

Bcl-2 protein is present in membranes of endoplasmic reticulum, nuclear capsule and mitochondrial external membrane. It has an anti-apoptic role by inhibiting the displacement of cytochrome c from the mitochondrial intramembranous area to the cytosol and prevents the pro-apoptotic actions of Bax, Bak proteins [31] without producing the cell dissemination. As a result, the proportion between Bcl-2 and Bax proteins plays an important role in the apoptosis process [32].

There are at present in literature studies of Bcl-2 protein only at eutopic endometrium where it was prev
alently identified in glandular cells. There has been noticed a high expression of Bcl-2 in cells from eutopic endometrium of women suffering of endometriosis, suggesting that the endometrial tissue of these patients has an increased dissemination capacity and rising outside the peritoneal cavity, a possible explanation of pathogenic mechanism in which endometrial tissue develops in ectopic places [33–35]. On the other hand, Jones et al. [36] noticed that there is no important statistical difference regarding the relation between apoptosis and the level of Bcl-2 proteins comparing the ectopic endometrium of patients suffering of endometriosis with the endometrial tissue from women without pathology.

IHC analysis of cases included in study pointed out an expression of Bcl-2 protein in endometriosis implants (ectopic endometrium) in both components epithelium with bigger expression and stroma with an anti-apoptotic index of maximum 20%. This index had a low value (less than 8%) in 50% of cases of mild (stage II) endometriosis.

Ki-67 antigen is a nuclear protein strictly related to cells proliferation, being involved in ribosome RNA transcrip
tion [37, 38]. The observing study that contained 56 patients diagnosed with endometriosis stages III and IV coordinated by Ali et al. [39], published in 2013, aimed to analyze the correlation between the expression of Ki-67 protein correlated with the size of endometriosis ovarian cysts, as well as the stage of endometriosis. The results of research show an important correlation between the expression of Ki-67 protein and the size of endometriotic cysts.

The Ki-67 marker proves an increasing of cell dissemina
tion activity, thus explaining its expression directly proportional with the severity stage of disease [40, 41]. When cells dissemination activity is raised, cells become independent and affect surrounding tissues. Thus, the anatomy of the area is modified and appears an important adhering process between the affected structures. The expression of Ki-67 marker at analyzed cases was variable, not being able to correlate it with the stage of the disease. The expression of this marker was low (less than 5%) in 50% of mild or moderate endometriosis to which intra-operative was identified a maximum 6 cm one-sided endometriosis cyst and minimal peritoneal implants. The Ki-67 expression was marked (35%) at epithelium components, both in moderate stages and in severe stages of endometriosis. Contrary to the data described in the specialty literature by Li et al. [40] and Kahyaoglu et al. [41], our study did not find a correlation between the Ki-67 expression and the stage of the disease, as it represents an aggressiveness marker that best describes the dissemination potential rather than the actual stage of the endometriosis. In one case of stage II endometriosis, we identified the Ki-67 protein in a very high percentage – over 90%. That is why in cases of aggressive forms of disease discovered in incipient stages the level of Ki-67 is as high as in cases diagnosed in advanced stages of the disease.

In choosing of the long-term medical consolidation treatment in order to avoid the relapses in endometriosis, we need to have a fully diagnostic profile of the endometriosis lesions that contains clinical data, ultrasound
and biomarkers that characterize the aggressiveness of these lesions (Table 1). Intraoperative standardization of cases according to the localization of the implants realizes a description of the disease in that precise moment, without bringing other data about the aggressiveness of the endometriosis implants and the evolution capacity of the disease.

### Table 1 – Therapeutic approach according to IHC profile and endometriosis stage

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>ER PR Bcl-2 Ki-67</th>
<th>Postoperative medical treatment</th>
<th>Endometriosis stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>↓ ↓ ↓ ↓</td>
<td>No treatment; Progestative; Estro-progestative.</td>
<td>II</td>
</tr>
<tr>
<td>5</td>
<td>↑ ↑ ↑ ↑</td>
<td>Progestative.</td>
<td>II, III, IV</td>
</tr>
<tr>
<td>4</td>
<td>↑ ↑ ↑ ↑</td>
<td>Estro-progestative.</td>
<td>III, IV</td>
</tr>
<tr>
<td>3</td>
<td>↑ ↓ ↓ ↓</td>
<td>Surgical treatment; GnRH agonist.</td>
<td>III, IV</td>
</tr>
</tbody>
</table>

The variable expression of anti-apoptotic markers (Bcl-2) and of cell proliferation marker (Ki-67) results from the fact that not all endometriosis lesions have the same progression capacity. Expression variability of estrogen and progesterone receptor is correlated with the response to hormonal medical treatment. Less expression of these hormonal receptors in some cases explain the evolution of lesions and the persistence of symptomatology under hormonal treatment and for these cases, the only reasonable treatment is the surgical excision of the endometriosis implants.

### Conclusions

Although there are many medical and surgical available therapeutic methods, the endometriosis treatment must be individualized considering each case, including IHC analysis of endometriosis implants. ER and PR identifying in endometriosis implants is useful in establishing the postoperative hormone medical treatment. The consolidation of surgical treatment by prescribing of a long-term medical treatment is crucial, because endometriosis is a chronic relapsing disease. The pointing out of Bcl-2 and Ki-67 apoptotic markers and cell proliferation marker results from the fact that not all endometriosis lesions have the same progression capacity. Expression variability of these hormonal receptors in some cases explain the evolution of lesions and the persistence of symptomatology under hormonal treatment and for these cases, the only reasonable treatment is the surgical excision of the endometriosis implants.

### Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors read and approved the final manuscript.

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### References

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