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Enclosures

## Rectovaginal Septum Endometriosis: An Immunohistochemical Analysis of 62 Cases

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**Abstract.** Deep infiltrating endometriosis of rectovaginal septum is a particular form of endometriosis located under the peritoneal surface. This kind of lesions are very active and strongly associated with pelvic pain symptoms. A study on 62 cases of rectovaginal septum endometriosis by means of immunohistochemistry was conducted in order to evaluate the oestrogen and progesterone receptor levels in these cases and to correlate them to the level of vascularization (CD34 expression) and the amount of nerve fibres (S100 expression). Data showed great heterogeneity in the expression of all the parameters analyzed. Nevertheless, by using Spearman correlation test to assess relationship among oestrogen and progesterone receptors, S100 and CD34 staining, a significant direct correlation was found between all the parameters analyzed. These observations sustain the hypothesis that oestrogen and progesterone play an important role in the genesis of endometriotic glands, in the vascularization and in the proliferation of nerves.

Endometriosis is a gynaecological disease defined by the histological presence of endometrial glands and stroma outside the uterine cavity, most commonly implanted over visceral and peritoneal surfaces within the female pelvis, but rarely also in the pericardium, pleura and even brain (1, 2). Although the exact prevalence of endometriosis in the population is not clear, the prevalence in the general female population is 6-10%; in women with pain, infertility or both, the frequency increases to 35-60% (3). Endometriosis is usually associated with infertility and pelvic pain such as

chronic dysmenorrhea, intermenstrual abdominal and pelvic pain, back pain, dysuria, dyschezia and dyspareunia (1). Deep infiltrating endometriosis of rectovaginal septum is a particular form of endometriosis located under the peritoneal surface (4). These kind of lesions are very active and are strongly associated with pelvic pain symptoms (5). The mechanisms by which deep infiltrating endometriosis lesions cause pain and hyperalgesia are poorly understood. A multifactorial pathogenetic mechanism could be hypothesized, where cyclical bleeding, anatomical structure and locations of the lesions, production of prostaglandins and inflammatory mediators by the endometriotic lesions themselves and local response of the damaged tissues with production of mast cells could be outlined (6, 7). Despite this, deep infiltrating endometriosis is rather poorly reflected in the R-AFS classification (8). Moreover, previous immunohistochemical analyses have demonstrated marked heterogeneity in the expression of oestrogen and progesterone receptors in endometriosis lesions from different patients with non-homogeneous results (9, 10). Indeed, studies addressed to the exhaustive morphological and molecular characterization of this peculiar type of endometriosis are needed to better define the impact of rectovaginal endometriosis on the pathogenesis and clinical course of this very common disease. To further analyse this topic, a study on 62 cases of rectovaginal septum endometriosis has been conducted. The aim of the study was to determine, by means of immunohistochemistry, the oestrogen and progesterone receptor levels in these cases and to correlate them to the level of vascularization and the amount of nerve fibres.

### Materials and Methods

*Patients and tissue samples.* Retrospective evaluation was performed on surgical specimens from patients who underwent surgery for infertility, pelvic pain symptoms (including dysmenorrhea, deep dyspareunia and no-menstrual pain) or adnexal masses between 2005 and 2007 at the "Centro Italiano Endometriosi" in Rome and that were diagnosed with deep infiltrating endometriosis. This

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*Key Words:* Endometriosis, immunohistochemistry, oestrogen, progesterone, S100, CD34.

determination was made during the diagnostic and therapeutic phase of laparoscopic surgery and was based on the macroscopic appearance of the lesions, using the following criteria: a) palpable and visible nodule or induration and retraction of peritoneum in the posterior and lateral area of cervix, at the level of the uterosacral ligaments and medial broad ligaments, and rectovaginal septum; b) dark blue nodule visible at the posterior vaginal wall at speculum examination. Only the cases where the presence of endometriotic glands was confirmed histologically were included in this study. Moreover, who had previously had endometriosis surgery and women no longer menstruating were excluded. After selection, 62 cases were eligible for the analysis. Main patients' characteristics are summarized in Table I.

**Immunohistochemistry.** Representative sections of each specimen were stained with haematoxylin-eosin. For immunohistochemistry 5-7  $\mu$ m specimen sections embedded in paraffin, were cut, mounted on glass and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide in aqueous solution and blocked with PBS-6% non-fat dry milk (Biorad, Hercules, CA, USA) for 1 h at room temperature. Slides were then incubated at 4°C overnight at 1:100 dilution with the following affinity-purified rabbit antibodies: ER $\alpha$  (Santa Cruz, Santa Cruz, CA, USA; cat. # sc-542); PR (Santa Cruz, cat. # sc-538); CD34 (Santa Cruz, cat. # sc-9095); S100 (Santa Cruz, cat. # sc-7851). After three washes in PBS to remove the excess of antiserum, the slides were incubated with diluted goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) at 1:200 dilution in PBS-3% non-fat dry milk (Biorad) for 1 h. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (Vector Laboratories) 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. All samples were processed under the same conditions. The percentage of nuclei positive for oestrogen or progesterone receptors in the epithelial and stromal cells per field (250 X) using light microscopy were calculated and compared in different specimens by two separate observers (A.B. and A.D.) in a double blind fashion and described as: score 0 (absent); score 1 (from 1% to 20%); score 2 (from 21% to 40%), score 3 (>40%). The intensity of S100 and CD34 expression was, instead, 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.

**Statistical analyses.** Spearman correlation test was used to assess relationship between immunohistochemical data. Differences between the groups of patients were compared according to Mann Whitney U-test or Chi square test, *p*-values <0.05 were regarded as statistical significant in two tailed tests. SPSS software (version 11.05, SPSS, Chicago, USA) was used for statistical analysis

## Results

Histologic examination of endometriotic lesions of the rectovaginal septum showed the typical presence of both endometriotic glands and stroma. The glands had a clear

Table I. Characteristics of the 62 women included in the study.

	No	Mean $\pm$ SD	%
Age (years)	62	33 $\pm$ 5	
Parity	12	0.19 $\pm$ 0.39	19.3
Chronic pelvic pain symptoms	57	0.92 $\pm$ 0.27	91.9
AFS stage <sup>1</sup>			
I	1		1.6
II	5		8.1
III	9		14.5
IV	47		75.8

<sup>1</sup>American Fertility Society stage, based on The American Fertility Society, 1985.

endometriod appearance, while the endometriotic stroma typically resembled eutopic inactive or proliferative endometrial stroma. Moreover, frequently there was an infiltrate of hystiocytes with lipofuscin and hemosiderin pigment, probably caused by hemorrhage and menstrual changes in endometriosis. Finally, the presence of a network of small arterioles, proliferation of nervous fibers and nodular aggregates of smooth muscle was common in the stroma. Figure 1 depicts an exemplary case of the microscopical appearance of endometriosis of the rectovaginal septum.

In Figure 2, examples of immunohistochemical staining for the parameters analyzed are depicted. There was a marked heterogeneity in the expression of oestrogen and progesterone receptors in the 62 endometriosis lesions studied. There were some lesions where the epithelial cells were positive for oestrogen receptor and negative for progesterone receptor or the opposite; consistently, there were cases where the receptors were expressed in the epithelium and were negative in the stromal cells or the opposite. The percentage of cells stained positively for oestrogen receptor varied between 0 and 93% and for progesterone receptor between 0 and 94%. Expression levels of both S100 and CD34 varied from absent to intense. All these data are summarized in Tables II and III. In order to analyze if there was any statistical correlation between the immunohistochemical data, a Spearman correlation test was performed to assess relationship among oestrogen and progesterone receptors, S100 and CD34 staining. Interestingly, a significant direct correlation between all the parameters analyzed was found (see Table IV). Therefore, for a marked heterogeneity in expression levels among the different patients, there was a corresponding a significant positive correlation between the various parameters analyzed. It is noted that no any significant correlation was found when contrasting the immunohistochemical parameters with the clinical data available, such as score of the disease, presence of symptoms or assumptions of oral contraceptive steroids (data not shown).

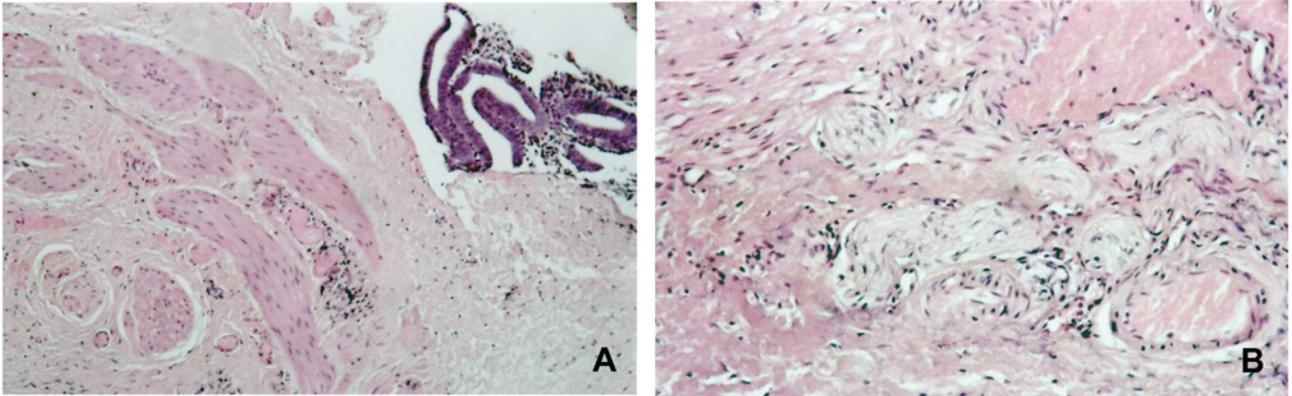


Figure 1. Microscopical appearance of deep infiltrating endometriosis. A) Typical endometriosis showing part of an endometriotic gland and a thin cut of periglandular endometriotic stroma containing dilated blood vessels and hypertrophic smooth muscle fibers (Hematoxylin and Eosin, original magnification  $\times 20$ ). B) Higher magnification of the same case showing hypertrophic nerve endings (Hematoxylin and Eosin, original magnification  $\times 40$ ).

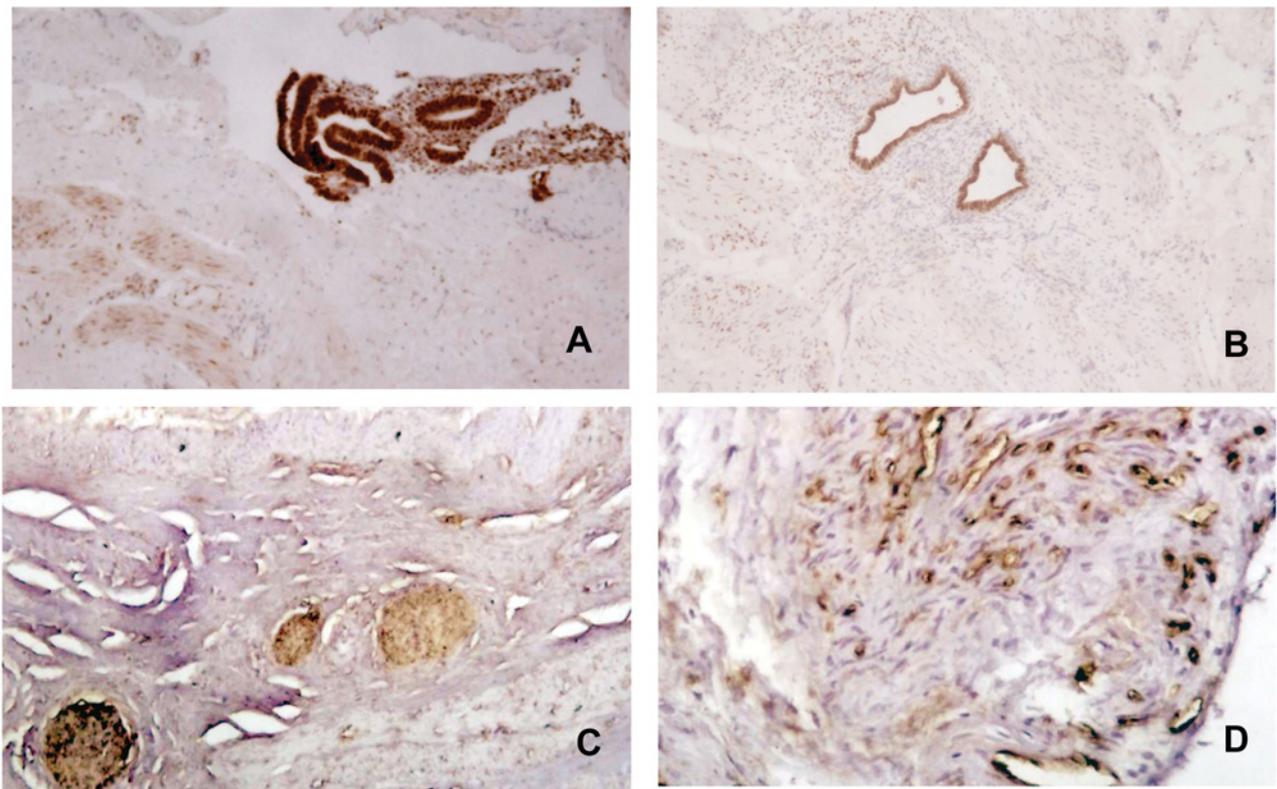


Figure 2. Examples of immunohistochemical staining for the parameters analyzed. A) Oestrogen receptors expression (ABC, original magnification  $\times 20$ ). B) Progesterone receptor expression (ABC, original magnification  $\times 20$ ). C) S100 expression (ABC, original magnification  $\times 20$ ). D) CD34 expression (ABC, original magnification  $\times 20$ ).

## Discussion

Endometriosis continues to remain a significantly under-diagnosed and under-treated disease (1, 2). Though there are several theories, researchers remain unsure as to the definitive

cause of endometriosis. One widely accepted mechanism for the development of peritoneal endometriotic lesions is the adhesion and growth of endometrial fragments deposited into the peritoneal cavity *via* retrograde menstruation (11). The coelomic metaplasia theory claims, on the other hand, that

Table II. Expression levels in epithelial and stromal cells for oestrogen (OR) and progesterone receptor (PR).

	Cells (%)	Score	Epithelium	Stroma
			Number of cases	
OR	0	0	13	18
	<20%	1	27	26
	20%-40%	2	6	8
	>40%	3	16	10
PR	0	0	13	15
	<20%	1	22	23
	20%-40%	2	11	10
	>40%	3	16	14

Table III. Expression levels in stromal cells for CD34 and S100.

	Level of expression	Score	Number of cases
CD34	absent to very low	1	8
	low	2	13
	moderate	3	14
	intense	4	27
S100	absent to very low	1	20
	low	2	12
	moderate	3	16
	intense	4	14

Table IV. Spearman correlation test (and statistical significance) between immunohistochemical parameters in recto-vaginal septum endometriosis patients.

	OR epithelium	OR stroma	PR epithelium	PR stroma	CD34	S100
OR epithelium		R: 0.656 p: 0.0001	R: 0.781 p: 0.0001	R: 0.627 p: 0.001	R: 0.693 p: 0.001	R: 0.529 p: 0.001
OR stroma	R: 0.656 p: 0.0001		R: 0.477 p: 0.0001	R: 0.589 p: 0.0001	R: 0.644 p: 0.001	R: 0.551 p: 0.001
PR epithelium	R: 0.781 p: 0.0001	R: 0.477 p: 0.0001		R: 0.731 p: 0.0001	R: 0.600 p: 0.001	R: 0.426 p: 0.001
PR stoma	R: 0.627 p: 0.001	R: 0.589 p: 0.0001	R: 0.731 p: 0.0001		0.665 p: 0.001	R: 0.544 p: 0.001
CD34	R: 0.693 p: 0.001	R: 0.644 p: 0.001	R: 0.600 p: 0.001	R: 0.665 p: 0.001		R: 0.572 p: 0.0001
S100	R: 0.529 p: 0.001	R: 0.551 p: 0.001	R: 0.426 p: 0.001	R: 0.544 p: 0.001	R: 0.572 p: 0.0001	

formation of endometriomas in the ovary or rectovaginal endometriosis is caused by metaplasia of the coelomic epithelium, perhaps induced by environmental factors (12-14). Altered cellular immunity is another proposed pathogenetic mechanism and a lack of adequate immune surveillance in the peritoneum is thought to be a cause of the disease (15). Finally, by linkage analysis, various groups have proposed that endometriosis could be considered a hereditary disease (16-21).

Concerning specifically the pathogenesis of deep endometriosis, it has been proposed that such lesions could originate from metaplasia of mullerian remnants located in the rectovaginal septum, thus consisting an entity different from peritoneal endometriosis (22, 23). This form of endometriosis has great clinical relevance, because it is strongly associated with pelvic pain symptoms (24). Therefore, a better understanding of the anatomical and biochemical characteristics of these lesions could be very

important in order to define new and more efficacious pharmacological treatments. It is well established that, similarly to what occurs for the endometrial growth, the proliferation and differentiation of endometriotic tissue is influenced by the interplay between oestrogen and progesterone (25, 26). The presented data showing great heterogeneity in the expression of oestrogen and progesterone receptors support the hypothesis that the hormonal input could not be the only factor implicated in the growth and proliferation of endometriotic tissue. Especially in the cases where a very low to undetectable expression level for oestrogen receptor was obtained, different pathways of growth would be activated to sustain the growth of endometriotic glands, may be through paracrine mechanisms, involving also the cells of the stroma, as it would be the case of the marked levels of aromatase P450 mRNA and activity detected in the stromal cells of endometriotic cells (27). Indeed, when the relationship between oestrogen and

progesterone receptor expression and level of vascularization and of nerve fibers proliferation was analysed, a significant direct correlation was found. Assuming that angiogenesis represents one of the crucial steps in the pathogenesis and persistence of endometriotic foci and that presence of nerve fibers in endometriotic lesions is implicated in the mechanisms of pain generation in this disease, this data sustains the hypothesis that oestrogen plays an important role in the genesis and maintenance of these biological processes (28-31). Nevertheless, additional studies at molecular levels are required in order to better define the molecular pathways activated or inhibited in endometriotic tissues; in particular, it would be of great interest to outline the paracrine mechanisms elicited in endometriotic cells in which the expression of oestrogen is absent. The authors are currently investigating this phenomenon by means of cDNA array technology with the goal of defining the molecular pathways responsible for the survival of endometriotic cells.

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