Original Article
Correlation of histological and macroscopic findings in peritoneal endometriosis

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Abstract: Context: In the last two decades, a color based concept of disease activity in peritoneal endometriosis has been in use in the clinical context, with red lesions being considered active and black or white lesions being interpreted as less active or dormant. Objective: Our aim was to analyze 4 main color categories of peritoneal endometriosis (black, white, red and brown) in one single patient group using histomorphological and immunohistochemical methods. Design: 65 endometriosis lesions (30 black, 17 white, 11 brown, 7 red) were resected from 47 premenopausal, nulliparous women which had not received exogenous hormones for at least six months prior to the operation. Specimen workup, histomorphological analysis and immunohistochemical analysis were performed in a standardized manner. Results: The color categories showed a broad overlap in proliferative activity and hormone receptor expression. Differences were found in lesion morphology. Adjacent stromal reaction in particular showed a marked increase from red through brown and black to white lesions. Differences were also seen in gland pattern and gland content. Conclusions: Lesion colors in peritoneal endometriosis seem to be determined by gland content and a varying adjacent stromal reaction and more likely reflect an aging process than different levels of disease activity.

Keywords: Endometriosis, colors, proliferation, hormone receptors, age

Introduction

Endometriosis affects between 4% and 30% of women of reproductive age, making this condition one of the most frequent benign gynecological diseases [1]. The disease is defined as the occurrence of patches of endometrial glands and/or stroma outside of the uterine cavity [2]. It was first described in 1860 by Rokitansky, who documented “new growth of uterine glands” in patients with “uterine and ovarian sarcomas” [3].

The etiology of endometriosis has not been fully explained yet despite a high clinical and pathological interest. The most widely accepted theory was proposed by Sampson who postulated in 1927 that endometriosis is caused by the retrograde flow of menstrual blood through the fallopian tubes with subsequent dissemination and implantation of endometrial cells in the peritoneum [4].

Standardized research on endometriosis has been hampered by the highly heterogeneous morphology of the disease. The spectrum of colors is quite broad, ranging from black through brown to red and white, transparent foci [5-7], rendering standardized description difficult. Sampson himself described “blueberry blue” and “raspberry red” lesions [7]. Clinical observation of the variable morphology of endometriosis lesions gave rise to the question of whether different colors in the lesions also reflect differing histology and biology in endometriosis. Several study groups addressed this issue with different methods. Redwine [8] and Goldstein et al. [9] reported, on the basis of laparoscopic observations, that red lesions precede other lesion colors. In 1991, Köhler and Lorenz were the first to present a color-related description of the histology of endometriosis. In a schematic overview, they described a decline in endometrial glands, endometrioid stroma, and hormone receptor expression along with a
simultaneous increase in collagenous fibers from colorless through red to blue-black lesions. Unfortunately, the underlying data and methodology were not presented in the study [6]. Nisolle et al. investigated vascularization and proliferation in red, black, and white lesions, finding the strongest vascularization and highest level of proliferative activity in red lesions and the lowest level of proliferative activity in white lesions [10]. Donnez et al. reported a higher level of vascular endothelial growth factor (VEGF) content in red lesions in comparison with black lesions [11]. On the basis of this data, Nisolle et al. [12] suspected - like Köhler and Lorenz [6] and Brosens [13] - that red lesions represent “fresh” implants, while white foci correspond to dormant lesions.

Based on these observations, it was postulated that the different lesion colors also reflect differing disease activity [14, 15]. However, up to now - to the best of our knowledge - the main color categories have not been comprehensively investigated in a single group of patients.

The aim of the present study was to investigate histology, proliferative activity and hormone receptor expression in black, white, brown and red endometriosis lesions obtained from a single group of patients.

Materials and methods

Patients

The study collective comprised 47 premenopausal, nulliparous women aged 19-52 years which had not received exogenous hormones for at least six months prior to the operation. Endometriosis was suspected clinically in all of the patients and a diagnostic laparoscopy with tissue resection was performed in all of the cases. All patients provided written informed consent to the histopathological workup of their endometriosis lesions. The local ethics committee approved the study. Any prior abdominal surgery was an exclusion criterion. Patients were recruited from September 2011 to November 2012.

Sampling and tissue processing

The patients’ pelvic and abdominal peritoneum was examined laparoscopically. Endometriosis lesions were photographed intraoperatively and their location, size, and color were documented. Lesions that could be clearly classified as “black”, “white”, “brown” or “red” were sharply resected without coagulation. All in all, 30 black, 17 white, 11 brown and 7 red endometriosis lesions were excised (total 65). Eutopic endometrium was also obtained in 27 patients (26 x streak curettage, 1 x hysterectomy).

After 24 hours of fixation, the peritoneal specimens were inked on the resection margin, laminated at right angles to the peritoneal surface and embedded in paraffin, oriented so that the entire width of the specimen was demonstrated on the cut surface. 3 µm sections were taken from all of the paraffin blocks and stained with hematoxylin-eosin (HE). On the basis of HE morphology, the block with the largest proportion of endometriosis lesions was selected for specialized histochemical staining (Berlin Blue (BB), Elastica van Gieson (EvG)) and immunohistochemistry.

Immunohistochemical staining

Immunohistochemical stains were performed on 1 µm sections using the fully automatic BenchMark ULTRA staining machine (Ventana Medical Systems, Inc., Tucson, Arizona, USA) in accordance with the manufacturer’s specifications. The immunohistochemical panel included: estrogen receptor (ER; CONFIRM anti-ER [SP1], alpha chain, ready-to-use; Ventana); progesterone receptor, (PR; lyophilized monoclonal mouse progesterone receptor [NCL-PGR-312], 1:200; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom); Moleculare Immunologie Bortsel1 (MIB1; monoclonal mouse anti-human Ki-67 antigen [clone MIB-1], 1:100, Dako Ltd., Glostrup, Denmark); cluster of differentiation 10 (CD10; lyophilized monoclonal mouse antibody CD10 [NCL-CD10-270], 1:20, Novoceastra); actin (monoclonal mouse anti-human smooth muscle actin [clone 1A4], 1:400, Dako); desmin (monoclonal mouse anti-human desmin [clone D33], 1:50, DakoCytomation); and caldesmon (monoclonal mouse anti-human caldesmon [clone h-CD], 1:100, Dako).

Histological and immunohistochemical analysis

Histological and immunohistochemical analyses were carried out by two pathologists (J.D.S. and D.L.W.) who worked independently and in
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ignorance of the clinically documented lesion colors. Divergent results were discussed until consensus was reached.

Hematoxylin-eosin analysis: Gland pattern, gland content and grade of endometrioid stroma were assessed. In addition, the presence or absence of neural structures and inflammatory infiltrates in the area of the endometriosis lesions were noted.

The gland pattern categories were defined as “large”, “medium”, “small” and “canalicular/collapsed” (Figure 1). “Large” endometrioid glandular structures characteristically were strongly dilated and showed irregular contours, whereas medium-sized and smaller glandular structures possessed round, regular shapes. Canalicular/collapsed glands had narrow, slit-like to completely obliterated glandular lumens.

The glandular content was defined as “bloody” or “serous/empty”, with the category “bloody” including both fresh erythrocytes and also old-blood liquid and/or pigment-storing macrophages.

The amount of endometrioid stroma was graded as “sparse” (+), “moderate” (++), and “abundant” (+++). “Sparse” represented only focal patches of endometrioid stroma incompletely surrounding the endometrioid glands. A wider rim of endometrioid stroma at least partly surrounding the endometrioid glands was described as “moderate”. The category “abundant” referred to a broad rim of endometrioid stroma completely enveloping the endometrioid glands.

Berlin blue stain: In the BB stain, the endometriosis lesions were examined for hemosiderin deposits. The quantity and distribution pattern of intracellular and extracellular hemosiderin pigment within the endometriosis lesions were documented.

Elastic van gieson stain: The adjacent stromal reaction in the endometriosis lesions was ana-

Figure 1. Gland patterns in peritoneal endometriosis; Hematoxylin-Eosin; 40x magnification. A: Large gland pattern, B: Medium gland pattern, C: Small gland pattern, D: Canalicular/collapsed gland pattern.
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Lyzed using the EvG stain, with the individual components “collagenous fibers”, “elastic fibers”, and “smooth-muscle metaplasia” being evaluated separately. In EvG stains collagen fibers are strongly pink, elastic fibers stain black, and smooth muscle appears yellowish to light brown (Figure 2). The grading of these three components of the stromal reaction was differentiated into the categories of “sparse” (+), “moderate” (++), and “abundant” (+++), as in the scoring system for endometrioid stroma detailed above. The grading of smooth muscle metaplasia was later refined with immunohistochemistry of smooth muscle markers.

**Immunohistochemical analysis**

*Smooth-muscle metaplasia:* Immunohistochemical staining for Actin, Desmin, and Caldesmon was carried out to confirm the smooth-muscle metaplasia previously identified using EvG staining. Fragmented, irregular courses of the smooth-muscle fibers were classified as evidence of smooth-muscle metaplasia. By contrast, a regular fascicular arrangement suggested localized smooth muscle.

*Estrogen receptor/progesterone receptor:* ER and PR expression were quantified separately in the endometrioid epithelium and stroma using the Immunoreactive Score (IRS). Hormone receptor expression was evaluated as “+” (low, scores 1-4), “++” (moderate, scores 5-8) or “+++” (high, scores 9-12). The entire endometriosis lesion was taken into account for quantification of hormone receptor expression.

*Proliferative activity:* Proliferative activity in the endometrioid epithelium and stroma was evaluated using MIB1 staining. Counting was performed in the areas of highest proliferation activity as identified in low magnification (25x). To determine proliferation activity in the endometrioid epithelium, the mean number of MIB1-positive cell nuclei in three areas of 100 adjoining epithelial cells was calculated. (“Number of MIB1-positive cells per 100 epithelial cells”). The proliferation activity in the endometrioid

![Figure 2. Components of the stromal reaction adjacent to endometriosis lesions; Elastica von Giessen; 40x magnification. A: Mostly collagen fibers (pink stain), B: Mostly smooth muscle metaplasia (yellowish stain), C: Mostly elastic fibers (grey-black stain), D: Mixed lesion.](image-url)
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Table 1. Overview of all of the results

<table>
<thead>
<tr>
<th>Subcategories</th>
<th>Black lesions (n=30)</th>
<th>White lesions (n=16)</th>
<th>Brown lesions (n=11)</th>
<th>Red lesions (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gland size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>16/30</td>
<td>5/16</td>
<td>2/11</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>8/30</td>
<td>6/16</td>
<td>5/11</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>2/30</td>
<td>3/16</td>
<td>1/11</td>
</tr>
<tr>
<td></td>
<td>Collapsed</td>
<td>4/30</td>
<td>2/16</td>
<td>3/11</td>
</tr>
<tr>
<td></td>
<td>Gland content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serous/empty</td>
<td>4/30</td>
<td>9/16</td>
<td>5/11</td>
</tr>
<tr>
<td></td>
<td>Bloody</td>
<td>26/30</td>
<td>7/16</td>
<td>6/11</td>
</tr>
<tr>
<td></td>
<td>Proliferation endometrioid epithelium</td>
<td>MIB + epithelial cells/100 epithelial cells</td>
<td>21.2 ± 19.3</td>
<td>14.2 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>Gland content</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Serous/empty</td>
<td>4/30</td>
<td>9/16</td>
<td>5/11</td>
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<tr>
<td></td>
<td>Bloody</td>
<td>26/30</td>
<td>7/16</td>
<td>6/11</td>
</tr>
<tr>
<td></td>
<td>Proliferation endometrioid stroma</td>
<td>MIB + stromal cells/hpf</td>
<td>13.2 ± 11.8</td>
<td>3.8 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>ER expression endometrioid epithelium</td>
<td>(+)</td>
<td>1/30</td>
<td>0/16</td>
</tr>
<tr>
<td></td>
<td>ER expression endometrioid stroma</td>
<td>(+)</td>
<td>2/30</td>
<td>1/16</td>
</tr>
<tr>
<td></td>
<td>PR expression endometrioid epithelium</td>
<td>(+)</td>
<td>4/30</td>
<td>2/16</td>
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<td></td>
<td>PR expression endometrioid stroma</td>
<td>(+)</td>
<td>9/30</td>
<td>2/16</td>
</tr>
<tr>
<td></td>
<td>Collagen fibers</td>
<td>(+)</td>
<td>3/30</td>
<td>1/16</td>
</tr>
<tr>
<td></td>
<td>Elastin fibers</td>
<td>(+)</td>
<td>12/30</td>
<td>3/16</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle metaplasia</td>
<td>(+)</td>
<td>11/30</td>
<td>4/16</td>
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<tr>
<td></td>
<td>Endometriotic stroma</td>
<td>(+)</td>
<td>11/30</td>
<td>4/16</td>
</tr>
<tr>
<td></td>
<td>Inflammatory infiltrates</td>
<td>present</td>
<td>4/30</td>
<td>2/16</td>
</tr>
<tr>
<td></td>
<td>Not present</td>
<td>26/30</td>
<td>14/16</td>
<td>9/11</td>
</tr>
<tr>
<td></td>
<td>Nerves</td>
<td>present</td>
<td>15/30</td>
<td>9/16</td>
</tr>
<tr>
<td></td>
<td>Not present</td>
<td>15/30</td>
<td>7/16</td>
<td>7/11</td>
</tr>
</tbody>
</table>
stroma was given as the mean number of MIB1-positive cell nuclei in three high power fields (hpf, at 400x) (“number of MIB1-positive cell nuclei per high-powered field with field number 25 (hpf/FN 25)”).

**Data processing and statistical analysis**

In addition to the 4 color categories, three menstrual cycle categories were defined: “proliferative phase” (day 1-14), “secretory phase” (day 15-28) and “irregular/absent cycle”. This categorization was based on the clinical history of the patients and the cycle day given by the patients. In 27 of the 47 cases, eutopic endometrium was available for examination and the cycle phase given by the patient could be corroborated histologically.

Histomorphological and immunohistochemical characteristics were analyzed in regard to color categories and menstrual cycle categories.

For proliferative activity and hormone receptor expression, means and standard deviation were calculated in the various categories. The color categories and menstrual cycle categories were assessed for significant differences using the Kruskal-Wallis test.

A P-value of 0.05 was considered significant. In case of significant results, the Levene test was used to check the equality of variances in the groups. In case of homogeneous variance, the Bonferroni test was carried out as a post-hoc test; if there was inhomogeneous variance, the Games-Howell test was used.

**Results**

**Table 1** provides an overview of all of the results.

**Histology**

Clear trends were evident for the histological criteria of “glandular growth pattern” and “glandular content” in black and red lesions. Black lesions frequently showed large glandular structures (16/30), and the glandular content was usually bloody (26/30). By contrast, red lesions with identifiable glandular lumina had serous content in all cases (6/6). White and brown lesions showed variable results in relation to both categories.

Trends were also evident in relation to the adjacent stromal reaction (Figure 3). Red lesions showed mainly sparse elastic fibers (5/7) and...
smooth-muscle metaplasia (5/7), with moderate collagenous fiber content (5/7). Many white lesions, in contrast, displayed an abundant amount of collagenous fibers (10/16), elastic fibers (7/16) and smooth-muscle metaplasia (8/16). Black lesions were in an intermediate position, with mainly moderate (16/30) to abundant (11/30) collagen content, variable smooth-muscle metaplasia, and low (12/30) to moderate (13/30) elastic fiber content. The brown lesions were also in an intermediate position. Overall, an increase in the degree of stromal reaction was evident from red lesions through black and brown lesions to white lesions.

Most red lesions showed a moderate degree (6/7) of endometrioid stroma. Black and brown lesions contained mainly moderate (15/30 and 7/11, respectively) or sparse (11/30 and 3/11, respectively) amounts of endometrioid stroma. In contrast, white lesions showed mostly sparse endometrioid stroma (10/17). A slight declining trend from the red lesions through black and brown lesions to white lesions thus became apparent in this category.

Inflammatory infiltrates were absent in the majority of cases in red and brown lesions (5/7 and 7/11, respectively). However, they were present in half of the black lesions (5/7) and in more than half (10/17) of the white lesions.

By contrast, no trends were observed in relation to the detection of nerves inside the endometriosis lesions, with the presence of nerval structures being rare in all color categories.

**Immunohistochemistry**

**Hormone receptors:** In the immunohistochemical analysis of ER expression, no major differences were observed between the color categories either with regard to endometrioid epithelium or stroma. ER expression was uniformly high in all color categories (endometrioid epithelium: 27/30, 15/16, 11/11, and 6/7, respectively; endometrioid stroma: 28/30, 15/16, 11/11, and 6/7, respectively). With regard to the menstrual cycle categories, a declining trend was observed from the proliferative to the secretory phase in white, black, and red lesions. However, this trend was only slight, as all lesions had at least moderate ER expression.

PR expression showed a more variable picture. In the red lesions, epithelial PR expression was mainly strong (6/7). The proportion of cases with strong epithelial PR expression was lower in black and white lesions (16/30 and 12/16, respectively). Moderate epithelial PR expres-
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Proliferative activity: No significant differences were observed with regard to epithelial proliferative activity in the four color categories. Stromal proliferative activity was significantly higher in black than in white lesions ($P=0.019$). No other significant differences were identified in this context (Table 2, Figure 4A, 4B).

The menstrual cycle categories were regarded separately within the individual lesion colors. A statistically significant difference ($P=0.037$) was found between the proliferative and the secretory phase in the black category in relation to epithelial proliferation. No further significant differences were observed in the individual color categories regarding the menstrual cycle categories (Table 2).

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Proliferative activity was also analyzed with in the menstrual cycle categories independently of lesion color (Figure 4C, 4D). A significant difference ($P=0.002$) was noted in epithelial proliferative activity between samples from the proliferative and the secretory phase and between samples from the proliferative phase and the group of absent/irregular cycle ($P=0.002$). There were no significant differences between the menstrual cycle categories with regard to stromal proliferation.

Discussion

Histology

Our analysis showed considerable overlapping of results in all of the color categories with regard to both histological and immunohistochemical characteristics. However, clear trends were also evident. Thus the black coloring of endometriosis lesions is well explained by the presence of large endometriosis glands with bloody content. However, on BB staining, only sparse hemosiderin deposits were found. Furthermore, no glandular or stromal fragments were evident in the glandular lumina. This contradicts the commonly held view that the blood seen in endometriosis lesions is caused by a process analogous to that of menstrual withdrawal bleeding [12] and points in the direction of simple lesional bleeding.

Regarding adjacent stromal reaction, there is an increase in the quantity of collagenous fibers, elastic fibers and smooth-muscle metaplasia from red lesions through brown and black to white lesions. Also, there is a slight declining trend in endometrioid stroma from red lesions through brown and black to white lesions. A strong adjacent stromal reaction is probably the reason why some endometriosis lesions appear macroscopically white, even though they contain endometrioid glandular structures that are partly filled with blood. Previously, it has been postulated that white lesions are almost completely scarified lesions which contain only sparse endometriosis foci [16, 17]. The white lesions in our collective did indeed show high-grade adjacent stromal reaction. However, easily identifiable patches of endometriosis were found in all cases. These findings are more compatible with endometriosis being “walled in” by a strong stromal reaction than with “burnt-out” endometriosis.

All in all, our histomorphological findings seem to reflect an “aging process” from red through brown and black to white lesions. The fact that inflammatory infiltrates are found comparatively more often in black and white lesions may be regarded as further evidence of a greater “age” of black and white endometriosis. The concept that lesion color reflects on lesion age was already put forward by Köhler and Lorenz in 1991 [6]. More recently, the aging of endometriosis has been demonstrated in an animal model with baboons [18]. Furthermore, Sohler et al. [19] have shown through molecular studies that tissue remodeling generally plays an important role in endometriosis lesions. However no distinctions were made between lesion colors in this publication.

Immunohistochemistry

Hormone receptors: Epithelial and stromal ER expression and stromal PR expression were generally high in all color categories. Only epithelial PR staining showed some variability, with moderate to low epithelial PR expression being demonstrated in over half of brown and black lesions. This is in contrast to the color concept of Köhler et al who postulated a decline in hormone receptor expression from non-pigmented to pigmented lesions [6].

With relation to menstrual cycle categories, we found only a slight decrease of ER and PR expression from the proliferative to the secretory phase. This finding contrasts somewhat with the results reported by Nisolle et al. [12], who observed significantly higher levels of ER and PR expression in the proliferative phase in comparison with the secretory phase in the endometrioid epithelium of black and red lesions and in the endometrioid stroma of red lesions. The persistence of hormone receptor expression throughout the menstrual cycle documented in the present study may be regarded as further evidence of a certain degree of endocrine autonomy within endometriosis lesions.

Proliferative activity: No significant differences between the color categories were observed with regard to epithelial proliferation activity. However, a significant difference was found between black and white lesions regarding stromal proliferation activity. Our results thus partly corroborate and partly contradict those of Nisolle et al., who documented significantly
lower proliferation activity in white lesions and significantly higher proliferation activity in red lesions compared to black lesions [10, 12]. When endometriosis lesions were regarded irrespective of lesion color, there was a significant difference between the proliferative phase and the secretory phase and also between the proliferative phase and the group of irregular/absent menstruation. When analysis by menstrual cycle categories was performed on individual colors, significant differences were seen for epithelial proliferation between the proliferative and the secretory phase in black lesions. The present data therefore provide some evidence of a cycle-related variability of proliferation activity in endometriosis lesions.

Study design: In the present study, the assignment of the menstrual cycle phase was based on clinical history and the cycle day given by the patient. It is possible that imprecise information may have been provided in some cases. In 27 of 47 cases, eutopic endometrium was available for histomorphologic study. All of the samples of eutopic endometrium from patients with a regular cycle corresponded histologically to the cycle phase given by the patients. However, additional clinical testing with analysis of hormone levels would have rendered the menstrual cycle categories more robust.

Conclusions

In the literature the impression is often given that the histology and the biology of peritoneal endometriosis lesions have been comprehensively elucidated. However, our knowledge of endometriosis lesions is still incomplete and based on a small number of publications. The results of our study contradict the concept that the different colors of endometriosis lesions reflect different degrees of biologic activity as defined by proliferation activity and hormone receptor expression. Our data suggest that lesion color in endometriosis is associated with lesion age, gland pattern and gland content rather than with biologic activity. Regarding the menstrual cycle, we documented consistently high levels of hormone receptor expression in endometriosis lesions in all three cycle categories, a finding which may be indicative of endocrine autonomy. However, we also saw significant differences in proliferation activity between the three cycle categories, suggesting that endometriosis lesions are, to a certain degree, responsive to the menstrual cycle.

To date, there has only been one study providing evidence of a connection between proliferation activity and hormone receptor expression and the symptoms of peritoneal endometriosis [14]. Further studies are needed in order to determine whether the severity of symptoms in endometriosis does indeed correlate with a particular morphological, histological or immunohistochemical characteristic of the endometriosis lesion.

Disclosure of conflict of interest

None.

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