

*Original article*

**Occult microscopic endometriosis: an undetectable finding by laparoscopy in normal peritoneum**

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***Running title:*** Occult endometriosis in normal peritoneum

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## **Abstract**

**Title:** Occult microscopic endometriosis: an undetectable finding by laparoscopy in normal peritoneum

**Study question:** Are there any occurrence and activity of hidden (occult) endometriotic lesions, an undetectable finding by naked eyes, in normal peritoneum of women with and without visible endometriosis?

**Summary answer:** We detected higher occurrence of occult microscopic endometriosis (OME) in normal peritoneum of women with visible endometriosis than in control women and confirmed that these OME lesions have variable amount of tissue activity as measured by proliferative potentiality of these lesions.

**What is known already:** Using small number of cases, the concept of invisible microscopic endometriosis or OME in visually normal peritoneum has been reported in more than a decade ago with a controversial opinion regarding their tissue activity and clinical significance.

**Study design, size and duration:** Case-controlled biological research using prospectively collected normal peritoneal samples from women with and without visible

endometriosis and their retrospective evaluation.

**Participants/materials, setting, methods:** Normal peritoneal biopsy specimens from different anatomical sites of pelvis were collected from 151 women with endometriosis and 62 control women during laparoscopy. A histological search of all peritoneal biopsy specimens for the detection of invisible endometriosis was done and confirmed by immunoreaction to Ber-EP4 (epithelial cell marker), CD10 (stromal cell marker), and Calretinin (mesothelial cell marker). Tissue expressions of estrogen/progesterone receptors (ER/PR) and cell proliferation marker, Ki-67 were performed by immunohistochemistry to identify tissue activity.

**Main results and the role of chance:** Three different patterns of OME were detected based on (I) presence of typical gland/stroma, (II) reactive hyperplastic change of endometrioid epithelial cells with surrounding stroma, and (III) single-layered epithelium-lined cystic lesions with surrounding stroma. A higher tendency in the occurrence of OME was found in women with visible endometriosis (15.2%, 23/151) comparing to control women (6.4%, 4/62) ( $p=0.06$ ,  $X^2$  test). The epithelial cells and/or stromal cells of OME lesions were immunoreactive to Ber-EP4/CD10 but non-reactive to

Calretinin. ER and PR expression was observed in all patterns OME lesions. Ki-67 index was significantly higher in pattern I/II OME lesions than in pattern III OME lesions ( $p < 0.05$  for each). We re-confirmed a decade long old concept of invisible (occult) endometriosis in visually normal peritoneum of women with visible endometriosis and found that a proportion of these occult peritoneal lesions displayed increased tissue activity.

**Limitations, reasons for cautions:** Bias in the incidence rate of OME lesions in this study cannot be ignored, because we could not analyze biopsy specimens from the Pouch of Douglas of women with r-ASRM stage III-IV endometriosis due to the presence of variable amount of adhesion in the pelvis.

**Wider implications of the findings:** Our time-consuming current findings indicate the acceptability of OME lesions in normal peritoneum even it is undetected by laparoscopy. The existence of a variable amount of tissue activity in these occult lesions may contribute to the recurrence/occurrence of endometriosis or persistence/recurrence of pain manifestation in women even after successful ablation or excision of visible lesions by laparoscopy.

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**Trial registration number:** not applicable.

**Key words:** visible endometriosis / occult endometriosis / ER / PR / Ki-67

## **Introduction**

Endometriosis is an estrogen-dependent chronic inflammatory disease mostly affecting women of reproductive age. Originally described over three hundred years ago, endometriosis is classically defined by the presence of endometrial glands and stroma in extrauterine locations (Burney and Giudice, 2012). There are some established hypotheses and regulatory factors supporting the development or maintenance of endometriosis (Burney and Giudice, 2012; Attar and Bulun, 2006). Recently it has been demonstrated that besides hormonal regulation, both secondary and initial inflammatory mediators are known to involve in the growth of endometriosis (Khan et al., 2008, 2009, 2010a). However, it is difficult to uniformly explain the pathogenesis of endometriosis by

a single factor. Even after long three hundred years, most of the literatures still claim that exact pathogenesis of endometriosis is unclear and stamp endometriosis as an enigmatic disease. Recurrence of pain and lesion is still occurring after effective medical or surgical therapies.

The detection and visible diagnosis of peritoneal endometriosis is usually performed by laparoscopy, a gold standard modality and is microscopically confirmed by histopathology. Even with the careful eyes of expert surgeons, there is obvious chance to miss or overlook hidden (occult) lesions in visually normal peritoneum. Therefore, immense interest could arise to randomly collect visually normal peritoneum from different anatomical location in pelvis and to investigate the nature of these visually undetectable lesions of endometriosis. The concept of microscopic endometriosis in visually normal peritoneum was first reported by Murphy (1986) and subsequently confirmed with an incidence rate of 6-13% (Nisolle et al., 1990, Balasch et al., 1996). With the elapse of more than one decade, further information on invisible or occult microscopic endometriosis (IME or OME) is lacking.

Therefore, we histologically examined all biopsy specimens derived from the

visually normal peritoneum of women with and without endometriosis to detect the possible occurrence of OME. The question still remains, if hidden endometriosis lesions could be detected in normal peritoneum, are these invisible lesions really inactive as proposed in a previous report? (Donnez and Langrndonckt, 2004) or they truly retain some tissue activity. If tissue activity of OME is there, this could be a clinically important issue. To address this question, we investigated the expression patterns of some tissue activity markers including ovarian steroid receptors and cell proliferation marker in histologically confirmed OME lesions. With the belief in mind that all cells of epithelial origin are embryologically derived from coelomic epithelium, we further extended our experiment to examine the immunoreaction of CA125/MUC16, a marker of cells derived from coelomic epithelium and its derivatives, in visible peritoneal lesions and in OME lesions. Finally we discussed the possible origin of OME, so called IME.

## **Materials and Methods**

*Subjects.* During the period between January 2005 and June 2010, peritoneal biopsy samples from different anatomical locations in pelvis were randomly and prospectively collected from women with and without visible endometriosis during

laparoscopy (Storz, Germany). With the concept of occult endometriosis in mind, we collected peritoneal biopsy samples together with visible peritoneal lesions. During this study period, we were able to collect a total of 895 peritoneal biopsy samples from 387 women with and without endometriosis.

The anatomical location in pelvis and quality of each collected peritoneal biopsy specimens were retrospectively reviewed and confirmed by video image and tissue observation (KNK, AF, MK). Visually normal peritoneum was examined from a distance of 3-4cm and an effort was made to select normal peritoneum based on the following criteria: (i) smooth peritoneal surface with no text irregularity, (ii) no abnormal vascular pattern, (iii) transparent peritoneum, (iv) no sub-peritoneal cystic structures, and (v) no superficial fibrosis. A sample of visually normal peritoneum is shown in Figure 1.

We used our laparoscopic technique where the laparoscope was 3-4cm away from the peritoneal surface and is almost similar to the technique described by Nisolle et al. (1990).

After careful observation and analysis, we could finally sorted out 227 visually normal peritoneal samples from 151 women with visible endometriosis and 78 samples from 62 women without any visible peritoneal lesions (control). We did not analyze remaining



peritoneal samples from the rest of 174 women, because these samples did not satisfy the criteria of normal peritoneum.

Women with visible endometriosis aged between 23 and 50 years were recruited by either elective laparoscopy for infertility or diagnostic laparoscopy for dysmenorrhea and subsequently confirmed by histology. The control group, between 19 and 48 years old, consisted of fertile women without any evidence of visible endometriosis and were operated on for benign ovarian cysts (dermoid cyst/serous cyst adenoma/mucinous cyst adenoma). The staging and the morphological distribution of peritoneal lesions were based on the revised classification of the American Society of Reproductive Medicine (r-ASRM) (1997). All control women and women with endometriosis had regular menstrual cycles (28-35 days) except cases with hormonal therapy. The phases of the menstrual cycle was determined by histological dating of eutopic endometrial samples taken simultaneously with pathological lesions during laparoscopy.

The distribution of biopsy specimens from visually normal peritoneum based on anatomical location in pelvis is as follows: visible endometriosis (n=227), Pouch of

Douglas (n=87), uterovesicle space (n=104), right sacrouterine ligament (n=20), left sacrouterine ligament (n=16); control women (n=78), Pouch of Douglas (n=32), uterovesicle space (n=35), right sacrouterine ligament (n=6), left sacrouterine ligament (n=5). We could not collect biopsy specimens from the Pouch of Douglas in women with r-ASRM stage III-IV endometriosis due to the presence of adhesion. Instead, we collected peritoneal biopsy specimens from other anatomical locations in these women. After holding up peritoneum with forceps, all peritoneal biopsy samples were collected from respective sites by cutting with scissor 1cm in depth and with a length of 1.0 to 2.5 cm. In our initial trial, we could not collect enough connective tissue just by punch biopsy from any of these anatomical sites.

All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board of Nagasaki University. An informed consent was obtained from all women.

***Antibodies used.*** We performed immunohistochemical studies to investigate immunoreaction of target antigen in the serial section of biopsies using following antibodies: Ber-EP4 (epithelial cell marker, 1:200), M0804, mouse monoclonal, Dako,

Denmark; CD10 (stromal cell marker, 1:40), 56C6, mouse monoclonal, Dako, Denmark; Calretinin (mesothelial cell marker, 1:50), SP13, rabbit monoclonal, Nichirei Bioscience, Japan; ER (estrogen receptor, 1:50), ER1D5, mouse monoclonal, Dako, Denmark; PR (progesterone receptor, 1:40), NCL-PGR, mouse monoclonal, Dako, Denmark; aromatase cytochrome P450 (estrogen metabolizing enzyme, 1:100), ab18995, rabbit polyclonal, abcam, Japan; Ki-67 (cell proliferation marker, 1:100), MIB-1, mouse monoclonal, Immunotech, Marseille, France; Toll-like receptor 4 (TLR4, a pattern recognition receptor, 1:50), ab22048, mouse monoclonal, abcam, Japan; D2-40 (lymphatic vessel marker, 1:100), M3619, mouse monoclonal, Dako, Denmark; von Willebrand factor (VWF, micro-vessel marker, 1:25), F8/86, M0616, mouse monoclonal, Dako, Denmark; CA125/MUC16 (marker of cells derived from coelomic epithelium, 1:20), M11, mouse monoclonal, Dako, Denmark. Non-immune mouse immunoglobulin (Ig) G1 antibody (1:50, Dako) was used as a negative control.

In an attempt to detect hidden lesions of endometriosis, all slides of biopsy specimens derived from visually normal peritoneum were histologically examined and any specimen with microscopic lesion suspected of endometriosis was re-examined and

confirmed by an expert histopathologist of our University (MN).

***Immunohistochemistry.*** The details of immunohistochemical staining were described elsewhere (Khan et al., 2003, 2004, 2005; Ishimaru et al., 2004). We used at least three slides per biopsy for immunohistochemical analysis. Briefly, five-micrometer thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After immersion in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol to block endogenous peroxidase activity, sections were pre-incubated with 10% normal goat serum to prevent nonspecific binding and then incubated overnight at 4°C with respective antibodies. The slides were subsequently incubated with biotinylated second antibody for 10 minutes, followed by incubation with avidin-peroxidase for 10 minutes and visualized with diaminobenzidine. Finally, the tissue sections were counterstained with Mayer's hematoxyline, dehydrated with serial alcohols, cleared in xylene, and mounted.

The immunoreactivities of ER/PR, aromatase cytochrome P450, TLR4, and CA125/MUC16 in biopsy specimens were quantified by a modified method of quantitative-histogram score (Q-H score) as described elsewhere (Khan et al., 2003, 2005; Ishimaru et al., 2004). The Q-H score was calculated using the following equation:

Q-H score =  $\sum P_i (i+1)$ , where  $i = 1, 2$  or  $3$  and  $P_i$  is the percentage of stained cells for each intensity. The staining intensity was graded as  $0 = \text{no}$ ,  $1 = \text{weak}$ ,  $2 = \text{moderate}$ , and  $3 = \text{strong}$ . We calculated the mean Q-H scores of five different fields of one section by light microscopy at moderate magnification ( $\times 200$ ).

The cell proliferation index (Ki-67 index) in each tissue section was calculated by measuring the mean percentage of Ki-67-positive nuclei among total cells in four different microscopic fields ( $\times 200$ ) as we described before (Khan et al., 2010b). The lymphatic vessel and micro-vessel density, as measured by total vessel number and as immunoreactive to D2-40 and VWF, respectively, were counted by light microscopy of those areas that contained the highest number of lymphatics, capillaries and venules, as described elsewhere (Khan et al., 2003). The total lymphatic vessel and micro-vessel counts were evaluated in the same specimen.

By the term “tissue activity” in this study, we mean the capacity of a tissue or OME lesion to express different molecular markers and to have variable proliferative potentiality in the examined tissue or lesion.

***Statistical Analysis.*** All results are expressed as either mean  $\pm$  SD or mean  $\pm$

SEM. The clinical characteristics of the subjects were compared with one-way analysis of variance and the  $X^2$  test was used for any difference between two groups. Mann-Whitney *U*-test or Student's *t*-test was used to analyze any difference in protein expressions between two groups. For comparisons among groups, the Kruskal-Wallis test was used. A box plot analysis of different protein expression was performed using the medians and inter-quartile range (IQR). A value of  $p < 0.05$  was considered to be statistically significant.

## **Results**

There were no significant differences in clinical characteristics between 151 women with visible endometriosis and 62 control women without endometriosis (Table 1). We could collect visually normal peritoneum from these two groups of women for our study purpose. All OME lesions were detected at a distance of 100-800  $\mu$  m beneath the mesothelium of normal peritoneum.

We detected three patterns of OME: (I) presence of typical gland/stroma, (II) reactive hyperplastic change of endometrioid epithelial cells with surrounding stroma, and (III) single-layered epithelium-lined cystic lesions with surrounding stromal cells.

We could detect variable patterns of OME in the peritoneum derived from 23 women with endometriosis (biopsy samples, n=27) and 4 control women (biopsy samples, n=4) without visible endometriosis. The detection rate of OME was as follows: for endometriosis, 15.2% (23/151) and 11.8% (27/227); for control women, 6.4% (4/62) and 5.1% (4/78) by the number of patients and number of collected samples, respectively. A higher tendency in the incidence of OME was found in women with visible endometriosis than in control women (p=0.06 by patient number and p=0.07 by sample number, analyzed by  $X^2$  test) (Table 2). With the concept of stromal endometriosis in mind, we found it in only 3 cases. We did not include them as a separate pattern (pattern IV) due to less sample number. All these 3 cases with endometriotic stroma were immunoreactive to CD10 and non-reactive to either Ber-EP4 or Calretinin.

The distribution of three patterns of OME was as follows: endometriosis, pattern I (n=18), pattern II (n=9), and pattern III (n=6); control women, pattern I (n=3), pattern II (n=0), and pattern III (n=1). A total of 33 and 4 IME lesions were detected in women with visible endometriosis and control women, respectively. The detection pattern of OME lesions based on anatomical site of peritoneum was as follows:

endometriosis (total sample, n=27), Pouch of Douglas (n=6), uterovesicle space (n=10), right sacrouterine ligament (n=5), left sacrouterine ligament (n=6); control women (total sample, n=4), Pouch of Douglas (n=1), uterovesicle space (n=2), right sacrouterine ligament (n=0), left sacrouterine ligament (n=1). A predominance of OME occurrence was observed in Pouch of Douglas and uterovesicle space.

Some normal peritoneal samples displayed more than one OME lesion. Therefore, a total of 33 OME lesions were detected in 27 peritoneal samples in women with visible endometriosis. We found peritoneal pockets in the peritoneum of two cases that were derived from rectovaginal pouch of women with visible endometriosis but these two cases lacked OME lesion anywhere in the pelvis including pockets.

The clinical profiles of patients with OME in 23 women with visible endometriosis and 4 control women are shown in Table 3. There was no difference in mean ages between them (34.4 vs. 34.2yrs). A dominant presence of r-ASRM stage I-II endometriosis, red/black lesions and complain of dysmenorrhea was observed in women with visible endometriosis harboring OME in their peritoneum.

***Immunostaining pattern of Ber-EP4/CD10/Calretinin in OME.*** We found



that glandular epithelial cells/rim-like epithelial cells and surrounding stromal cells of three patterns of OME lesions were immunoreactive to Ber-EP4 and CD10, respectively but non-reactive to Calretinin, which is a marker of mesothelial cells (Figure 2). Only three OME lesions of pattern II/III showed negative immunoreaction either to Ber-EP4, CD10 or Calretinin. Flat mesothelial cells derived from visually normal peritoneum were immunoreactive to Calretinin, non-reactive to CD10 and partially reactive to Ber-EP4. We used biopsy specimens from mesothelioma as a positive control and found that these cells were strongly immunoreactive to Calretinin (inset, Figure 2).

***Immunostaining pattern of ER and PR in OME.*** A variable pattern of ER and PR immunoexpressions was observed in all OME lesions detected in women with visible endometriosis and control women (Figure 3, A, B). The immunoreactivity of PR as measured by Q-H score appeared to be higher in all patterns of OME lesions detected in women with visible endometriosis comparing to ER expression (Figure 3, C). In contrast to OME lesions, ER/PR expressions did not show any apparent difference in the corresponding eutopic endometria of these two groups of women across the phases of the menstrual cycle (data not shown).

***Immunostaining pattern of aromatase in OME.*** In an attempt to examine the possibility of local estrogen production by OME lesions, we found that all these OME lesions express a variable amount of aromatase P450. An apparent immunoreaction to aromatase was found in visible peritoneal lesions and in OME lesions derived from women with visible endometriosis and in control women (Supplementary Fig. 1).

***Immunostaining pattern of TLR4 in OME.*** As a pattern recognition receptor, immunoexpression of TLR4 was found in red/black lesions, in all patterns of OME diagnosed in women with visible endometriosis and control women (Figure 4, A-C). In addition to visible and occult lesions of endometriosis, TLR4 immunoreactivity was also found in micro-vessels as observed in different anatomical sites of peritoneum derived from women with and without endometriosis (Figure 4, D).

***Immunoexpression of Ki-67 in OME.*** In order to examine the cell proliferation potentiality of OME lesions, we investigated immunoexpression of Ki-67, a cell proliferation marker, in visible peritoneal lesions and in OME lesions (Figure 5). A strong immunoreaction of Ki-67 was found in red lesions and pattern I/II OME lesions in women with visible endometriosis. A weak Ki-67 expression was found in pattern I/III

OME lesions diagnosed in control women (Figure 5, A, B, C). Ki-67 index was significantly higher in red lesions than in black lesions ( $p < 0.05$ ) and in pattern I/II OME lesions than in pattern III OME lesions found in women with visible endometriosis ( $p < 0.05$  vs. pattern I or pattern II) (Figure 5, D).

***Immunostaining pattern of D2-40 and VWF in OME.*** As a marker of lymphatic vessels and vascular micro-vessels, we examined immunoreaction of D2-40 and VWF, respectively, in serial sections derived from visually normal peritoneum. A variable immunoexpression of D2-40 and VWF was found in lymphatic cells and micro-vessels of all examined biopsies derived from women with visible endometriosis and in control women. Number of D2-40/VWF immunoreactive lymphatic/vascular channels appeared to be higher in women with endometriosis than in controls in the peritoneum adjacent to OME lesions. In contrast, lymphatic/vascular numbers appeared to be higher in controls than in women with endometriosis in the peritoneum distant from OME lesions. There was no significant difference in lymphatic or micro-vessel count between these two groups of women in either area (Supplementary Fig. 2 and Fig. 3).

***Immunostaining pattern of CA125/MUC16 in OME.*** A variable

immunoexpression of CA125/MUC16 was found in red/black lesions, pattern I/II/III OME lesions detected in women with visible endometriosis and in pattern I/III OME lesions detected in control women (Figure 6, A, B, C). It was interesting to observe that all three Ber-EP/CD10/Calretinin had non-reactive OME lesions (pattern II/III) were also immunoreactive to CA125/MUC16 (Figure 6, D). We did not find any difference in the immunoreactivity of CA125/MUC16 among these visible and occult lesions as measured by Q-H scores (data not shown). Normal peritoneal mesothelium was also found to be immunoreactive to CA125/MUC16 (inset, Figure 6, D).

## **Discussion**

We re-established more than a decade old concept of IME (Redwine, 1988, 1990; Balasch et al., 1996) using increased number of visually normal peritoneal samples. In order to avoid confusion in the use of the term “invisible” or “non-visible” microscopic endometriosis among laparoscopists, here we used the term “occult” microscopic endometriosis (OME) instead of IME to indicate any hidden lesion in visually normal peritoneum. We demonstrated that occurrence of OME in visually normal peritoneum was higher in women with visible peritoneal endometriosis than in control women. Our

detection rate of OME in women with visible endometriosis (15.2%) and in control women (6.4%) is in consistence with the published incidence rate of 6-13% (Nisolle et al., 1990) and of 6-11% as reported by Balasch et al. (1996). Although rejected previously by Redwine (2003), we re-established the controversial debate once more that the concept of IME or OME indeed exists. Our findings coincide with the findings of Nisolle et al., (1990), Balasch et al., (1996) and Walter et al., (2001) and have further reinforced the concept of OME, the so called IME.

We support the opinion of Donnez et al (2004) and oppose the proposal made by Redwine (2003) that OME is a rare and clinically unimportant entity. We followed some criteria of visually normal peritoneum and collected increased number of samples during a period of five years to re-confirm the previous concept of IME. Unlike previous studies (Donnez and Langrndonckt, 2004; Balasch et al., 1996), we confirmed our findings by random collection of peritoneal biopsy specimens from different anatomical locations and not from a single site. In fact, we found a higher incidence rate of OME in dependent part of pelvis and not in sacrouterine ligaments as reported previously.

The classical histological diagnosis of endometriosis differs in point of views

between gynecologists and histopathologists. All clinical gynecologists accept diagnosis of endometriosis based on the findings of typical endometrial glands with peripheral rim of stromal cells. But the majority of histopathologists disagree with this definition and define endometriosis just by the presence of stromal cells around cells of coelomic epithelium such as gland cells, endometrioid epithelial cells, or mesothelial cells. Since this is a histopathology-based study, we defined endometriosis according to the opinion of an expert pathologist of our University (MN).

Similar to the opinion of our pathologist (MN), Redwine and Yokom (1990) reported that any glandular structure within peritoneal tissue cannot be accepted as true endometriosis without the presence of stroma around glands. With this knowledge in mind, we classified OME lesions into three categories based on the presence of stromal cells in and around glandular structure (pattern I), hyperplastic endometrioid epithelial cells (pattern II) and single-layered epithelium-lined cystic lesions (pattern III). All these three patterns of OME lesions were immunoreactive to Ber-EP4/CD10 but non-reactive to Calretinin. The immunoreaction of Ber-EP4 in pattern II and III lesions may dictate the usefulness of Ber-EP4 staining in differentiating rim of epithelial cells that might be

confused with mesothelium-like cells by hematoxylin and eosin stain. In fact, antibody against Ber-EP4 has been reported to distinguish epithelia from mesothelia (Latza et al., 1990). Only three OME lesions of pattern II/III showed negative immunoreaction either to Ber-EP4/CD10 or Calretinin. Lack of cellular differentiation or improper histogenesis of cells may explain this negative immunoreaction. But we cannot exclude the possibility of endosalpingiosis for these three lesions. Further in depth investigation is needed to clarify this issue.

Even the laparoscopic tip differed between our study and study of Redwine and Nezhat, it is quite reasonable to detect OME lesions under microscope rather than sharp visible power of laparoscopic tip or sharp visual acuity of surgeon's eyes. We presume that it is not always possible to identify an OME lesion located at 100-800  $\mu$  m beneath the mesothelium of normal peritoneum even the laparoscopic lens is in close proximity to the peritoneal surface. In fact, our findings clearly supported findings as depicted in Figure 2 of Redwine's review article (2003). It is clear from findings of Redwine (2003) that with a distance of 3-4 cm from the peritoneal surface, the incidence of IME (here OME) ranges from 5-10% that coincides with our findings and findings of Nisolle (1990)

and Balasch (1996). Since our sample size was larger than that of Redwine's study, our samples were more likely to contain some retroperitoneal glandular inclusions. We believe that further study with contact laparoscope is necessary to carefully differentiate normal from abnormal peritoneum and also to strengthen our current findings.

We found a variable amount of tissue expressions of ER, PR, aromatase, and TLR4 in OME lesions and were detected in women with both visible endometriosis and control women. Although, PR expression appeared to be higher than ER expression in OME lesions, there was no obvious difference in the expression of steroid receptors in the corresponding endometria across the phases of menstrual cycle. Higher PR expression and lower ER expression in OME lesions could be due to the difference in the binding affinities of the antibodies to their respective antigens. The higher PR expression may be involved in proper decidual reaction and slow progression of these OME lesions once they are established. The immunoexpression of aromatase in all OME lesions could be another interesting findings. With the influence of both systemic and local estrogen, these OME lesions, even it is minute in size, may time-dependently increase in size to be recognized by histology.



We previously reported from our laboratory (Khan et al., 2010a) that a small amount of lipopolysaccharide (LPS) is available in pelvis across the phases of the menstrual cycle. This LPS derived from higher colony formation of *Escherichia coli* in menstrual blood (Khan et al., 2010a) may promote the growth of OME lesions after its binding with TLR4. In fact, we found moderate expression of TLR4 in all OME lesions. This growth promoting effect was clearly supported by significantly higher Ki-67 index, a cell proliferation marker, in pattern I and pattern II OME lesions diagnosed in women with endometriosis than in pattern III OME lesions or in control women. We are against the argument by Donnez et al., (2004) that IME (here OME) lesions are quiescent and they are non-active or inactive and that these lesions are clinically irrelevant. From our findings we can at least argue that OME lesions are indeed active and retain variable growth potentiality in response to cyclic estrogen and/or various inflammatory mediators in pelvis, even their concentration is minimal. This could be obviously responsible for the subsequent recurrence or occurrence of endometriotic lesions even after successful excision or ablation of visible peritoneal lesions by laparoscopy. From the logical point of view, it is difficult to trace these growing lesions on the peritoneal surface by repeated

surgical procedures in human. Therefore, consistency with Figure 2 of Redwine's article (2003) and existence of variable biological activity of OME lesions in our study can be considered as something new and important in both biological and clinical science. We can at least speculate from our current findings that OME lesions are not clinically irrelevant rather more or less clinically significant, because these subtle lesions of OME displayed some degree of biological activity. .

The most alarming questions may arise now, 'how can we decide the origin of OME lesions?' or 'Is Sampson's theory enough to explain OME lesions?' (Sampson, 1927). There is no definite answer at this moment. But we argue that we can link each and every theory supporting the origin of visible endometriosis (Burney and Giudice, 2012) to the pathogenesis of OME lesions. If Sampson's theory does not directly support the origin of OME lesions, it can be indirectly explained by lymphatic or hematogenous spread of menstrual debris and its subsequent localization within lymphatic/vascular channels deep into peritoneum as already proposed (Sampson, 1927; Javert, 1952; Hey-Cunningham et al., 2011). But our current findings did not support this phenomenon for OME. We failed to locate any OME lesion within lymphatic channel or vasculatures. Instead, we found an

increased angiogenic and lymphangiogenic response in and around OME lesions. This could be due to local inflammatory reaction in the pelvis of women with visible endometriosis or activity response of OME lesions.

We cannot exclude the possibility of genetic factor or metaplastic transformation of peritoneal mesothelial cells in response to inflammation or environmental factors (Burney and Giudice, 2012). Despite possible origin of OME as a result of epithelial-mesenchymal transition/mesenchymal-epithelial transition or from stem cells (Gaetje et al., 1997; Sasson and Taylor, 2008; Maruyama and Yoshimura, 2012), embryonic development (Mülleriosis) within peritoneum may be another possible mechanism to explain the origin of IME as described by Redwine in 1988.

In addition to recognize müllerian tissue and epithelial ovarian cancer cells, CA125/MUC16 can be used as a marker to identify cells derived from coelomic epithelium (embryonic origin) and its derivatives. With this concept in mind, we extended our experiment to immunolocalize cells of embryonic origin by immunoreaction to CA125/MUC16 in OME lesions. CA125 is a high molecular weight mucin-type glycoprotein encoded by MUC16 gene. CA125/MUC16 is expressed in epithelial ovarian

cancer cells and in apical surface of coelomic epithelia and its derivatives such as endometrium, mesothelial cells lining body cavities and fallopian tube (Cheon et al., 2009). As a marker of coelomic epithelial cells, we found immunoreaction of CA125/MUC16 in visible endometriosis and in all patterns of OME lesions. Three cases with OME lesions, had non-reactive to Ber-EP4/CD10/calritinin, were equally immunoreactive to CA125/MUC16. Our findings were further supported by the immunoreaction of CA125/MUC16 to normal peritoneal mesothelium. These findings may support the notion that cells of visible and occult endometriosis could be of embryonic origin (Mülleriosis) and CA125/MUC16 can be expressed irrespective of the presence of peritoneal pockets and anatomical sites of OME lesions. We speculate that the transformation of peritoneal mesothelial cells (metaplasia theory) or time-dependent activation of coelomic epithelium (induction theory) within peritoneum may explain the origin of OME lesions in our study.

Although the theory of müllerianosis cannot be ignored as proposed by Batt (2013) as an additional factor in the origin of OME lesions from müllerian tissue associated with peritoneal pockets, our findings did not support this theory. Because even

we could detect two cases with peritoneal pockets derived from rectovaginal pouch of women with visible endometriosis, both of these cases lacked OME lesions anywhere in the pelvis, including peritoneal pockets. .

Finally, we conclude that existence of OME, previously described as IME, in visually normal peritoneum is true and acceptable. We rule out the previous argument and confirmed here by serial experiments that OME lesions are indeed biologically active and retain their growing potentiality. This may be involved in the persistence and/or recurrence of pain, recurrence of endometriosis after successful surgery or possible development of time-dependent overt endometriosis. Further studies are needed to strengthen our current findings.

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**Authors' roles:** KNK was involved in concept, study design, experiments, data

analysis and manuscript writing; AF, MK, and KH contributed equally to sample collection and experimental assistance; MN was involved in histopathological reading and experimental advice and HM was involved in draft reading.

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## **Figure Legends**

**Figure 1.** Photographs of visually normal peritoneum observed during laparoscopy (Storz, Germany) and were taken from the area of Pouch of Douglas (**A**) and uterovesical space (**B**). The criteria of visually normal peritoneum are described in materials and methods.

**Figure 2.** Microscopically detected three patterns of occult microscopic endometriosis in visually normal peritoneum. Pattern I shows presence of typical gland/stroma; pattern II shows reactive hyperplastic change of endometrioid epithelial cells with surrounding stroma; and pattern III shows single-layered epithelium-lined cystic lesions with surrounding stromal cells (all in HE stain, upper column). The

identification of glandular epithelial cells, stromal cells and peritoneal mesothelial cells was confirmed by the immunoreaction to Ber-EP4, CD10 and Calretinin, respectively and are shown against each HE stained slides. Flat mesothelial cells derived from normal peritoneum and mesothelioma cells as a positive control (inset) immunoreactive to Calretinin are shown at the right panel. The immunoreactions to non-immune mouse IgG as a negative control are shown on the extreme right panel. HE stain, hematoxylin and eosin stain. Magnification of slides (x200).

**Figure 3.** Immunohistochemical staining of estrogen receptor (ER, upper column) and progesterone receptor (PR, middle column) in different patterns of occult microscopic endometriosis (OME) detected in women with visible endometriosis (**A**) and in control women (**B**). The immunoreactions to non-immune mouse IgG as a negative control are shown in the lower panel of **A** and **B**. Quantitative histogram (Q-H) scores indicated that comparing to ER expression (white bar), immunoexpressions of PR (hatching bar) appeared to be higher in all patterns of OME lesions detected in women with endometriosis (**C**). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median

values. Magnification of **A** and **B** (x200 and x400).

**Supplementary Figure 1.** Hematoxylin and eosin stain (upper column), immunohistochemical staining of aromatase cytochrome P450 (middle column), and non-immune mouse IgG-stained negative control (lower column) in visible peritoneal endometriosis (red lesion), in patterns I and III lesions of occult microscopic endometriosis (OME) detected in women with visible endometriosis, and in control women. A variable amount of aromatase immunoexpression was found in patterns I/III OME lesions and also in red lesions.

**Figure 4.** Hematoxylin and eosin stain (upper column) and immunohistochemical staining of Toll-like receptor 4 (TLR4, lower column) in peritoneal endometriosis (**A**), different patterns of occult microscopic endometriosis (OME) detected in women with visible endometriosis (**B**) and in control women (**C**). TLR4 immunoreaction was also observed in micro-vessels of normal peritoneum derived from pouch of Douglas (**D**, upper column) and uterovesicle space (**D**, lower column) of women with endometriosis and control women. Magnification of **A**, **B**, **C**, **D** (x200).

**Figure 5.** Hematoxylin and eosin stain (upper column) and

immunohistochemical staining of Ki-67 (lower column), a cell proliferation marker, in lesions derived from visible peritoneal endometriosis (**A**), different patterns of occult microscopic endometriosis (OME) detected in women with endometriosis (**B**), and in control women (**C**). The Ki-67 index (percentage of Ki-67 immunoreactive cells among total cells) in the respective lesions are shown in the lower right panel (**D**). Ki-67 index was significantly higher in red lesions (white bar) than in black lesions (hatching bar, \* $p < 0.05$ ). Ki-67 index was also significantly higher in patterns I or II OME lesions (white/hatching bar) than in pattern III lesions (gray bar,  $p < 0.05$  for each) detected in women with visible endometriosis. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. Magnification of **A**, **B**, and **C** (x200).

**Supplementary Figure 2.** Hematoxylin and eosin stain (far left column) and immunohistochemical staining of D2-40, a lymphatic vessel marker, in occult microscopic endometriosis (OME) detected in women with endometriosis (**A**, upper column) and in OME lesions of control women (**A**, lower column). Analysis of total lymphatic vessel count indicated that number of D2-40 immunoreactive lymphatic vessels

appeared to be higher in women with endometriosis than in controls in the peritoneum adjacent to OME lesions. In contrast, lymphatic vessel numbers appeared to be higher in controls than in women with endometriosis in the peritoneum distant from OME lesions. There was no significant difference in lymphatic vessel count between these two groups of women in either area (**B**). No OME lesions were found within lymphatic channels. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. Magnification of **A** (x200).

**Supplementary Figure 3.** Hematoxylin and eosin stain (far left column) and immunohistochemical staining of von Willebrand factor (VWF), a micro-vessel marker, in occult microscopic endometriosis (OME) detected in women with endometriosis (**A**, upper column) and in OME lesions of control women (**A**, lower column). Analysis of micro-vessel density indicated that number of VWF immunoreactive micro-vessels appeared to be higher in women with endometriosis than in controls in the peritoneum adjacent to OME lesions. In contrast, micro-vessel densities (MVD) appeared to be higher in controls than in women with endometriosis in the peritoneum distant from OME



lesions. There was no significant difference in MVD between these two groups of women in either area (**B**). No OME lesions were found within vascular channels. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. Magnification of **A** (x200).

**Figure 6.** Hematoxylin and eosin stain (upper column) and immunohistochemical staining of CA125/MUC16, a marker of cells derived from coelomic epithelium, (lower column) in visible peritoneal endometriosis (red/black lesions, **A**), different patterns of occult microscopic endometriosis (OME) detected in women with visible endometriosis (**B**) and in control women (**C**). Pattern II and III OME lesions that were non-reactive to Ber-EP4/CD10/Calretinin were also immunoreactive to CA125/MUC16 (**D**). Normal peritoneal mesothelium appeared to be immunoreactive to CA125/MUC16 (inset, **D**). Magnification of **A**, **B**, **C**, and **D** (x200 and x400).

Figure 1.

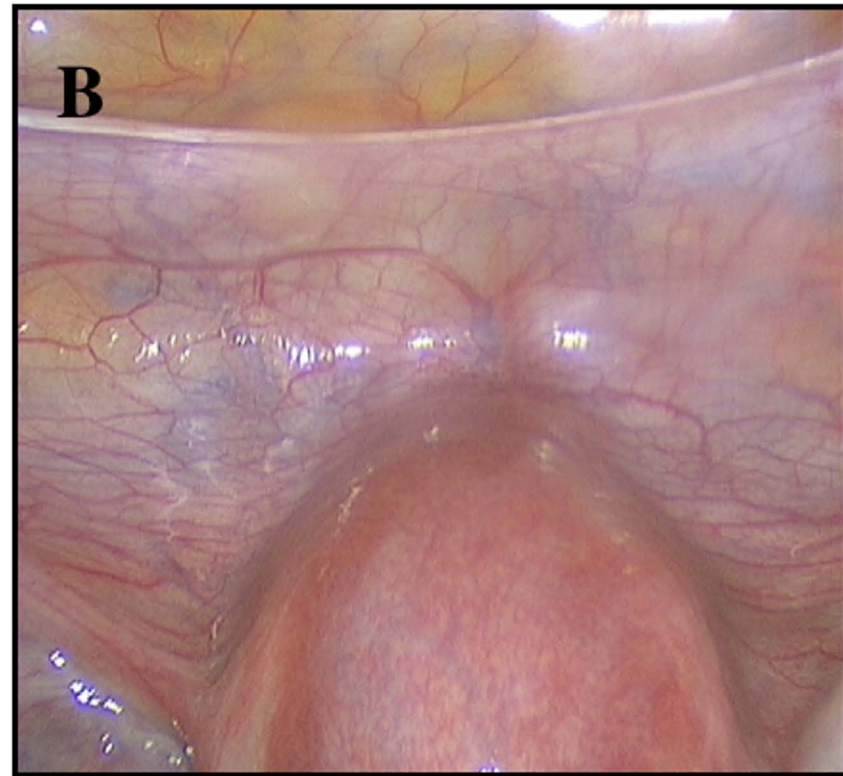
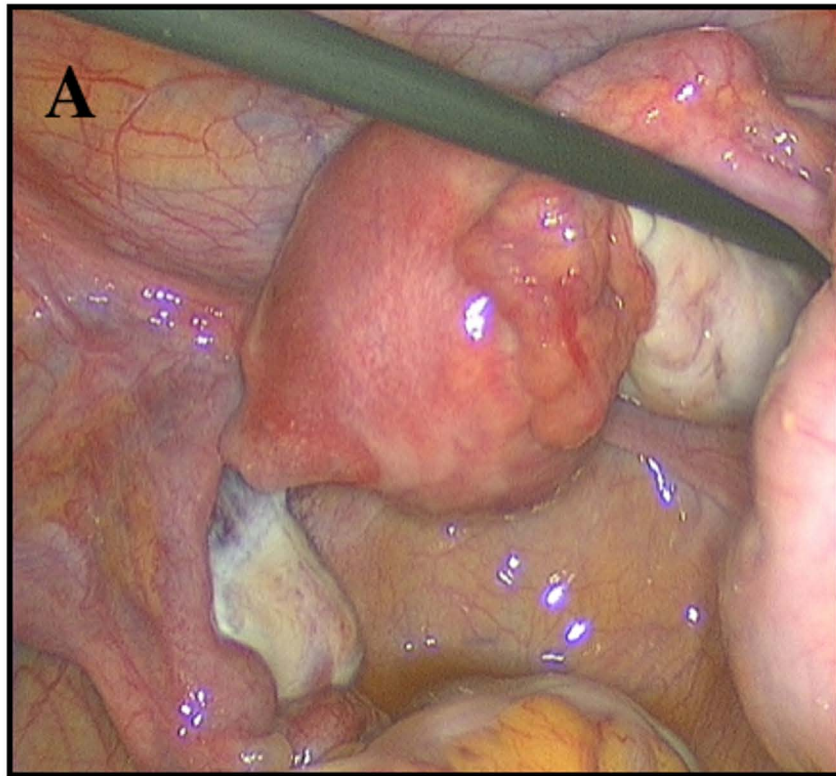




Figure 2.

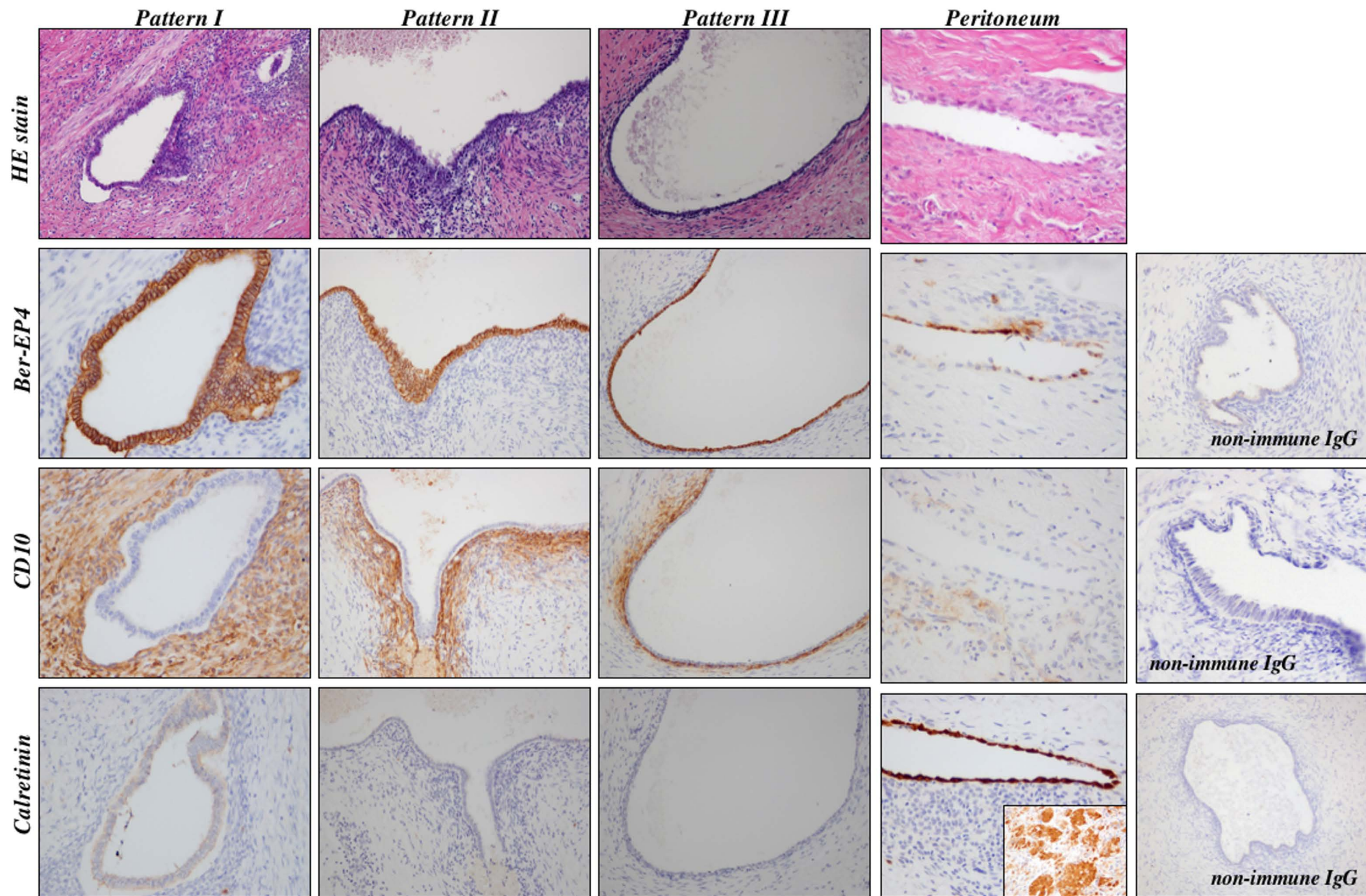


Figure 3.

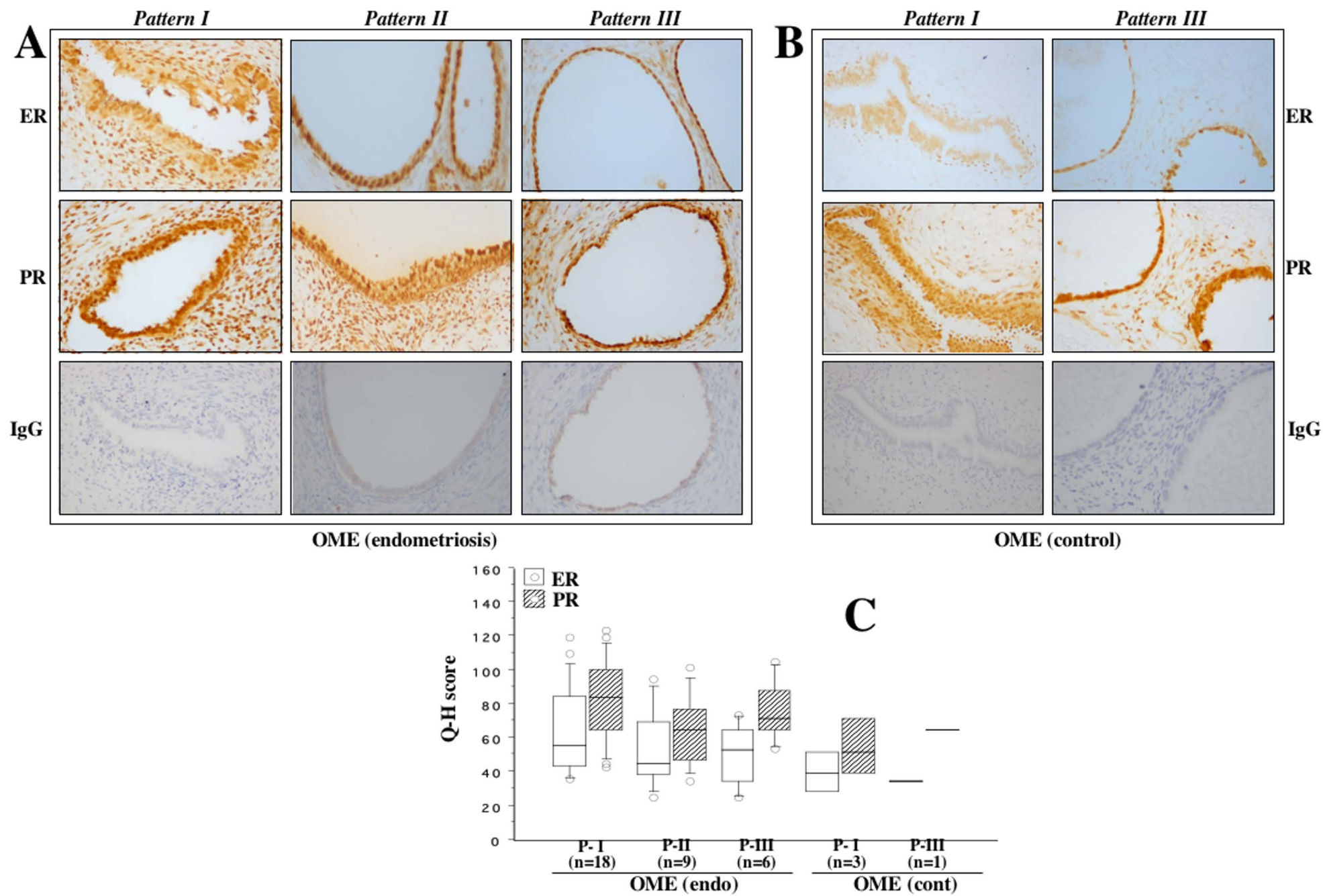




Figure 4.

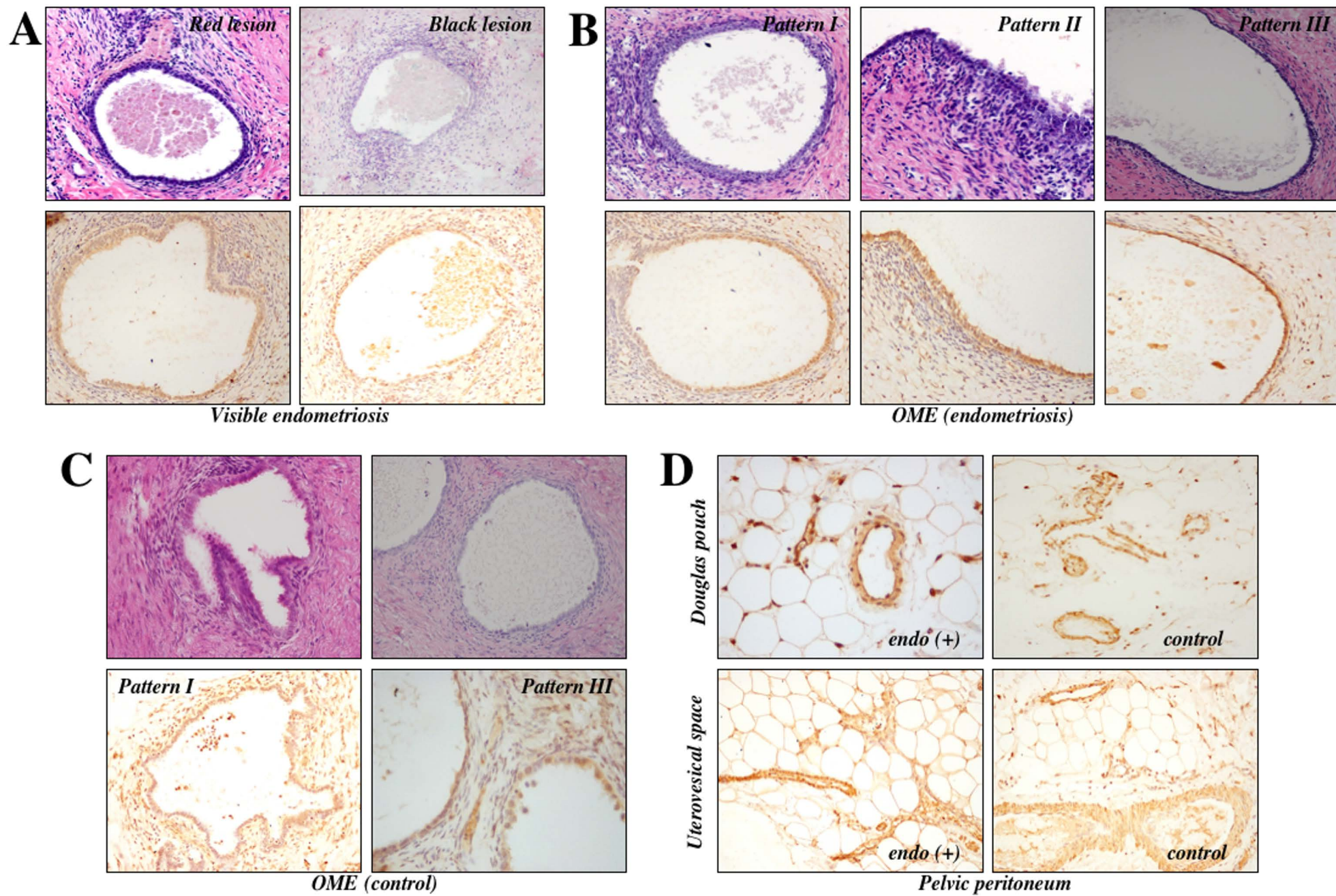




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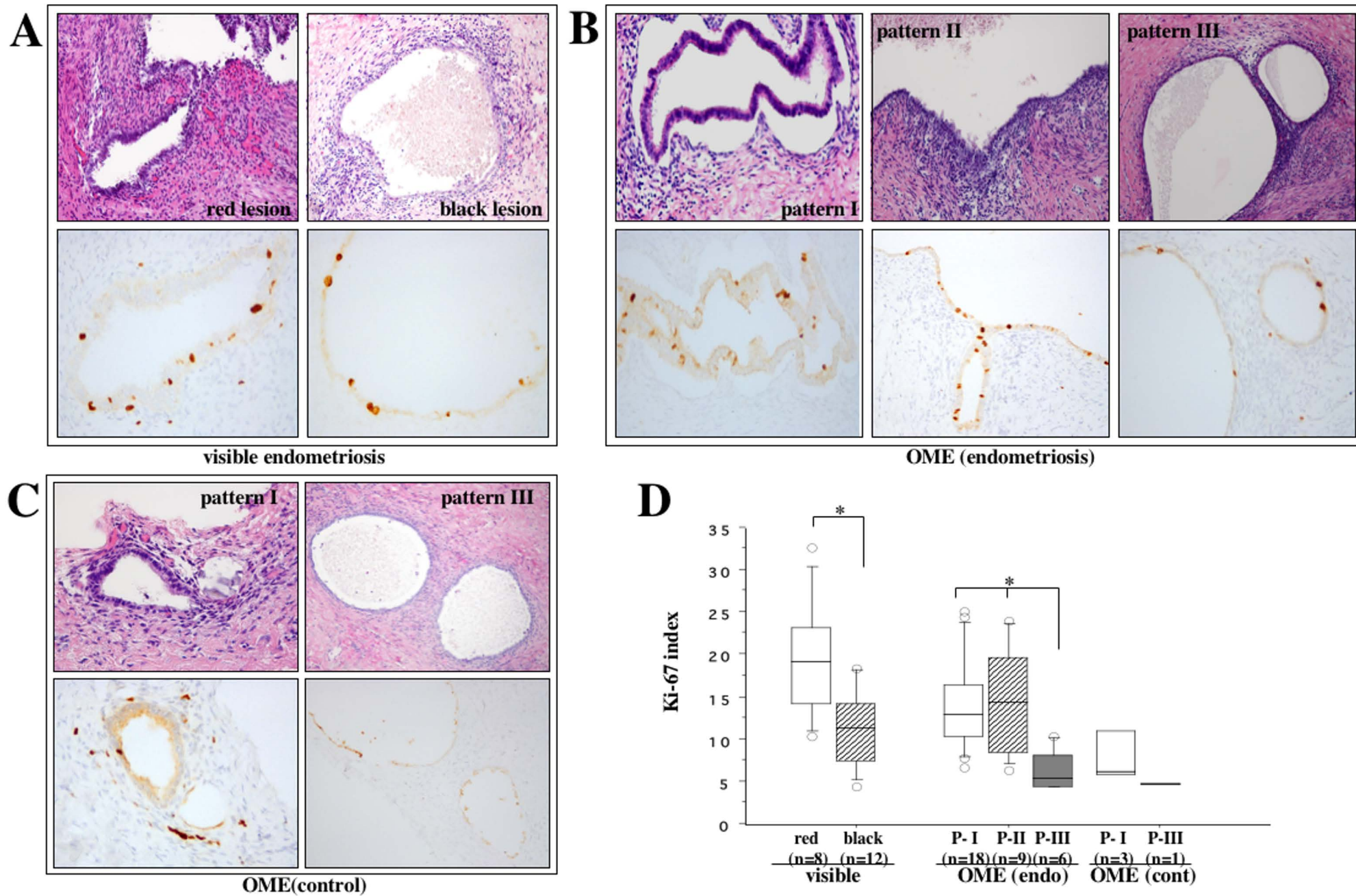
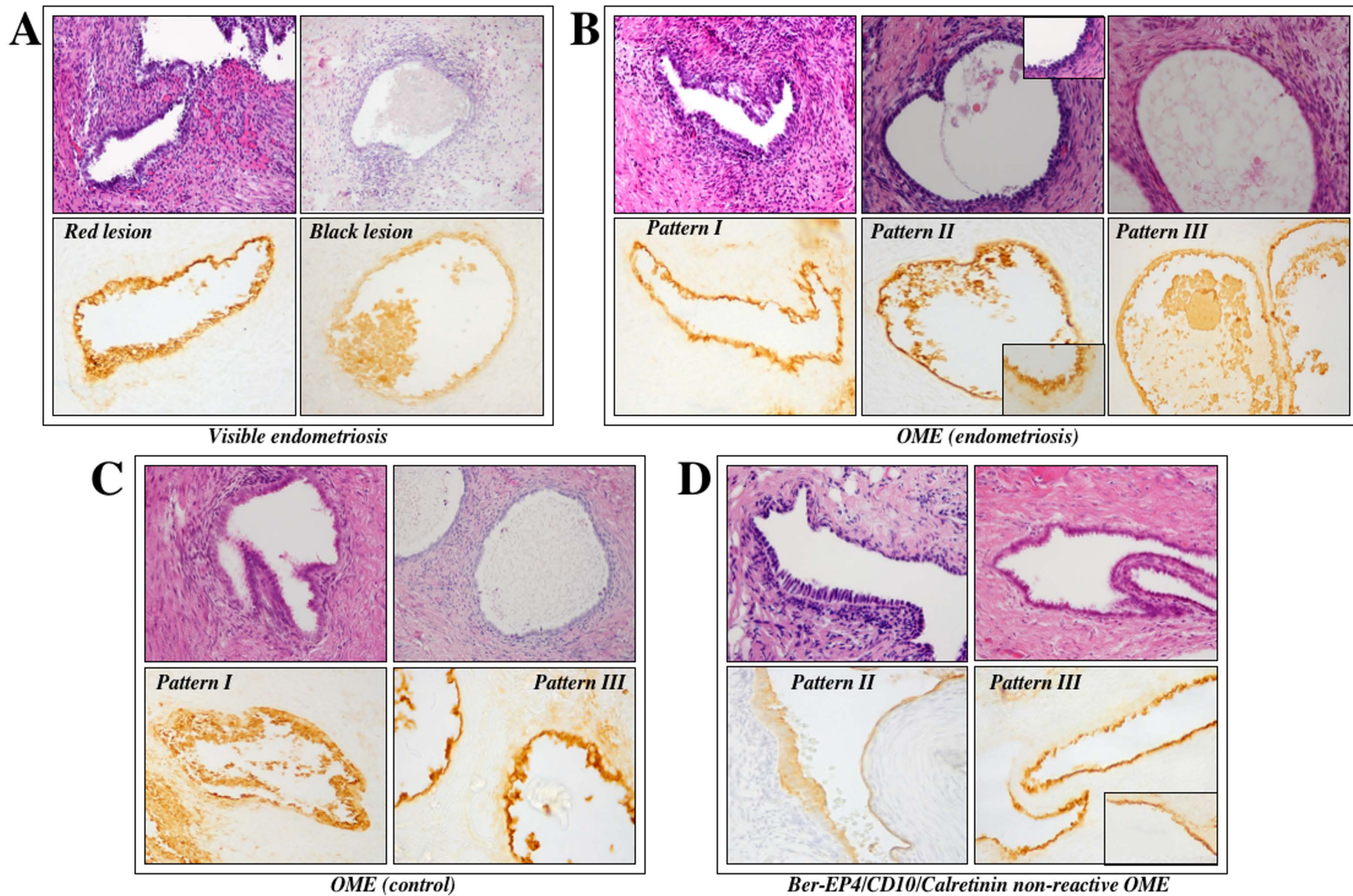


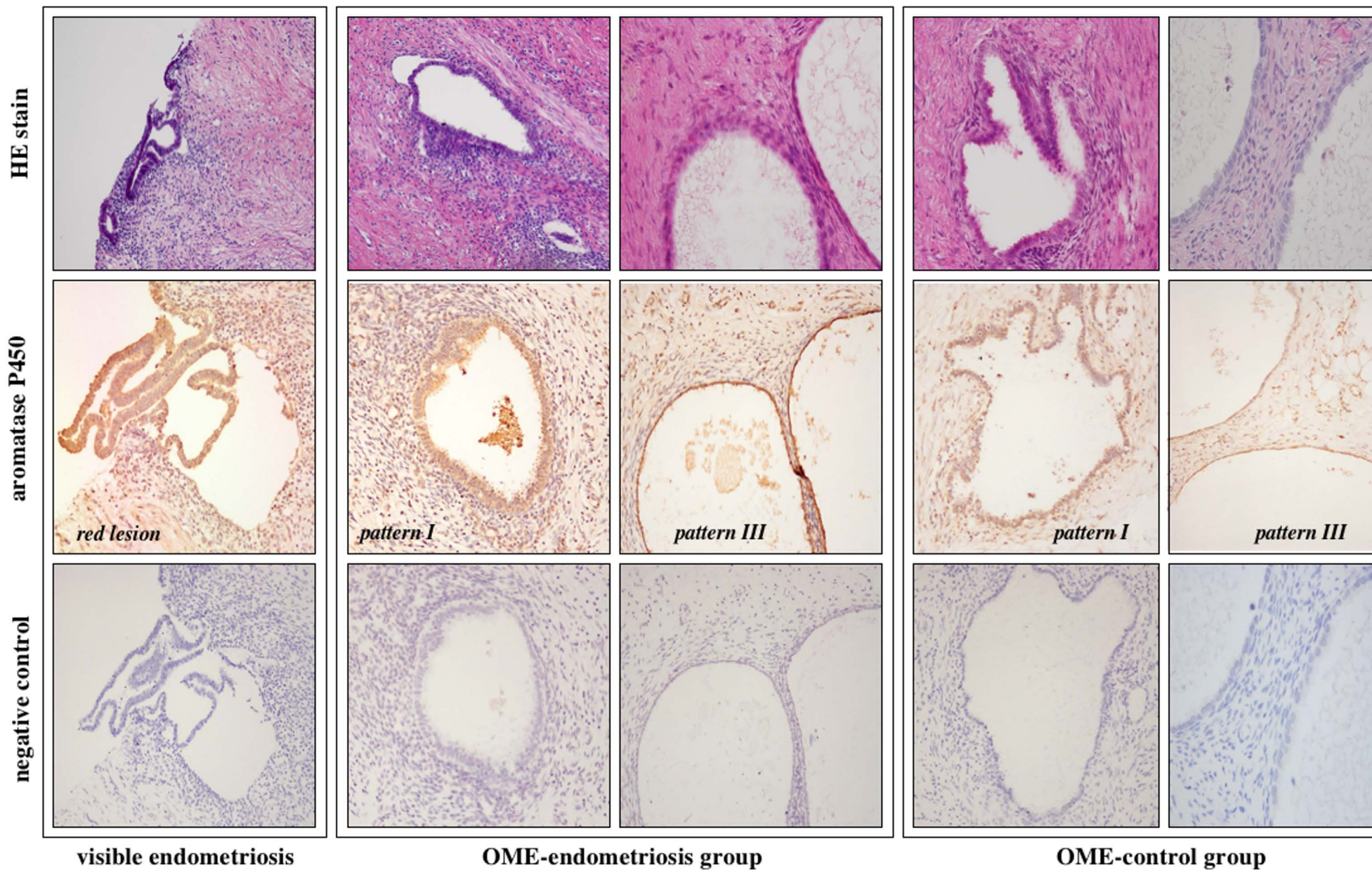


Figure 6.



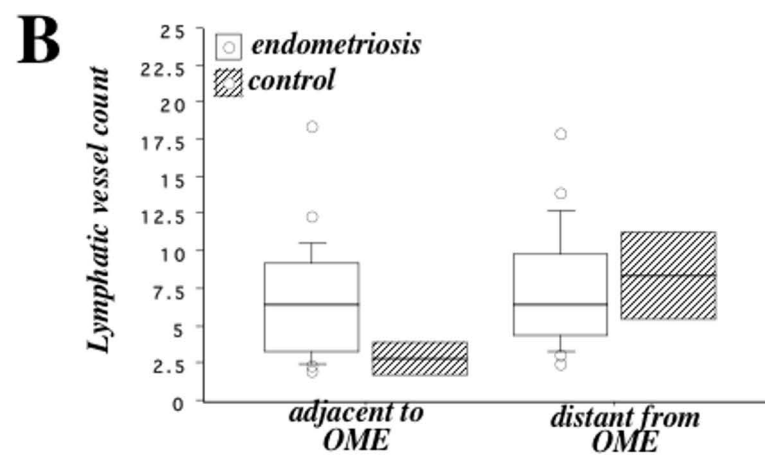
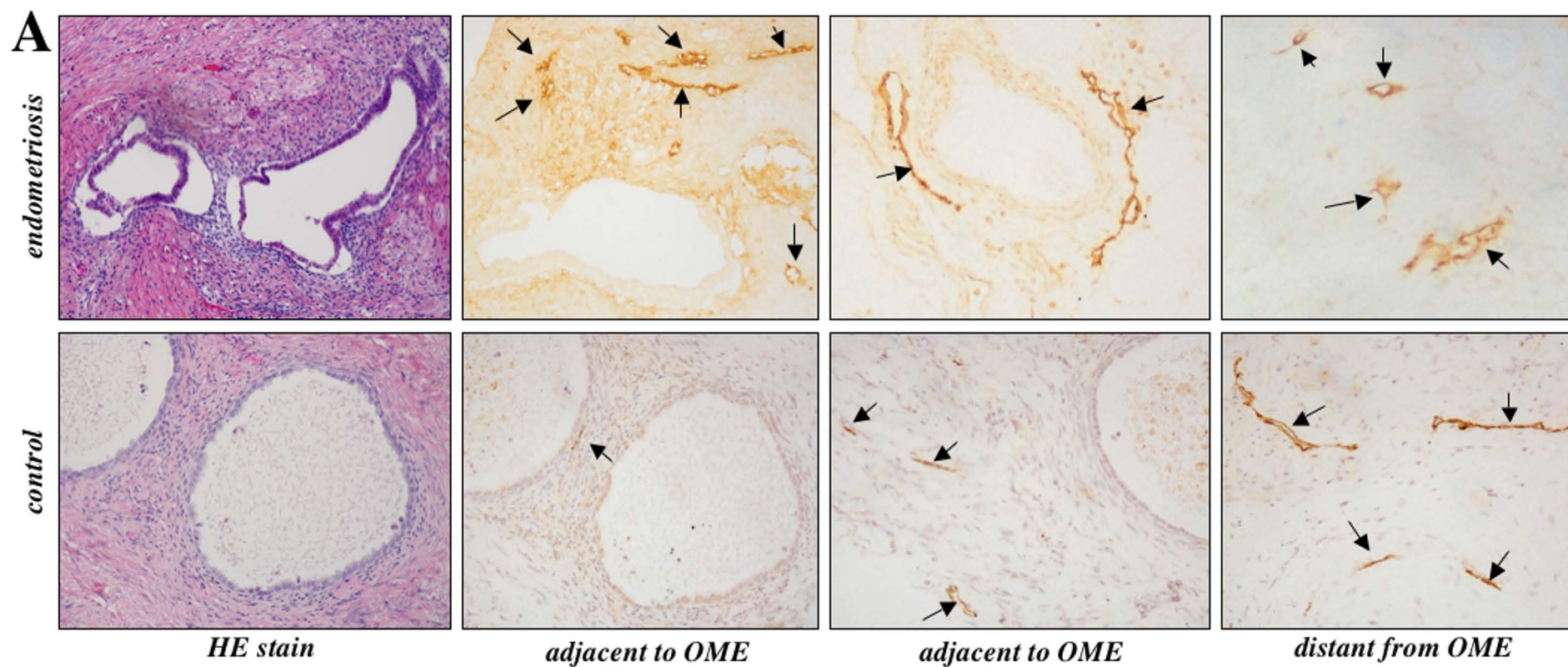


Supplementary Figure 1.





Supplementary Figure 2.



Supplementary Figure 3.

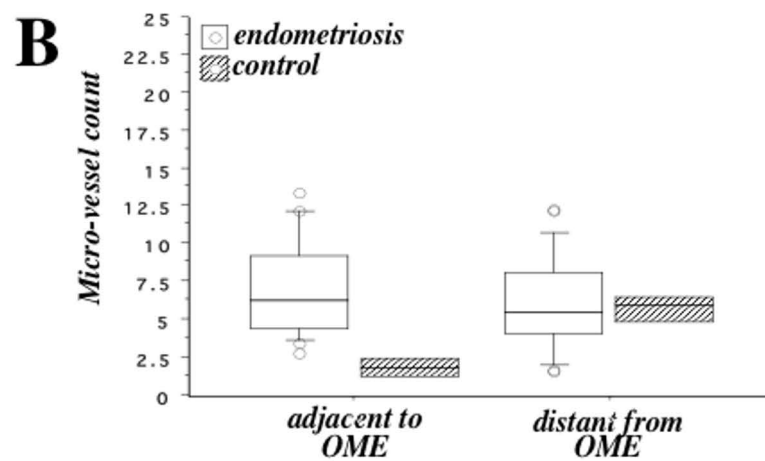
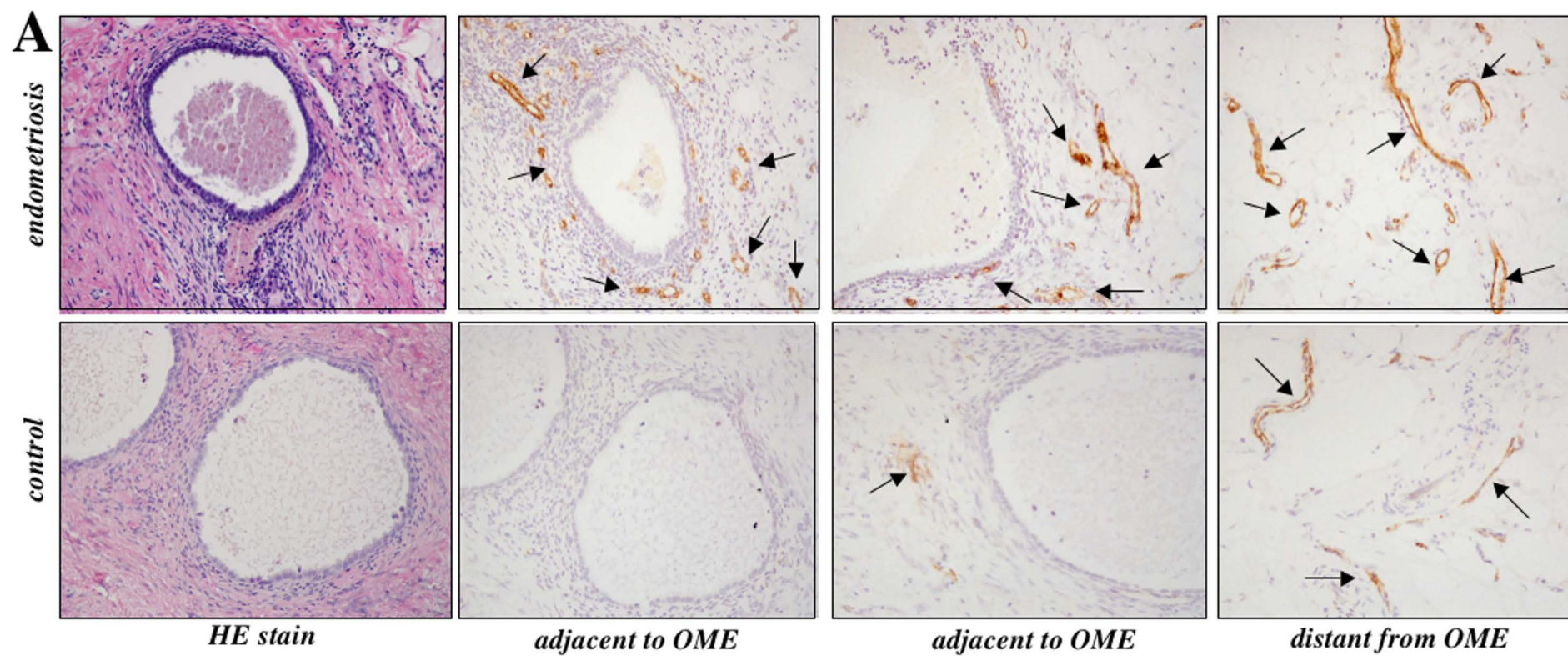


Table 1. Clinical profiles of enrolled patients with and without endometriosis

	control (n=62)	endometriosis (n=151)
age in years: (mean $\pm$ SD)	33.9 $\pm$ 7.4	35.1 $\pm$ 6.9
range of age: (years)	19-48	23-50
menstrual cycle: P/S/M/A (n)	13/40/6/3	29/68/16/38
r-ASRM staging: I/II/III/IV (n)	none	83/30/14/24
distribution of peritoneal lesions: red/black/white/mixed (n)	none	16/94/7/34
complaint of dysmenorrhea: present/absent (n)	9/53	93/58
GnRHa treatment before surgery: yes/no (n)	none	38/113
coexisting diseases: adenomyosis/myoma (n)	1/4	5/24

The results are expressed as mean  $\pm$  SD. r-ASRM, revised classification of the American Society of Reproductive Medicine; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; GnRHa, gonadotropin releasing hormone agonist.

Table 2. Detection rate of OME based on number of patients and biopsy samples

	control (n=62/78) (No. patients/samples)	endometriosis (n=151/227) (No. patients/samples)	p value
OME based on No. patients	6.4% (4/62)	15.2% (23/151)	0.06
OME based on No. biopsy samples	5.1% (4/78)	11.8% (27/227)	0.07

OME, occult microscopic endometriosis; Statistics were analyzed by Chi square test.

Table 3. Clinical profiles of patients with occult microscopic endometriosis

	control (n=4)	endometriosis (n=23)
age in years: (mean $\pm$ SD)	34.2 $\pm$ 9.7	34.4 $\pm$ 6.3
range of age: (years)	24-44	23-45
menstrual cycle: P/S/M/A (n)	1/3/0/0	5/12/3/3
r-ASRM staging: I/II/III/IV (n)	none	14/2/2/5
distribution of peritoneal lesions: red/black/white/mixed (n)	none	5/14/1/3
complaint of dysmenorrhea: present/absent (n)	0/4	16/7
GnRHa treatment before surgery: yes/no (n)	none	3/20
coexisting diseases: adenomyosis/myoma (n)	none	0/3

The results are expressed as mean  $\pm$  SD. r-ASRM, revised classification of the American Society of Reproductive Medicine; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; GnRHa, gonadotropin releasing hormone agonist.