Original article:

Pelvic pain in women with ovarian endometrioma is mostly associated with coexisting peritoneal lesions

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Running title: Pain in ovarian endometrioma

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Abstract

Study question: Is occurrence of pelvic pain in women with ovarian endometrioma associated with coexisting peritoneal lesions?

Summary answer: Pelvic pain in women with ovarian endometrioma is mostly associated with coexisting peritoneal lesions. An increased tissue inflammatory reaction with elevated prostaglandin production may be responsible for the generation of pain.

What is known already: Severe pelvic pain in women with ovarian endometrioma is reported to be associated with deeply infiltrating endometriosis. However, information on pelvic pain in women with ovarian endometriosis with and without coexistent peritoneal superficial lesions is limited.

Study design, size and duration: Retrospective clinical study with case-controlled biological research using prospectively collected tissue samples derived from women with and without endometriosis and their retrospective evaluation.

Participants/materials, setting, methods: We performed a retrospective cohort study conducted in 2988 cases had laparoscopic surgery for the indication of ectopic pregnancy, tubal infertility and other benign gynecologic diseases. We analyzed occurrence of pelvic

pain in the cases with ovarian endometrioma according to the distribution of coexisting peritoneal lesions and pattern of intrapelvic adhesions. Inflammatory reaction of eutopic and ectopic endometria was measured by immunoreaction to macrophage marker, CD68. The tissue expression of cyclooxygenase (COX) 2 was examined by immunohistochemistry and tissue concentrations of prostaglandin (PG) F2α was measured by ELISA.

Main results and the role of chance: Among 2988 cases of surgery, 350 cases (11.7%) were found to have ovarian endometrioma during laparoscopy. Among 269 women with endometrioma had coexisting peritoneal lesions, 85.4% of cases experienced pelvic pain and 14.6% had no pain and this difference was statistically significant (p<0.01). Among 81 women with ovarian endometrioma only, 38.3% cases experienced pelvic pain and 61.7% cases had no pain. The infiltration of Mϕ was significantly higher in the eutopic and ectopic endometria of women with peritoneal endometriosis than in ovarian endometrioma. The tissue expression of COX2 and levels of PGF2α were significantly higher in both eutopic and ectopic endometria derived from women with peritoneal endometriosis than in similar tissues derived from women with ovarian endometrioma.

Limitations, reasons for cautions: Lack of evaluation in the detection of general or disseminated DIE in pelvic cavity could be a bias or limitation in this study. Further multi-center prospective studies are needed to strengthen our current findings.

Wider implications of the findings: Our findings may provide some new insights to understand the physiopathology of pelvic pain in women with ovarian cystic endometriosis and may hint proper surgical manipulation to prevent recurrence of pelvic pain in these women. .

Trial registration number: not applicable.

Introduction

Endometriosis is an estrogen-dependent chronic inflammatory disease affecting 6-10% women of childbearing age (Giudice and Kao, 2004). Endometriosis is histologically characterized into three types: peritoneal superficial endometriosis, ovarian endometrioma and deeply infiltrating endometriosis (DIE) and is associated with infertility and a variable degree of pelvic pain (de Ziegler et al., 2010; Fauconnier et al., 2005). Different mechanisms have been proposed to explain the relationship that exists between endometriosis and pelvic pain such as tissue inflammatory reaction, production of prostaglandins (PGs) with consequent uterine contraction and nerve entrapment within lesions (Anaf et al., 2000; Berkley et al., 2005; Khan et al., 2004a; Miura et al., 2006). For DIE, entrapment of nerves within the endometriotic nodules represents a possible mechanism explaining pain (Anaf et al., 2000). For peritoneal superficial endometriosis and ovarian endometrioma, bleeding within the lesion, tissue inflammatory reaction, production of PGE2/PGF2α and associated intra-pelvic adhesion may explain their association with pain (Khan et al., 2004a; Miura et al., 2006; Khan et al., 2009). The severity of pain in women with endometriosis is associated with color appearance of

peritoneal lesions, depth of infiltration, anatomic location of lesions and intensity of inflammatory reaction with resultant pelvic adhesions (Fauconnier et al., 2005; Khan et al., 2004a; Fauconnier et al., 2002).

The relationship between ovarian endometrioma (chocolate cyst) and painful symptoms is not well established and is still controversial (Fauconnier et al., 2005). This pain could be due to the presence of endometrioma itself or due to coexisting peritoneal lesions or associated pelvic adhesions. Currently little is known about the mechanisms by which pain might be associated with ovarian endometrioma. Therefore, first of all, we aim to retrospectively review recorded files of laparoscopic surgery and analyzed the possible association of pelvic pain in women with ovarian endometrioma had coexisting peritoneal lesions or without any visible peritoneal lesions. Secondly, we plan to investigate the mechanistic basis of pelvic pain using prospectively collected biopsy specimens derived from women with peritoneal endometriosis, ovarian endometrioma, and women without endometriosis during laparoscopic surgery.

Materials and Methods

We retrospectively searched cases with ovarian endometrioma from the recorded files of laparoscopic surgery during the period between September 1982 and April 2008 and had surgery for the indication of ectopic pregnancy, tubal infertility and other benign gynecological diseases. In order to exclude the bias of pain as a result of previous surgery or type of surgery, we excluded all cases had previous surgery or had laparotomy from our current study. In this cohort study, we analyzed cases with ovarian endometrioma as follows: (1) according to coexisting peritoneal lesions, (2) according to pattern of pelvic adhesions, (3) association of pain with and without peritoneal lesions, and (4) association of pain based on pattern of adhesion. In addition to cases with ovarian endometrioma, laparoscopic surgery was also performed for cases with other benign gynecological diseases such as dermoid cysts, serous/mucinous cyst adenoma, uterine myoma and adenomyosis during this time period. Since it is not possible to diagnose DIE in general by their dissemination pattern over the peritoneum before surgery, we excluded cases only with DIE in the pouch of Douglas or in the recto-vaginal septum or in bowel. We excluded patients with deep endometriosis by subjective and objective (either of USG

or CT or MRI) evaluation. Any woman with the complaint of dyspareunia or presence of painful nodules by physical examination was excluded from our current study. In addition, we also excluded all cases had associated with any bacterial/viral infection, Chlamydia infection or PID. Therefore, we included cases only with ovarian endmetrioma (chocolate cysts) with and without coexisting peritoneal superficial endometriosis for our current study. Based on recorded files, complain of pain was described as menstrual pain (tolerable cyclic pelvic pain), dysmenorrhea (intolerable cyclic pelvic pain requiring NSAIDS) or chronic pelvic pain (pain longer than 6 months) either alone or in combination. We excluded any case with the complaint of dyspareunia, because it could be a manifestation of either PID or DIE.

The staging and the morphological distribution of peritoneal lesions were based on the revised classification of the American Society of Reproductive Medicine (ASRM, 1997). Peritoneal lesions of endometriosis were diagnosed by their macroscopic color appearances according to published criteria (Jansen and Russel, 1996) and categorized as red, black and white lesions as proposed in the latest revision of the ASRM classification (ASRM, 1997). The diagnosis of all cases with ovarian endometrioma and peritoneal

endometriosis was confirmed morphologically during operation and subsequently by histopathology.

We prospectively collected biopsy specimens from the eutopic and ectopic endometria of 15 women with pelvic endometriosis, 22 women ovarian endometrioma and 10 control women with dermoid cysts without any evidence of peritoneal lesions. Besides tissue sampling from 10 control women, we also collected biopsy specimens from unaffected normal peritoneum from six women each with and without endometriosis. Ten biopsy specimens were collected from red lesions and 15 from black lesions. Twelve women with ovarian endometrioma had coexisting peritoneal lesions (red, black or mixed) (CC+PL group) and 10 women had only ovarian endometrioma without any coexisting visible peritoneal lesions (CC only group). The distribution of biopsy samples based on menstrual cycle in these two groups of women is as follows: for CC+PL group, proliferative phase (n=4), secretory phase (n=6), and menstrual phase (n=2); for CC only group, proliferative phase (n=3), secretory phase (n=5), and menstrual phase (n=2). All collected biopsy specimens were prepared for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and

immunohistochemical study. All biopsy specimens were collected in accordance with the

guidelines of the Declaration of Helsinki and with the approval by the Institutional

Review Board of Nagasaki University. An informed consent was obtained from all

women.

Immunohistochemistry. Immunohistochemical analysis was performed to

immunolocalize CD68, a macrophage (Mϕ) marker and cyclooxygenase 2 (COX2), a

rate-limiting enzyme for the production of prostaglandins (PGs) in the eutopic and

ectopic endometria derived from women with and without endometriosis. The following

first antibodies were used for immunohistochemistry: (1) CD68 (KP1), a mouse

monoclonal antibody from Dako, Denmark and a 1:50 dilution was used, (2) COX2

(sc-1745), a goat polyclonal antibody from Santa Cruz Biotechnology and a 1:100

dilution was used. Non-immune mouse immunoglobulin (Ig) G1 antibody in 1:50

dilution was used as a negative control. The details of immunohistochemical procedure is

described elsewhere (Khan et al., 2004a; Miura et al., 2006).

The CD68 immunoreactive spots (brown spots) were counted in five different

fields of one section (x200 magnification) by light microscopy and expressed as the mean

Mϕ number per field in one specimen.

Quantification of COX2-immunostained cells by Q-H score. The immunoreactivity of COX2-stained gland cells, stromal cells and endometrioma cyst wall was quantified by a computer analyzed modified method of quantitative-histogram (Q-H) scores as described previously (Khan et al., 2003, 2005a; Ishimaru et al., 2004). The Q-H score was calculated using the following formula: Q-H score = ΣPi (I + 1), where I = 1, 2 or 3 and Pi is the percentage of stained cells for each intensity. The staining intensity was graded as 0 = no staining, 1 = weak, 2 = moderate, and 3 = strong. We calculated the mean Q-H scores of five different fields of one section by light microscopy at moderate magnification ($\times$ 200).

Measurement of PGF2α in tissue samples. A fraction of biopsy specimens from peritoneal lesions/cyst walls and autologous eutopic endometria were homogenized in a homogenizing buffer using a Polytron homogenizer (Kinematics, Luzern, Switzerland). The respective tissue suspension was centrifuged at 1500 rpm for 5 minutes to obtain the supernatant and stored at -80°C for the subsequent measurement of prostaglandin F2α (PGF2α). The tissue concentrations of PGF2α in the homogenized

supernatant of respective samples were measured in duplicate using a commercially available sandwich enzyme-linked immunosorbent assay (Quantikine; R&D System, Minneapolis, MN) according to the manufacturer's instructions The protein concentration of samples was measured by the method of Bradford (Bradford, 1976) to standardize PGF2α levels. The antibodies used in PGF2α determination do not cross-react with other cytokines. The limit of detection was less than 6.78 pg/mL for PGF2α. Both the intra-assay and inter-assay coefficients of variation were <10% for this assay. The tissue concentrations of PGF2α was expressed as pg/μ g protein.

Statistical analysis. All results are expressed as either mean ± SEM or mean ± SD or medians. The clinical characteristics of the subjects were compared with one-way analysis of variance and the X^2 test for any difference between two groups. Any differences in Mϕ numbers or Q-H scores between two groups was analyzed by the non-parametric Mann-Whitney *U*-test. For comparisons among groups, the Kruskal-Wallis test was used to assess the differences. A box plot analysis of tissue levels of PGF2α was performed using the medians and inter-quartile range (IQR). A value of p<0.05 was considered statistically significant.

Results

During the period between September 1982 and April 2008, we could search 2988 cases had laparoscopic surgery for a variable indication as mentioned in materials and methods. Among 2988 cases had surgery, 350 cases (11.7%) were found to have ovarian endometrioma during laparoscopy. The clinical characteristics of 350 cases with ovarian endometrioma are shown in Table 1. A predominance of ovarian endometrioma was found in 20th (31.4%) and 30th (51.7%) years of age. Occurrence of left sided endometrioma was dominant comparing to either right sided or bilateral occurrence of ovarian endometrioma.

The association of coexisting peritoneal lesions in women with endometrioma was as follows: no lesions (23%), red lesions (8.4%), black lesions (23.5%), white lesions (5.3%) and mixed lesions (39.7%)(Table 1). The distribution of associated intra-pelvic adhesions was as follows: no adhesion (15.7%), filmy adhesion (61.8%) and dense adhesion (22.5%)(Table 1). Among 81 women with ovarian endometrioma only, 38.3% cases experienced pelvic pain and 61.7% cases had no pain (Table 2). Among 269 women harboring endometrioma with coexisting peritoneal lesions, 85.4% cases experienced pain and 14.6% had no pain. Women with CC had coexisting peritoneal lesions

complained of significantly higher occurrence of pain than in women with CC had no coexisting peritoneal lesions (p<0.01) (Table 2). When we distributed occurrence of pain symptoms based on the size of ovarian endometrioma (<5cm vs. >5cm), we did not find any significant difference between these two groups of endometrioma (46.2% vs. 53.8%, respectively).

The association of pain in women with ovarian endometrioma was more remarkable when these cases were coexistent with dominant red lesions (88.2%), black lesion (85.5%) and mixed lesion (87.7%) comparing to white lesions (63.6%) (data not shown). The incidence of pain in women with endometrioma according to pattern of pelvic adhesion was as follows: no adhesion, 46.8%; filmy adhesion, 81.4% and dense adhesion, 86.9% (Table 3). Most of the women with endometrioma who had no adhesion complained of pain due to coexisting variable peritoneal lesions (data not shown).

Tissue infiltration of CD68-immunostained Mϕ in the eutopic and ectopic endometria. We found a differential infiltration of CD68-positive infiltration of Mϕ in the peritoneal lesions, cyst walls and their corresponding eutopic endometria, and unaffected normal peritoneum (Figure 1, A). The infiltration of Mϕ was significantly

higher in the red lesions and corresponding eutopic endometria comparing to biopsy samples derived from black lesion/eutopic endometria, cyst wall of chocolate cyst (CC)/eutopic endometria or to samples of eutopic endometria derived from control women (Figure 1, B). The amount of Mϕ was significantly higher in the eutopic endometria derived from women with black lesions and with CC + coexistent peritoneal lesions (PL) than in similar tissues derived from control women and women with CC only (Figure 1, B). No difference in Mϕ infiltration was found among black lesions, cyst wall of CC + PL and cyst wall of CC only group. As a negative control, unaffected normal peritoneum derived from women with and without endometriosis was examined and found that normal peritoneum also displayed minimal inflammatory reaction without showing any significant difference between women with and without endometriosis (Figure 1A, B).

Immunoexpression of COX2 in the eutopic and ectopic endometria. The immunostaining of COX2 was found in both glandular epithelial cells and stromal cells and cyst wall lining cells. The immunoexpression of COX2 was the strongest in the red lesion, moderate in the black lesion, cyst wall of CC had coexisting peritoneal lesions

(PL) and weak in the cyst wall derived from CC only cases (Figure 2A, lower column). A

similar strong immunoexpression of COX2 was found in the corresponding eutopic

endometria derived from women containing red lesions, black lesions and from women

with CC + PL but COX2 expression appears to be weak in the eutopic endometrium

derived from CC only cases (Figure 2A, upper column). The tissue expression of COX2

in the eutopic endometria derived from control women was similar to that in CC only

group (data not shown).

Quantitative-histogram (Q-H) scores of COX2 immunoreaction. When we

combined the Q-H scores of COX2 immunoexpression in gland cells and stromal cells,

we found a significantly higher Q-H scores of COX2 in the red lesions than in either black

lesions or in the cyst walls of CC had coexisting peritoneal lesions or in cyst walls of CC

without coexisting peritoneal lesions (Figure 2B). Although Q-H scores of COX2

immunoexpression was almost similar in the eutopic endometria of women containing

red lesions, black lesions, cyst wall + peritoneal lesions, these Q-H scores of COX2 were

significantly higher when compared with the corresponding eutopic endometria derived

from CC only women or control women (Figure 2B). The Q-H scores of COX2 in the cyst

wall of CC/eutopic endometria derived from women had coexisting peritoneal lesions were also significantly higher comparing to corresponding tissues derived from CC only women (Figure 2B).

We analyzed immunoexpression and Q-H scores of COX2 in the eutopic endometria derived from women with CC had coexisting peritoneal lesions and without peritoneal lesions and based on the phases of menstrual cycle. We found a maximum immunoexpression/Q-H scores of COX2 during the menstrual phase, intermediate expression in the secretory phase and less expression in the proliferative phase of these two groups of women (Figure 3, A and B). We could not find any significant difference in COX2 expression among phases of the menstrual cycle, may be due to the less number of cases in each phase. However, Q-H scores of COX2 expression were found to be higher in endometria derived from CC + PL group than in CC only group during any phase of the menstrual cycle (Figure 3B).

Tissue levels of PGF2 α in the eutopic and ectopic endometria. The tissue levels of PGF2 α were significantly higher in the eutopic endometria derived from women with red lesions, black lesions and from women with CC had coexisting PL than

in control women or in CC only women (p<0.05 vs. control women or CC only women,

white box, Figure 4). Although no apparent difference of PGF2α levels was found among

black lesions, cyst wall of CC had PL and cyst wall of CC without coexistent PL, a

significantly higher PGF2α level was found in red lesions than in other three groups of

lesions (p<0.05 vs. each of other lesion, hatched box, Figure 4).

Similar to the immunoexpression of COX2, we found a similar pattern of PGF2

α tissue levels in the eutopic endometria and cyst walls derived from women with CC +

PL and CC only women when we distributed the findings according to the phases of the

menstrual cycle (Figure 5, A and B).

Discussion

In this retrospective cohort study using the collective data during the past 25

years, we demonstrated that women with only ovarian endometrioma experience less

pelvic pain and most of the pain manifestations in women with chocolate cysts are mostly

associated with variable coexisting peritoneal lesions. When we extended our study to

explain the mechanistic basis of pain in women with peritoneal and ovarian

endometriosis, we found that tissue inflammatory reaction, expression of COX2 and

tissue levels of PGF2α were remarkably higher in peritoneal lesions and their autologous eutopic endometria derived from women with pelvic endometriosis than in tissues samples derived from women with only chocolate cysts. An interesting finding in our current study was that tissue inflammation, COX2 immunoexpression and levels of PGF2α was higher in the cyst walls and corresponding eutopic endometria of women with chocolate cyst had different coexisting peritoneal lesions when compared with similar tissues derived from women harboring only chocolate cysts without any visible evidence of peritoneal lesions in their pelvic cavity.

The information regarding association of pelvic pain in women with ovarian endometrioma is scanty and is still controversial (Berkley et al., 2005). Most recently it has been demonstrated that severe pelvic pain in women with cystic endometrioma is mostly associated with deeply infiltrating endometriosis (Chapron et al., 2012). We could not analyze the association with DIE or severity of pain in our current study, because information was not enough on either DIE or severity of pain in the old recorded files of laparoscopic surgery. This could be a bias in our study when analyzing association between pelvic pain and ovarian endometriosis.

Studies that found an association between ovarian endometrioma and pelvic pain were performed using univariate analysis (Fedele et al., 1992; Muzii et al., 1997). However, the methodological approach was poor to establish the fact that ovarian endometrioma are frequently associated with other peritoneal lesions (Chapron et al., 2009; Redwine, 1999), which themselves could cause pain. In studies that used multivariate analysis, results were less clear with pain symptoms apparently not correlated with the presence of ovarian endometrioma (Koninckx et al., 1991; Porpora et al., 1999). In this controversial situation, we demonstrated for the first time that pelvic pain in women with ovarian endometrioma is mostly associated with coexisting peritoneal lesions. In fact, we found that women with ovarian cystic endometriosis who had variable peritoneal lesions in their pelvic cavity complained of higher occurrence of pelvic pain (85.4%) than in women without coexisting visible peritoneal lesions (38.3%). The incidence of no complain of pain was higher among women with CC whose pelvic cavity was free of peritoneal lesions (61.7%) than in women had coexistent peritoneal lesions (14.6%).

When we investigated the association between the occurrence of pain

symptoms and size of ovarian endometrioma (<5cm vs. >5cm), we did not find any significant difference in pain manifestations between these two groups of women. This indicates that occurrence of pain in these women is independent of the size of ovarian endometrioma. This could be due to the fact that both of these two groups of women with ovarian endometrioma (<5cm and >5cm size) had variable patterns of coexisting peritoneal lesions.

In clinical practice, women with ovarian endometriosis are presented with different peritoneal lesions of pelvic endometriosis and with variable pattern of inflammation-induced pelvic adhesions. The associated intra-pelvic adhesion could be a cause of pain symptoms in addition to peritoneal lesions. As a matter of fact, we found that association of pain in women with ovarian endometriosis was higher in women having filmy or dense adhesion when compared with cases with no adhesion (Table 3). A proportion of cases with no adhesion also complained of pelvic pain. Most of the cases without any pelvic adhesion were coexistent with a variable distribution of peritoneal lesions. This indicates that women with ovarian endometrioma may complain of pain due to associated peritoneal lesions irrespective of the presence of intra-pelvic adhesion.

We previously demonstrated that tissue activity of peritoneal lesions, as evidenced by the higher tissue infiltration of macrophages (Mϕ) and production of different cytokines/growth factors, was significantly higher in red lesions, specially in blood-filled opaque red lesions, than in other transparent/translucent peritoneal lesions or in cyst walls of ovarian endometriosis (Khan et al., 2004a, 2004b). The tissue infiltrations of Mϕ in the endometria and peritoneal fluid content of Mϕ were found to be significantly higher during the late secretory phase or during the menstrual phase in women with peritoneal endometriosis than in control women (Khan et al., 2004a, 2005b). These findings of intra-uterine or pelvic inflammation may promote the occurrence of pelvic pain during the perimenstrual period. Macrophages and eutopic/ectopic endometrial cells have been reported to be the predominant cell types exhibiting increased expression of COX2 and production of PGE2/ PGF2α (Benedetto, 1989; Herath et al., 2006). In our current study we found an increased tissue infiltration of macrophages, increased tissue expression of COX2 and consequently increased tissue concentration of PGF2α in different peritoneal lesions and corresponding eutopic endometria comparing to cyst walls/eutopic endometria of women who did not harbor

any peritoneal lesion in their pelvic cavity. This may enhance PGF2α-induced uterine

contraction and may explain the mechanistic basis of increased prevalence of pelvic pain

in women with ovarian endometrioma had coexistent variable peritoneal lesions.

The association between higher production of PGs and severity of pelvic pain

or between application of PGE2/ PGF2α and dose-dependent increase in intrauterine

pressure secondary to uterine contraction has been demonstrated (Dawood et al., 1984;

Koike et al., 1992; Dittrich et al., 2009). In parallel with the higher Mϕ infiltration, both

PGE2 and PGF2α levels in endometrial tissues were found to be maximum during the

menstrual phase, intermediate in the secretory phase and less in the proliferative phase

(Willman et al., 1976). These previous results coincided with our current findings of

higher COX2 expression and PGF2α levels in the endometria/cyst walls of women with

CC had coexisting peritoneal lesions than in women with CC only whose pelvic cavity

was free of any visible peritoneal lesion. Although not significant with either proliferative

or secretory phase, we also found a modest increase of PGF2α in the eutopic

endometria/cyst walls during the menstrual phase. This increase of PGF2α was

dominant in women with CC had coexisting peritoneal lesions than in women with CC

without any visible peritoneal lesion.

The link between the higher expression of COX2/PGF2α in the eutopic endometria of women with CC+PL group and pelvic pain secondary to PG-induced uterine contraction was confirmed by one later study. By using cine magnetic resonance imaging (cine MRI), Kido et al. (2009) found that presence of sustained uterine contraction and frequency of uterine peristalsis were predominant during the menstrual phase, moderate in the secretory phase and were less visible during the periovulatory phase of the menstrual cycle. This enhanced uterine contraction during the menstrual phase was more frequently identified in women with endometriosis than in control women (Kido et al., 2009). These previously published reports of uterine contraction and our current findings may explain the role of COX2/PGF2α in the generation of higher incidence of pelvic pain in women with CC + PL than in women with only CC without any coexisting visible peritoneal lesion.

In conclusion, our retrospective analysis of cases with ovarian endometrioma and prospective biological investigation indicated that women with only ovarian endometrioma experience less pain and pelvic pain in women with ovarian endometrioma

is mostly associated with coexisting peritoneal lesions. An increased tissue inflammatory

reaction with elevated prostaglandin production may be responsible for the generation of

pain in these women. Lack of evaluation in the detection of general or disseminated DIE

in pelvic cavity could be a bias or limitation in this study. Further multi-center

prospective studies are needed to strengthen our current findings.

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Conflict of interest: The authors declare that there is no conflict of interest related to this article.

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Figure legends

Figure 1. Shows the immunohistochemical staining of CD68-postive macrophages (Mϕ) (brown dots) in the red lesion, black lesion and chocolate cyst (CC) wall, their corresponding eutopic endometria and in unaffected normal peritoneum derived from women with and without endometriosis (**A**). The mean Mϕ numbers per

field was significantly higher in red lesions (black bar) and corresponding eutopic

endometria (white bar) than in control endometria (white bar) and other peritoneal lesions

and cyst wall of CC (black bar) and their corresponding endometria (white bar) (**B**). A

significantly higher Mϕ infiltration was observed in the eutopic endometria derived

from women with CC plus coexistent peritoneal lesions (PL) and from women harboring

black lesions than in control women and in CC only group (**B**). No difference in Mϕ

infiltration was found among black lesions, CC + PL group, and CC only group or in

unaffected normal peritoneum (**B**). The results are expressed as mean $\pm$ SEM of Mϕ

counts in five different fields of one specimen (x200). *p<0.05 vs. control/black/CC;

**p<0.001 vs. black/CC, ¶p<0.05 vs. control/CC only group; endo (+) denotes normal

peritoneal samples derived from women with endometriosis, endo (-) denotes normal

peritoneal samples derived from women without endometriosis.

Figure 2. Shows immunolocalization of cyclooxygenase 2 (COX2), a

rate-limiting enzyme of prostaglandin production, in the biopsy specimens derived from

red lesion/black lesion, cyst wall of chocolate cyst (CC) + coexisting peritoneal lesion

(PL) and cyst wall from CC only women (**A**, lower column) and their corresponding

eutopic endometria (**A**, upper column). A significantly higher quantitative-histogram

(Q-H) scores of COX2 immunoexpression was found in the red lesions than in either

black lesions or in cyst wall of CC + PL or in cyst walls without coexisting PL (black bar,

B). Although Q-H scores of COX2 immunostained cells was almost similar in the eutopic

endometria (white bar) of women containing red lesions, black lesions and CC + PL

group, these Q-H scores were significantly higher when compared with the eutopic

endometria derived from control women or from CC only women (**B**). The Q-H scores of

COX2 in the black lesions and cyst walls derived from women with CC + PL were also

significantly higher comparing to cyst walls derived from CC only women (Figure 2B).

The results are expressed as mean ± SEM of five different fields of one section by light

microscopy at moderate magnification (× 200). *p<0.01 vs. control/CC only group;

**p<0.01 vs. black/CC+PL/CC only group, ¶p<0.05 vs. CC only group.

       Figure 3. Shows immunolocalization of cyclooxygenase 2 (COX2), in the

biopsy specimens of eutopic endometrium derived from women with chocolate cyst +

coexisting peritoneal lesion (CC + PL group, upper column of **A**) and women with

chocolate cyst only without any coexisting peritoneal lesions (CC only group, lower

column of **A**) based on the phases of menstrual cycle (**A**). The quantitative-histogram

(Q-H) scores of COX2 in the eutopic endometria were found to be higher during the

menstrual phase than in either proliferative phase or secretory phase of the menstrual

cycle (**B**). These menstrual phase dependent findings of COX2 expression were more

prominent in women with CC + PL than in CC only women (**B**). The results are expressed

as mean ± SEM of five different fields of one section by light microscopy at moderate

magnification (× 200).

       Figure 4. Shows tissue concentrations of prostaglandin F2α (PGF2α) in the

eutopic (white box) and ectopic endometria (hatched box) of control women, women with

peritoneal endometriosis and women with chocolate cyst (CC). The tissue concentration

of PGF2α was significantly higher in the eutopic endometria derived from women

containing red lesions, black lesions and from women with CC + coexisting peritoneal

lesions (CC+PL group) than in control women and in CC only women. Although no

difference in tissue levels of PGF2α was observed among black lesions, cyst wall of CC

+ PL and cyst wall of CC only group, PGF2α level in red lesions was significantly higher

than in other lesions. Boxes represent the distance (interquartile range) between the first

(25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. *$p<0.05$ vs. CC only group/control women; **$p<0.05$ vs. other lesions.

Figure 5. Shows tissue concentrations of prostaglandin F2α (PGF2α) in the eutopic endometria (**A**) and cyst walls (**B**) derived from women with chocolate cyst + coexisting peritoneal lesions (CC+PL, white box) and from CC only women (hatched box) according to the phases of the menstrual cycle. The levels of PGF2α in both cyst walls (**B**) and corresponding eutopic endometria (**A**) derived from women with CC +PL and CC only women was maximum during the menstrual phase comparing to others phases of menstrual cycle (**A, B**). CC + PL group women showed a relatively higher tissue levels of PGF2α than in CC only group women across the menstrual cycle. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.
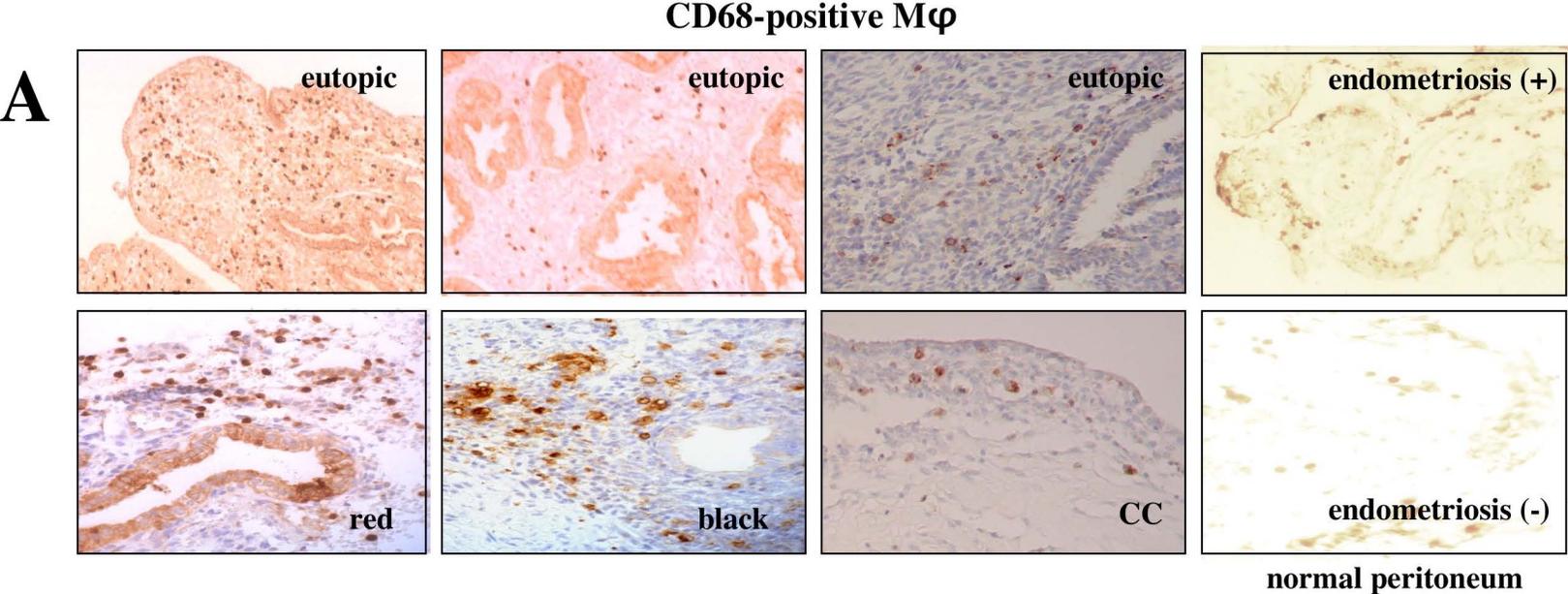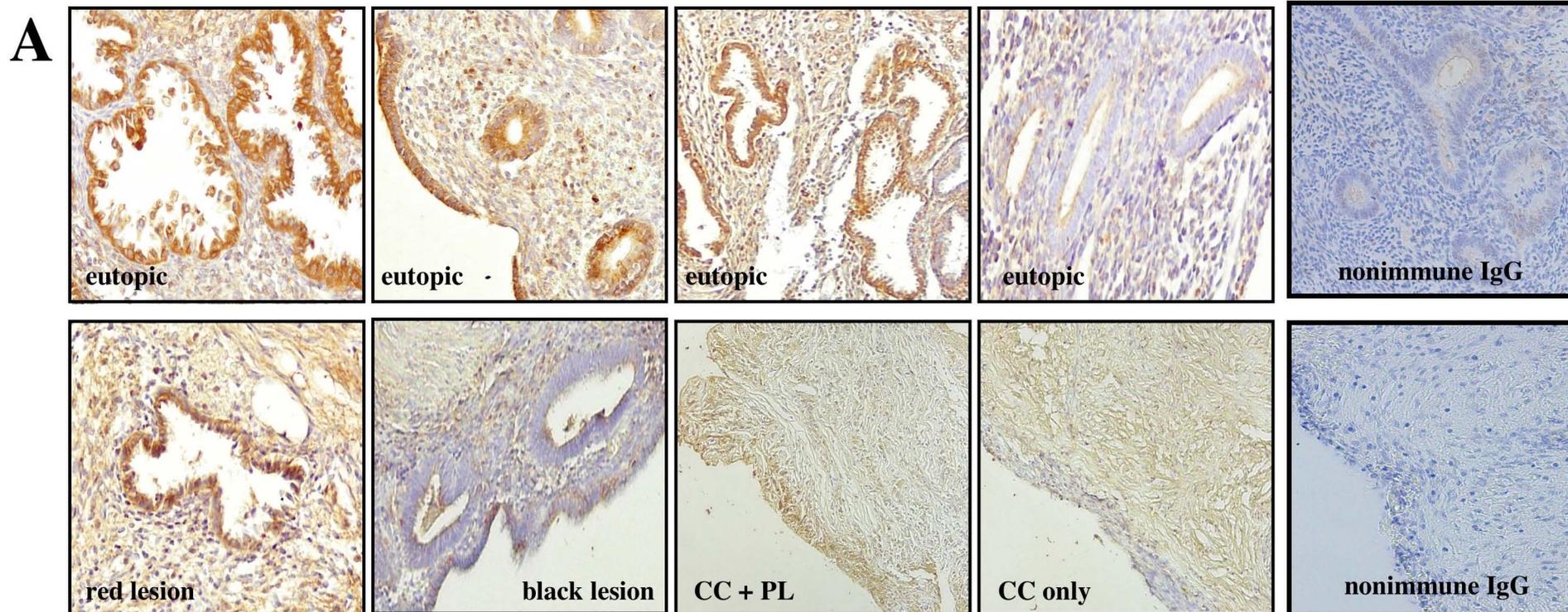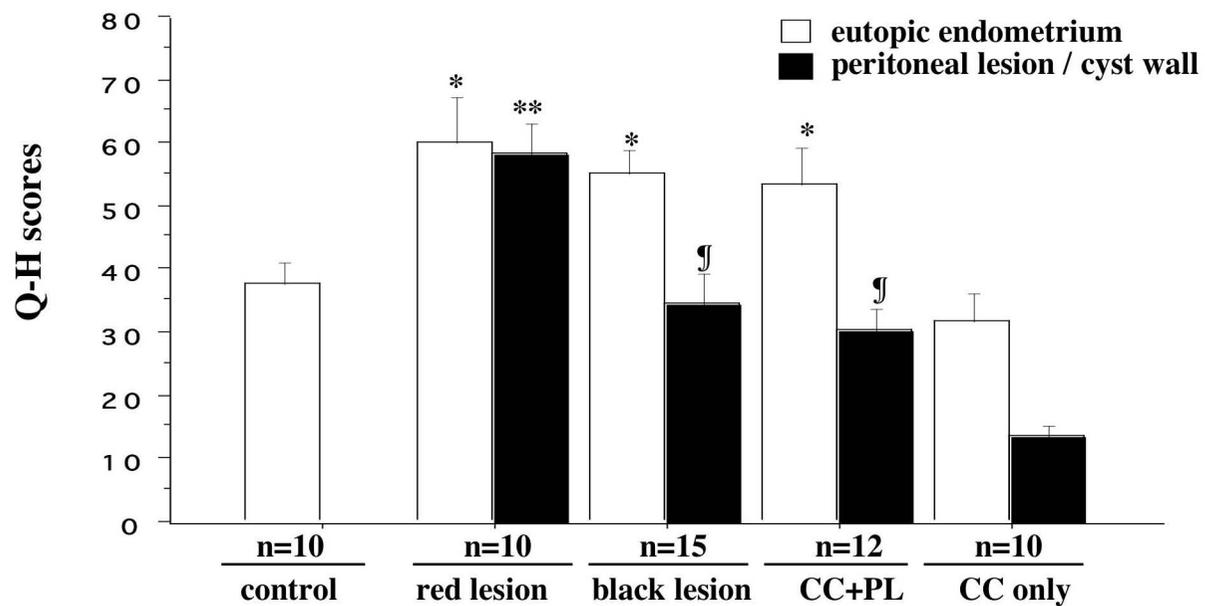
Figure 1.

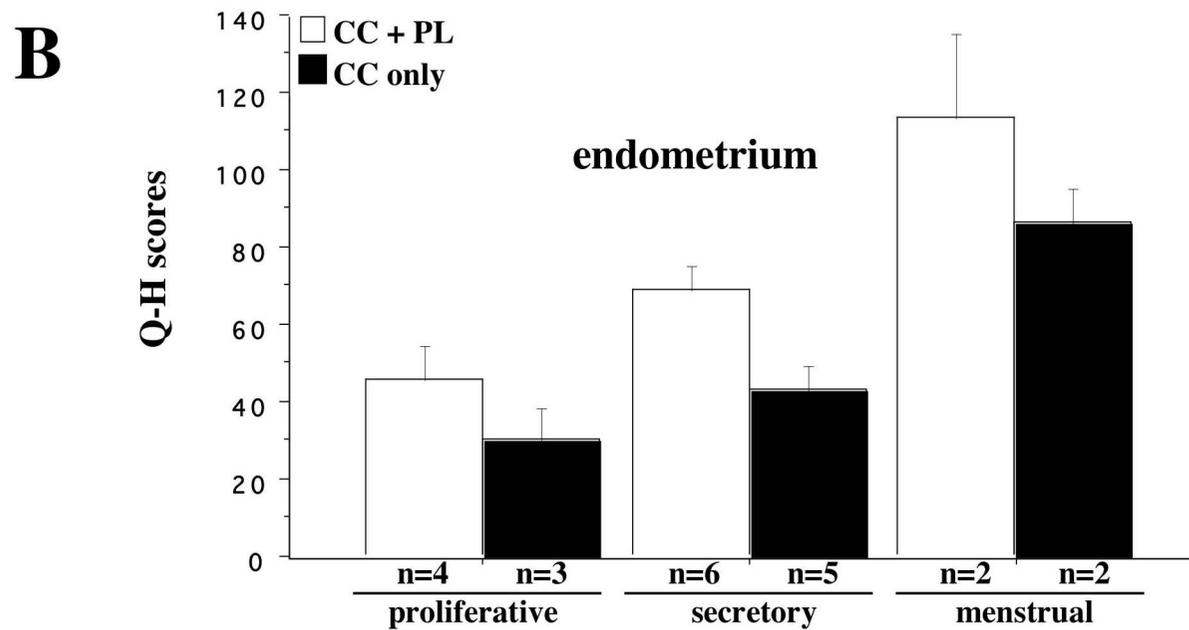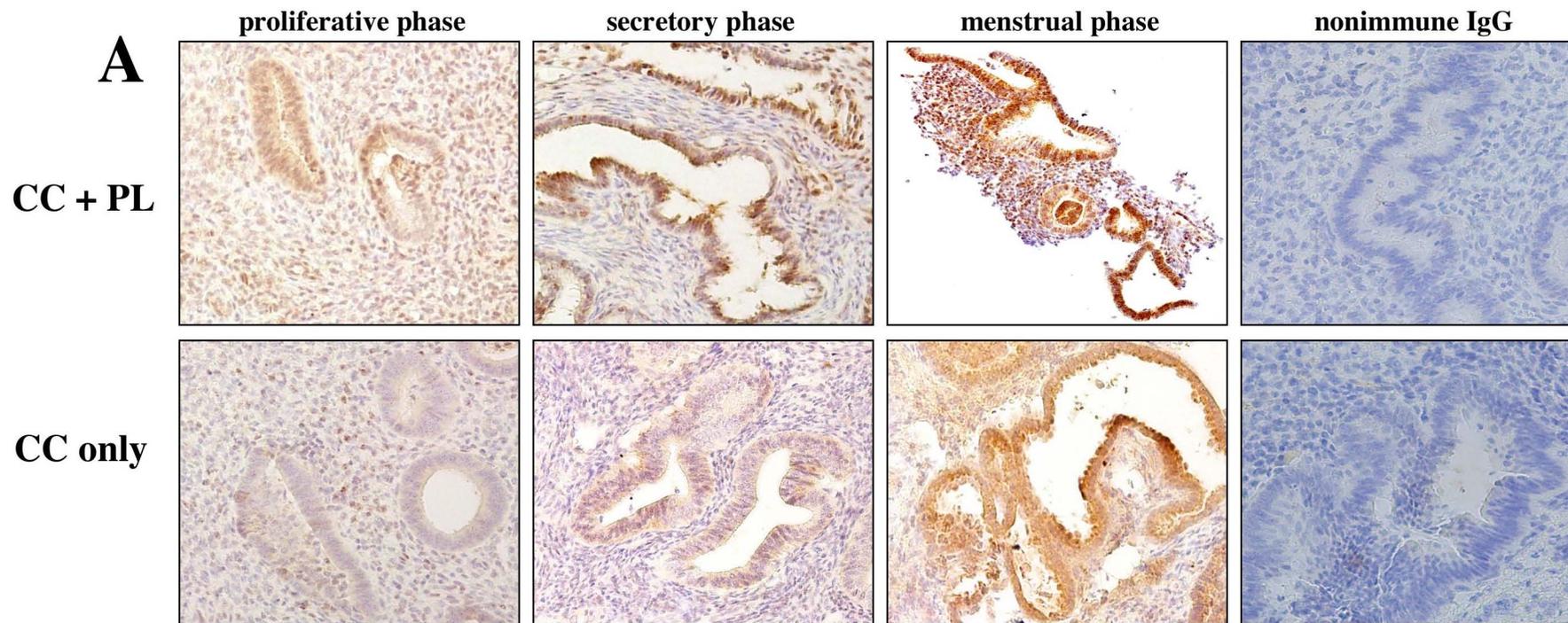**CD68-positive Mφ**
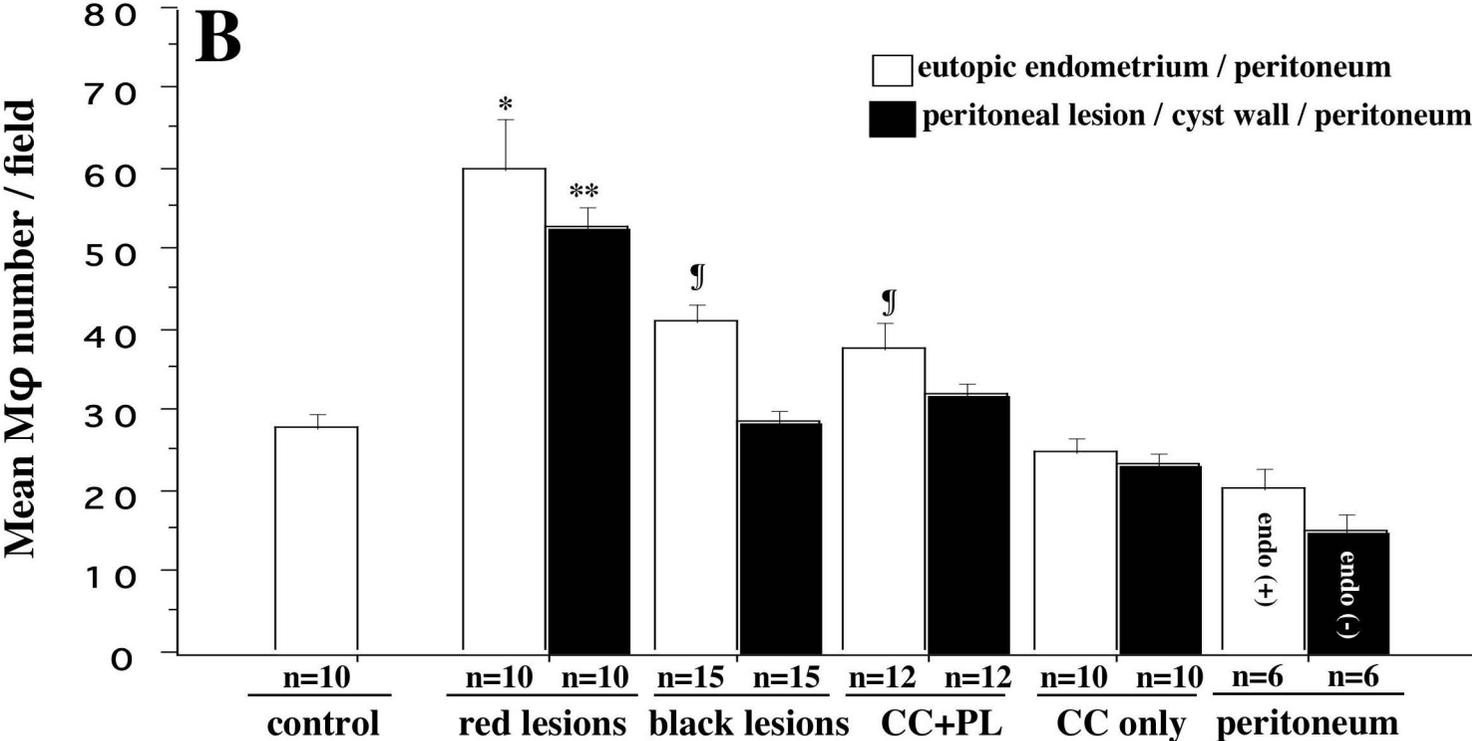
Figure 2.

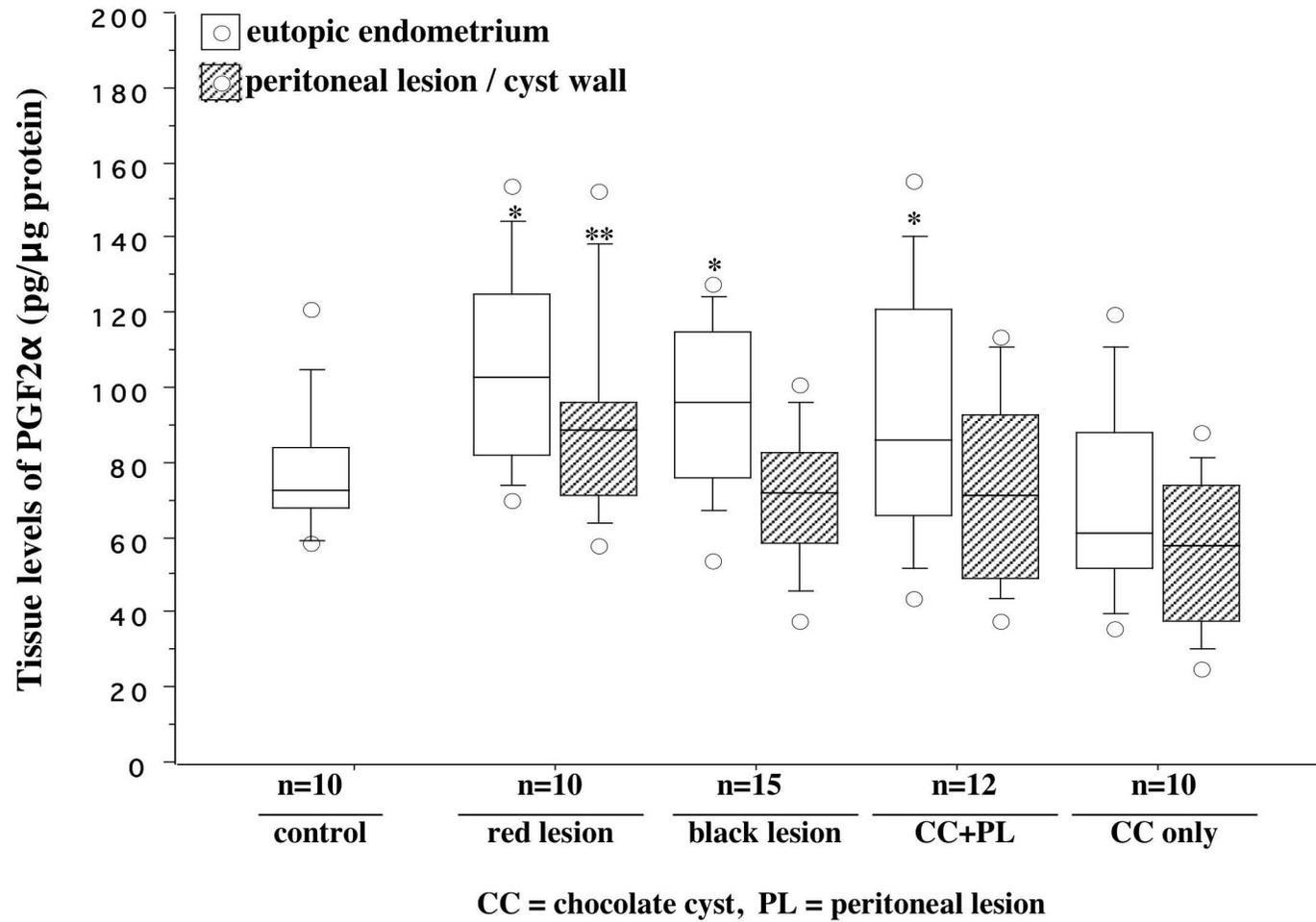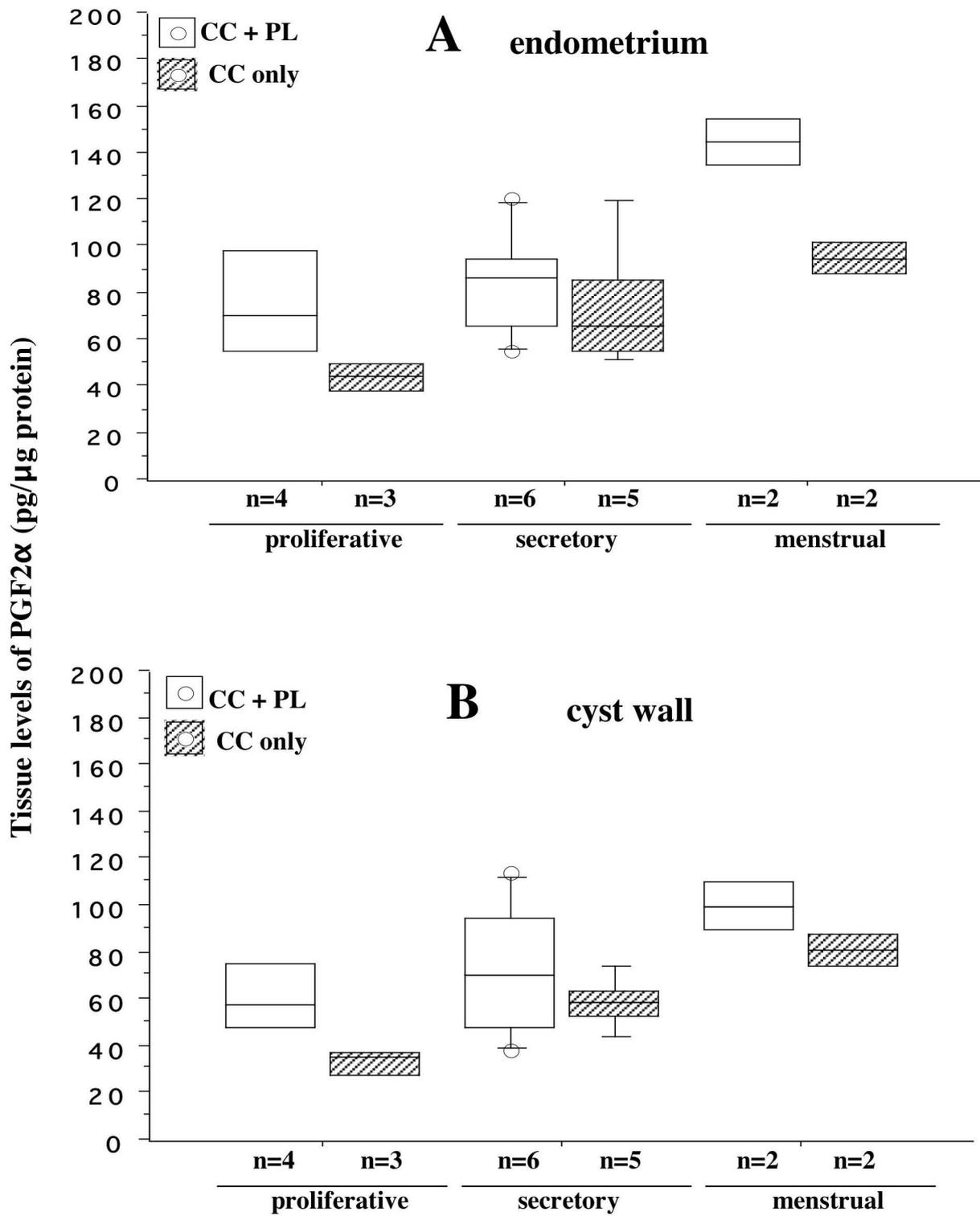# Figure 3.

Figure 4.

Figure 5.

Table 1. Clinical characteristics of 350 cases with chocolate cyst that were detected among 2988 cases of laparoscopic surgery during the period of 1982-2008.

	n (%)
Distribution according to age (years):	
<20	**3 (0.85)**
20-29	**110 (31.4)**
30-39	**181 (51.7)**
40-49	**51 (14.6)**
50-59	**5 (1.4)**
Distribution according to size (cm):	
<3	**8 (2.3)**
>3-5	**185 (52.8)**
>5-8	**133 (38.0)**
>8-10	**21 (6.0)**
>10	**3 (0.85)**
Laterality:	
left sided	**154 (44.6)**
right sided	**92 (26.3)**
bilateral	**102 (55.2)**
revised ASRM staging:	
stage II	**6 (1.7)**
stage III	**151 (43.1)**
stage IV	**193 (55.2)**
Phases of menstrual cycle: (P/S/M/A)	**87/212/36/15**
Coexisting peritoneal lesions:	
no lesion	**81 (23.1)**
red lesions	**29 (8.4)**
black lesions	**83 (23.5)**
white lesions	**18 (5.3)**
mixed lesions	**139 (39.7)**
Pattern of pelvic adhesion:	
no adhesion	**55 (15.7)**
filmy adhesion	**216 (61.8)**
dense adhesion	**79 (22.5)**

P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea

Table 2. Association of pain in 350 cases with chocolate cysts with and without coexisting peritoneal lesions.

	Cases with chocolate cyst without peritoneal lesions (n=81)	Cases with chocolate cyst with peritoneal lesions (n=269)
no complain of pain, n (%)	50 (61.7)	39 (14.6)
complain of pain,　n (%)	31 (38.3)	230 (85.4)*

Pain was defined by the presence of menstrual pain, dysmenorrhea, and chronic pelvic pain either alone or in combination. *p<0.01 vs. cases without peritoneal lesions by chi square test.

Table 3. Association of pain in 350 cases with chocolate cysts according to pattern of pelvic adhesion.

	No adhesion (n=55)	filmy adhesion (n=216)	dense adhesion (n=79)
no complain of pain, n (%)	29 (52.2)	44 (20.6)	10 (13.1)
complain of pain, n (%)	26 (46.8)	172 (79.6)	69 (86.9)

Pain was defined by the presence of menstrual pain, dysmenorrhea, and chronic pelvic pain either alone or in combination.