Int J Clin Exp Med 2016;9(11):20856-20865
www.ijcem.com /ISSN:1940-5901/IJCEM0030127

Original Article

Immunoreactivity of CD68, granulocyte-macrophage colony-stimulating factors receptor and vonWillebrand factor and its association with dysmenorrhea severity and the amount of menses in adenomyosis

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Received April 8, 2016; Accepted September 7, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Symptomatic adenomyosis often presents as a soft and diffusely enlarged uterus with dysmenorrhea and menorrhagia. It is reported that higher myometrial infiltration depth is associated with dysmenorrhea in adenomyosis, pinpointing inflammationand angiogenesis is of critical importance in the growth, survival, and myometrial infiltration of endometrial cells. Therefore we investigated the pattern of changes in inflammatory reaction and micro-vessel density in women with and without adenomyosis. For these experiments, Ectopic and homologous eutopic endometrium from 50 women with adenomyosis and endometrium from age 18 and above and menstrual phase-matched women without adenomyosis were used for immunohistochemical analysis. Tissue sections were immunostained with CD68 (a macrophage marker), granulocyte-macrophage colony-stimulating factors receptor (GM-CSFR, binding the GM-CSF produced by macrophages), and vonWillebrand factor (vWF, a vessel marker). GM-CSFR expression was higher in ectopic endometrium than in control endometrium, while both macrophage and micro-vessel number was higher in eutopic and ectopic endometrium than in control endometrium. Both macrophage number in eutopic and ectopic endometrium and GM-CSFR expression in ectopic endometrium were positively correlated with the severity of dysmenorrhea, and found to be significant predicators for dysmenorrhea severity. Increased micro-vessel number in both eutopic and ectopic endometrium were positively associated with heavier menses. These findings suggest that inflammation and angiogenesis may be involved in the genesis of adenomyosis. They also suggest the possibility that inflammation and angiogenesis be involved in dysmenorrhea and menorrhagia rendering them as potential therapeutic targets in the treatment of adenomyosis.

Keywords: Adenomyosis, dysmenorrhea, menorrhagia, CD68, GM-CSFR, vWF

Introduction

Much of adenomyosis has been discovered only from an anatomical perspective as we know it is benign and features an invasive nature that the glands and stroma can penetrate deep into the myometrium. However, little is known about its pathogenesis and hysterectomy presents as the main treatment option.

Symptomatic adenomyosis often presents as a soft and diffusely enlarged uterus with dysmenorrhea, heavy menstrual bleeding, and poor fertility [1]. Among women with adenomyosis who complain of dysmenorrhea, the prevalence rate is estimated to range between 15-30%, but could be as high as 65-78% [2]. Consequently dysmenorrhea is the most prevalent symptom besides abnormal uterine bleeding and can be very debilitating having a negative impact on people's life and work. It is surmised that even in women with endometriosis, persistent dysmenorrhea after surgery or dysmenorrhea of long duration may be indicative of adenomyosis [3, 4], anuntapped population worth further investigations. Obviously there is a lack of weaponry of medications and the medical community has to resort to hysterectomy as the treatment of choice for symptomatic adenomyosis, a procedure with huge impact on patients' reproductive expectations and family planning. It is becoming imperative to gain further insight to the pathogenesis and search for the identification of key targets involved in adenomyosis-associated dysmenorrhea. We believe our contributing efforts may offer prom-

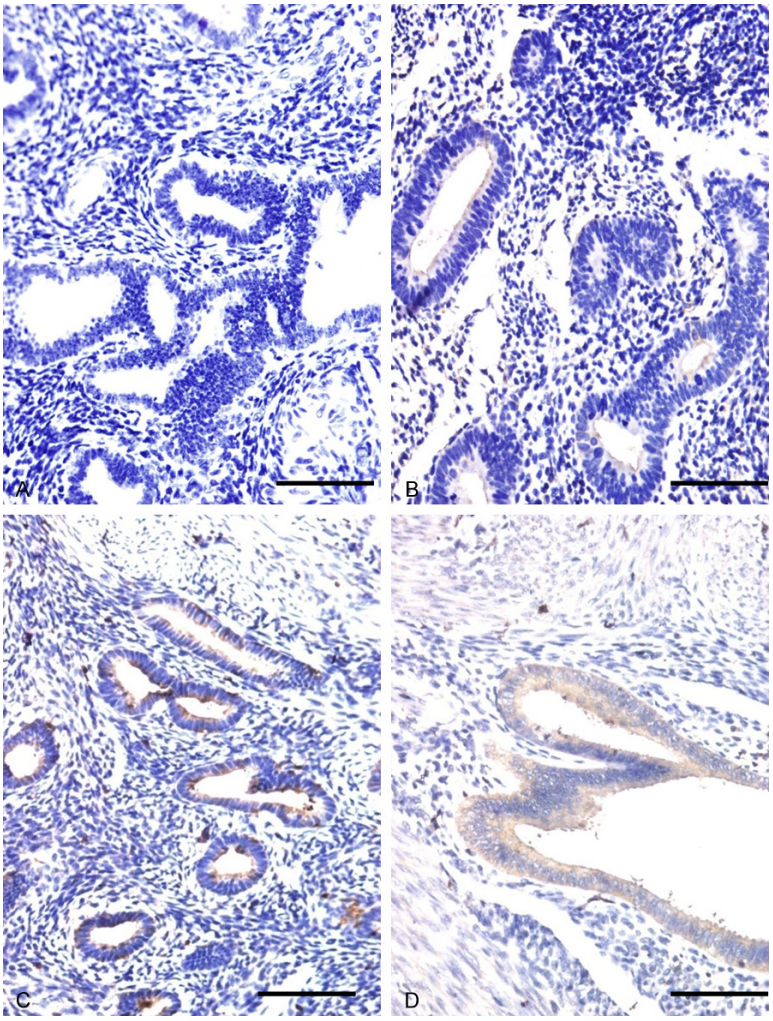

Figure 1. Immunohistochemical staining of GM-CSFRα. A. Staining in a normal endometrial tissue, using preimmune IgG, instead of the primary, antibody, that serves as a negative control. B. Immunohistochemical staining in a normal endometrial tissue. C and D. Staining in epithelial cells of eutopic and ectopic endometrium from a woman with adenomyosis. All magnifications were ×400. Scale bars represent 50 μm.

ises or at least point to workable directions for more effective treatment for adenomyosis-related dysmenorrhea and perhaps chronic pain as well.

Added to the list of treatment targets included the findings that decreased immunoreactivity to progesterone receptor isoform B (PR-B) and increased immunoreactivity to NF-κB, oxytocin receptor (OTR), and transient receptor potential vanilloid type 1 (TRPV1) were correlated with the severity of dysmenorrhea in women with adenomyosis [5-8], demonstrating their intimate involvement in the dysmenorrhea in adenomyosis. In addition, it is reported that higher myometrial infiltration depth is associated with dysmenorrhea in adenomyosis [9], pinpointing angiogenesis is of critical importance in the growth, survival, and myometrial infiltration of endometrial cells. Further, as in endometriosis, the involvement of vascular endothelial cell growth factor (VEGF) and other angiogenic mediators has long been recognized [10-14], and it has been shown that SLIT, a secretory glycoprotein and its derivatives, plays a role in increasedimmunoreactivity in endometriosis and overexpression of SLIT may be a biomarker candidate for recurrence of endometriosis [15, 16]. Both SLIT immunoreactivity in ectopic endometrium and microvessel density (MVD) in eutopic endometrium were positively correlated with the severity of dysmenorrhea and found to be significant predicators for dysmenorrhea severity in women with adenomyosis [7].

The research for treatment targets recently led us to the in vivo findings that bone cancer pain was abrogated by knockdown of granulocyte-macrophage colony-stimulating factors receptor (GM-CSF receptor) on primary afferent nerve fibers [17]. Considering that GM-CSF was primarily expressed by the monocyte-macrophage system [18, 19], and that CD68 positive macrophages were significantly increased in eutopic endometrium in women with endometriosis during all stages of the proliferative phase compared with normal controls [20], we analyzed GM-CSFR expression in adenomyosis and hypothesized that blockage of this receptor might be helpful in treating dysmenorrhea. VonWillebrand factor (VWF), a vessel marker, using respective antibodies was also brought to the research screen because of its reported infiltration of CD68-

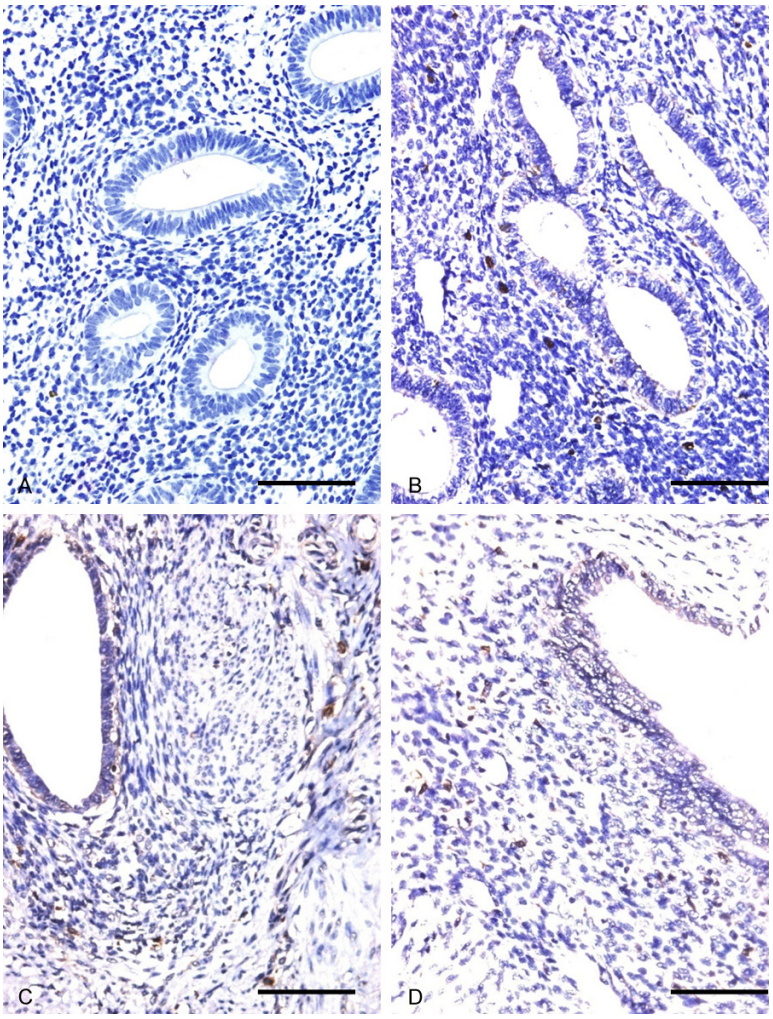

Figure 2. CD68 immunoreactivity. A. CD68 staining in a normal endometrial tissue, using preimmune IgG antibody instead of the primary antibody, that serves as a negative control. B. CD68 staining in epithelial cells from a control eutopic endometrium. C and D. CD68 staining in epithelial cells of eutopic and ectopic endometrium. All magnifications were ×400. Scale bars represent 50 μm.

positive Mf and VWF-positive micro-vessel density in the endometrial of women with endometriosis, adenomyosis and uterine myoma [21].

Liu et al maintained as adenomyosis and endometriosis bear many resemblances in that they are both estrogen-dependent, anatomically similar in definition with similar symptom profile and treatment and the two often occur concurrently [15, 22, 23]. Indeed, it has been proposed recently that both endometriosis and adenomyosis may result from tissue injury and repair [24]. This led us to believe that the above triad may also be closely involved in and contribute to the pathogenesis of adenomyosis and is worthy of further research.

We hypothesized that, as in endometriosis, GM-CSFR, CD68 and VWF expressions are also elevated in adenomyosis, and may contribute to heavy menstrual bleeding and dysmenorrhea in adenomyosis. Hence, in this study we sought to investigate the expression and localization of the triad in eutopic and ectopic endometrium of women with adenomyosis and in endometrium of women without adenomyosis. We also sought to determine the relationship among the amount of menses, and severity of dysmenorrhea and the triad immunoreactivity before we claim they are significant predicators for adenomyosis-associated dysmenorrhea severity [23].

Materials and methods

Patients and tissue samples

The patients recruited to this study were reported previously [5-7]. Briefly, 50 women with histological confirmed adenomyosis (excluding endometriosis) seen at Shanghai OB/GYN Hospital, Fudan University Shanghai Medical College, from 2004 to 2005, were recruited for this study. All of them had diffuse adenomyosis, the most common subtype seen in China. Their diagnoses were made by transvaginal ultrasound before surgery and histologically confirmed post-operatively, based on the presence of endometrial glands and stroma at least one lower-power field of view (about 10×10, or about 2-3 mm) away from the endometrial-myometrial junction [25]. All patients' ectopic, along with their homologous eutopic, endometrial tissue samples, were collected after hysterectomy and fixed in 10% buffered formalin and routinely processed for paraffin embedding. For controls, we also collected, after informed consent, endometrial tissue samples through curettage from 18 wo-

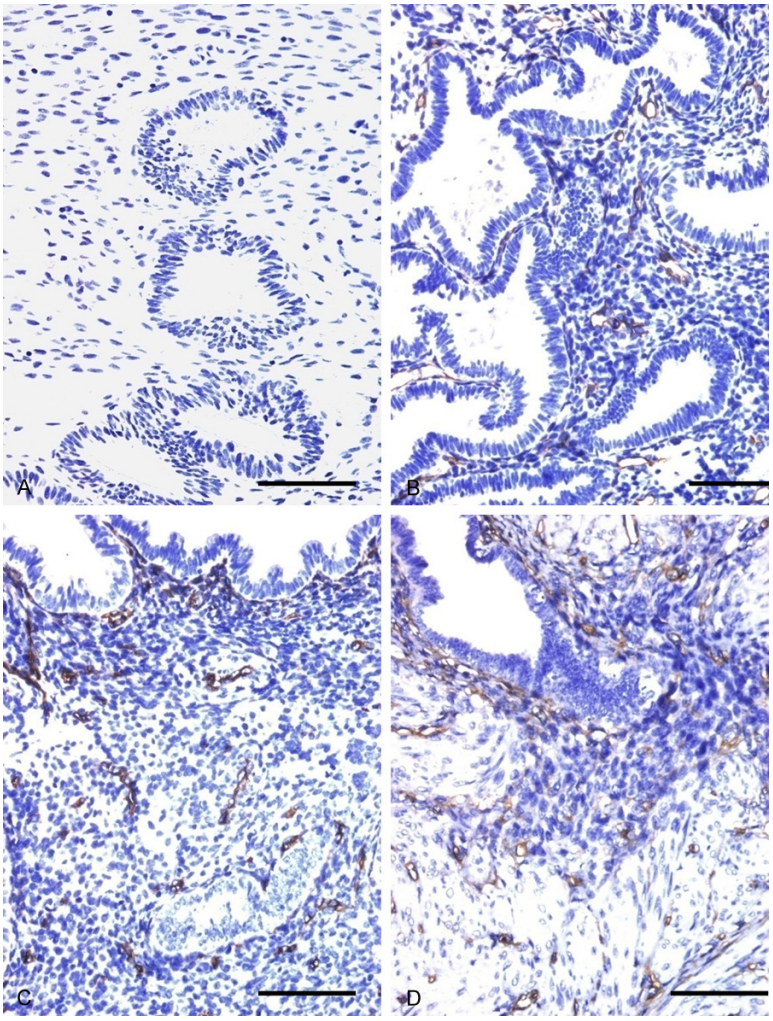

Figure 3. vWF immunoreactivity. A. vWF staining in a normal endometrial tissue, using preimmune IgG antibody instead of the primary antibody, that serves as a negative control. B. vWF staining in epithelial cells from a control eutopic endometrium. C and D. vWF staining in epithelial cells of eutopic and ectopic endometrium. All magnifications were ×400. Scale bars represent 50 μm.

men with surgically diagnosed benign ovarian cysts, but none had endometriosis, adenomyosis, or myoma. The selection of the controls was based on menstrual phase and age besides disease status.

All women in both study and control groups were premenopausal and had regular menses (range of the menstrual cycle lengths: 21--35 days), with no history of hormone therapy or intrauterine device use for ≥ 6 months prior to the surgery or tissue collection. The menstrual phase in which the patient was at the time of surgery was determined based on the day elapsed since the last period. All endometrial samples were grouped either in proliferative (days 1-14 of the cycle) or secretory (days 15-28 of the cycle) phase. In both study and the control groups, exactly half of women were in the proliferative phase while the rest half were in the secretory phase. Depending on whether they changed their sanitary pads < 3, between 3 and 6, or > 6 times a day, respectively, as we reported previously [5-7], their amount of menses during menstruation was grouped into three classes: light, moderate, and heavy. The severity of dysmenorrhea was classified as mild, moderate, and severe, as reported previously [5-7] and roughly equivalent to the verbal descriptor scale (VDS). This study was approved by the institutional ethics review board of Shanghai OB/GYN Hospital.

Tissue sample, antibodies, and immunohistochemistry (IHC)

Archived, formalin-fixed, paraffin-embedded tissue blocks were retrieved from the Department of Pathology, Shanghai OB/GYN Hospital. Serial 4-μm sections were obtained from each block, with the first resultant slide being stained for H&E to confirm pathologic diagnosis, and the subsequent slides stained fort CD68, vWF, and GM-CSFRα. Routine deparaffinization and rehydration procedures were performed [23].

The mouse monoclonal antibodies against CD68 and vWF (#MS-397-P0, #MS-722-P0, Lab Vision Corporation, Fremont, CA, USA) and GM-CSFRα (sc-21764, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:100, 1:50 and 1:50 respectively, were used as primary antibodies. For antigen retrieval, the slides were heated at 98°C in an EDTA buffer (pH 9.0) for a total of 30 min and cooled na-

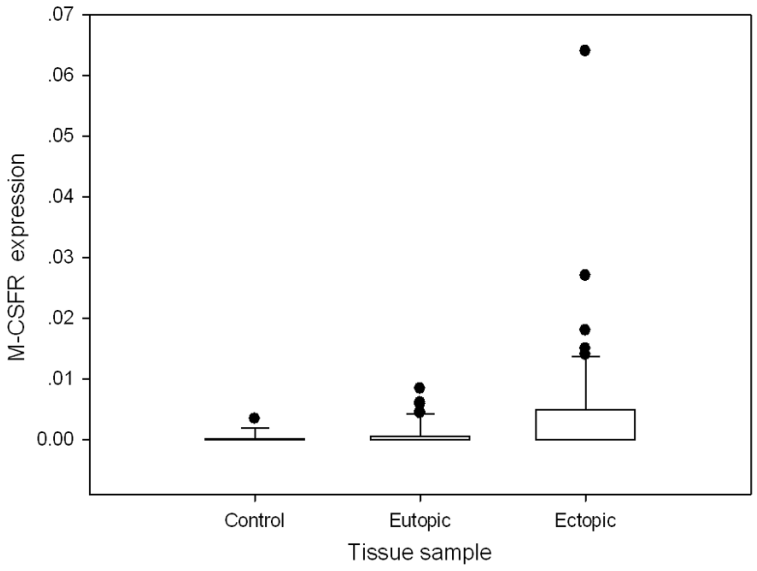

Figure 4. Boxplot of GM-CSFRα immunoreactivity levels in normal endometrium from controls and eutopic and ectopic endometrium from women with adenomyosis.

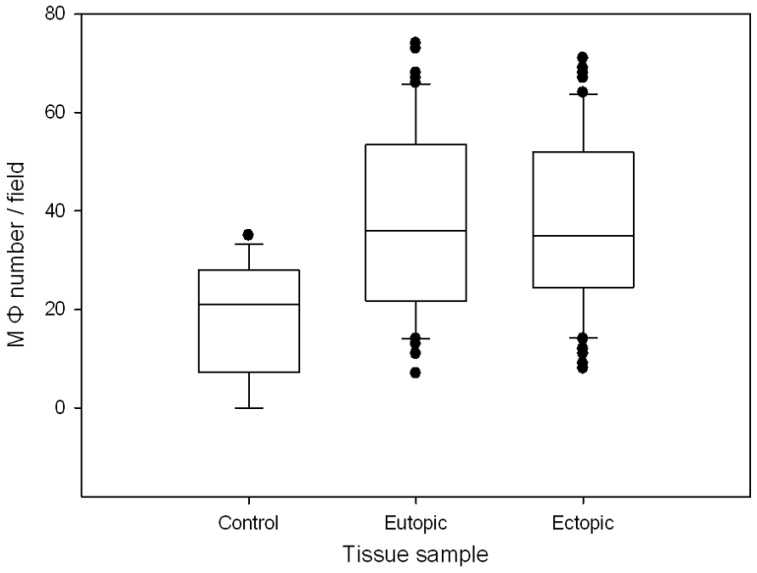

Figure 5. Boxplot of macrophage number in normal endometrium from controls and eutopic and ectopic endometrium from women with adenomyosis.

complexes were stained for 3-5 min or until appropriate for microscopic examination with diaminobenzidine and then counterstained with hematoxylin and mounted.

A negative control was also incorporated using preimmune IgG instead of the primary antibody. The scoring of the immunoreactivity of GM-CSFRα was evaluated by digital image analysis using the Image Pro-Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA) as reported in [26] without prior knowledge of any of the clinicopathological information. Briefly, images were obtained with the microscope (Olympus BX51, Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70, Olympus, Tokyo, Japan). A series of 10 random images on several sections were taken for each immunostained parameter to obtain a mean value. Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images, and measurements were obtained. Immunohistochemical parameters assessed in the area detected included (a) integrated optical density (IOD); (b) total stained area (S); and (c) mean optical density (MOD), which is defined as MOD=IOD/S, equivalent to the intensity of stain in the positive cells.

turally at room temperature. Sections were then incubated overnight with the primary antibody at 4°C. After slides were rinsed, the biotinylated secondary antibody, Supervision TM Universal (Anti-Mouse/Rabbit) Detection Reagent (HRP) (GK500705, Shanghai Gene Tech Company, Shanghai), was incubated at room temperature for 30 min. The bound antibody

The immunoreactive CD68 spots were counted in five different fields ofone section (*400 magnification) by light microscopy and expressed as the mean macrophage number per field in one specimen, as reported previously [21]. Micro-vessel numberwas assessed on vWF stained slides by light microscopy in the areas having the highest numbers of capil-

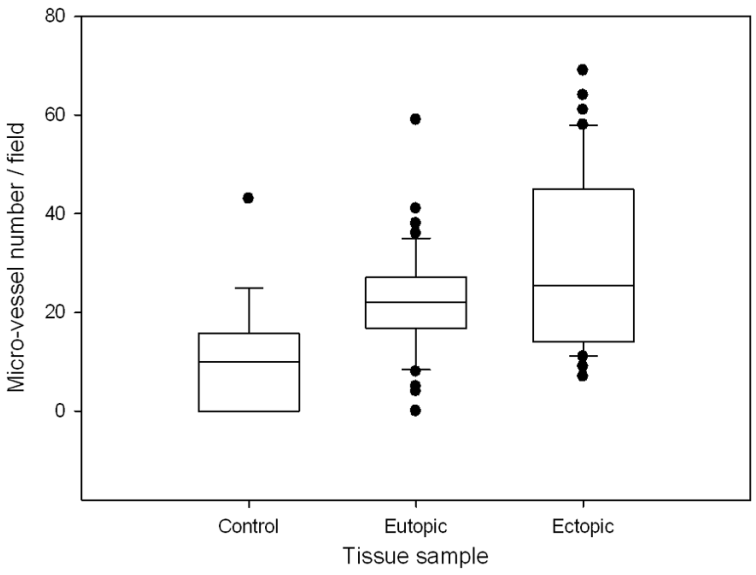

Figure 6. Boxplot of micro-vessel number in normal endometrium from controls and eutopic and ectopic endometrium from women with adenomyosis.

laries and small venules (neovascular hot spots). Then microvessel counting followed on 5 chosen 400× fields of the "hot plot" by the same investigator who did not know the history of the patients. Endothelial cells or cell cluster clearly separated from adjacent microvessels, ectopic endometrial cells, and other connective tissue elements were taken into account for microvessel counting. Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. The micro-vessel number was defined to be the mean of the vessel counts obtained in these fields, as reported previously [21].

Results

Clinicopathological data

The clinicopathological data were reported previously [27]. Briefly, among the 50 patients with adenomyosis, 6 (12%), 13 (26%), 17 (34%), and 14 (28%) complained having no, mild, moderate, and severe dysmenorrhea, respectively. The mean age and its standard deviation (SD) in women with adenomyosis and without were 43.4 (SD=3.9, range=32-50) years and 43.9 (SD=5.9, range=30-51) years, respectively. There was no significant difference in age between the cases and controls (P=0.34, Wilcoxon's test).

One (2%) patient reported only light menses during menstruation while the other 30 (60%) and 19 (38%) patients complained of having moderate and heavy menses, respectively. No relationship was found between the severity of dysmenorrhea and the amount of menses (Spearman's rank correlation r= 0.15, P=0.31), between severity of dysmenorrhea and the uterus size (r=0.19, P= 0.19), or between uterus size and the amount of menses (r=0.07, P=0.64). The time elapsed from the first diagnosis of adenomyosis to surgery ranged from 0.25 month to 144 months, with a median length of 36 months or 3 years [23].

GM-CSFRα, CD68 and vWF immunohistochemistry in normal, eutopic and ectopic endometrium

Representative immunostaining for GM-CSFRα in eutopic and ectopic and control endometrium is shown in **Figure 1**. As can be seen from **Figure 1**, GM-CSFRα immunoreactivity was seen mostly in glandular epithelial cells and was localized both in cytoplasm and membrane. CD68 staining was seen in macrophage and vWF immunostaining was seen mostly in vascular endothelial cells in both control and eutopic and ectopic endometrium (**Figures 2**, **3**).

In control endometrium, no difference in GM-CSFRα immunoreactivity or macrophage number or micro-vessel number was found between proliferative and secretory phases (all P > 0.16). In eutopic and ectopic (all P > 0.23) endometrium, no such difference was found, either.

The GM-CSFRα expression in ectopic, but not in eutopic endometrium, was significantly higher than that in the normal endometrium (P= 0.033; **Figure 4**). In contrast, the macrophage number and micro-vessel number in both eutopic and ectopic tissue was higher than that in the normal endometrium, and micro-vessel number was the highest in ectopic endometrium and the lowest in the normal endometrium (all *P* values < 0.05, **Figures 5** and **6**). Macrophage number both eutopic and ectopic

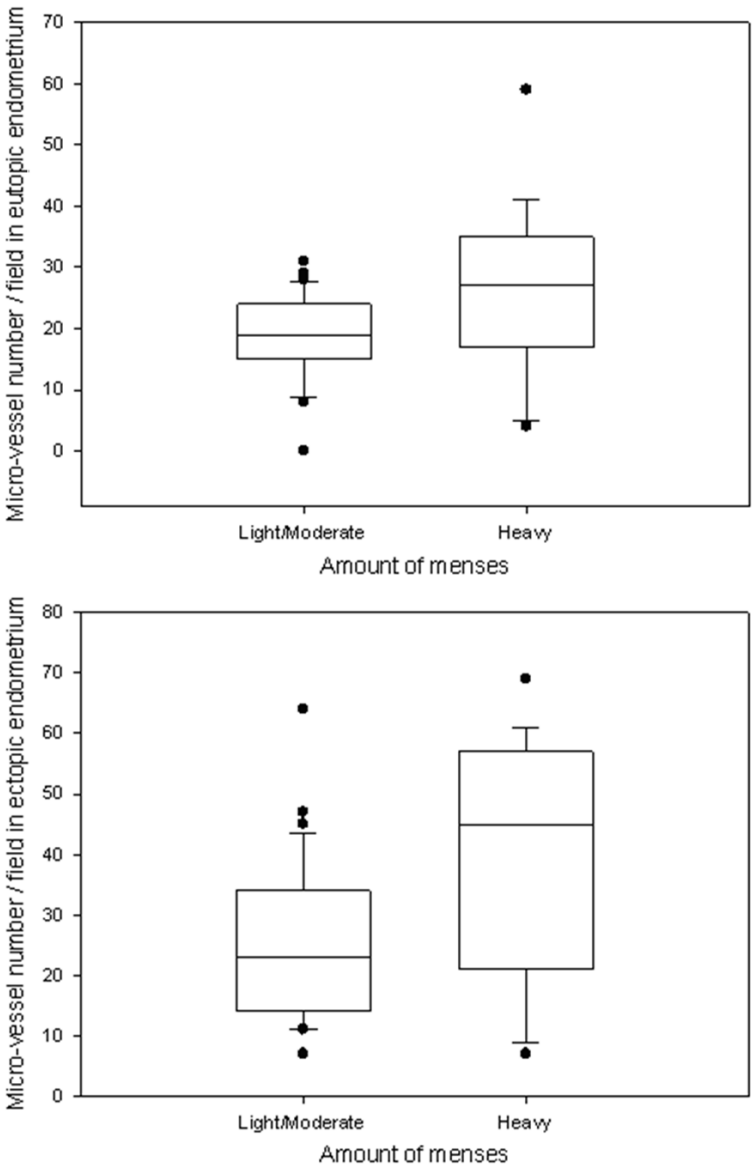

Figure 7. Boxplot of micro-vessel number andamount of menses in adenomyosis. "Mod." is an abbreviation of "Moderate".

endometrium were significantly correlated with micro-vessel number in eutopic tissue. (r= 0.738 and r=0.643, both *P* values < 0.05). These results suggest that inflammation and angiogenesis was higher in eutopic and ectopic endometrium as compared with control endometrium.

Amount of menses and micro-vessel number

Since there was only one woman who reported light menses, we divided all cases into two groups: women who reported light and moderate menses, and those who reported heavy menses. We found that micro-vessel number staining in both eutopic and ectopic endometrium was significantly higher in women with heavy menses than those with light/moderate menses (P= 0.011, P=0.004; **Figure 7**).

Relationship between dysmenorrhea severity and macrophage number and GM-CSFRα immunohistochemistry

Since dysmenorrhea is another major complaint in women with adenomyosis, we next attempted to examine as whether these biomarkersare associated with the severity of dysmenorrhea in women with adenomyosis. We found that macrophage number in both eutopic and ectopic endometrium were significantly correlated with the severity of dysmenorrhea (r= 0.308 P=0.029 and r=0.405 P=0.004; respectively). We also found that GM-CSFRα expression in ectopic endometrium was significantly correlated with the severity of dysmenorrhea (r=0.306 P= 0.031) (**Figure 8**).

Discussion

Presentingcy heavy menstrual bleeding and dysmenorrhea as the top two complaints [28], optimal treatment modality for symptomatic adenomyosis remains elusive. We have seen reports that heavy bleeding and dysmenorrhea are both associated with the depth of invasion of adenomyotic glands into the myometrium and with the density of deep endometrial glands with the myometrium [9, 29-33]. However these findings are far from conclusive as other studies report that adenomyosis-related symptoms could be non-specific and related to other associated pathological conditions [34, 35]. Few biomarkers for heavy bleeding or dysmenorrhea or its severity in adenomyosis have been identified and the hunt for the pre-

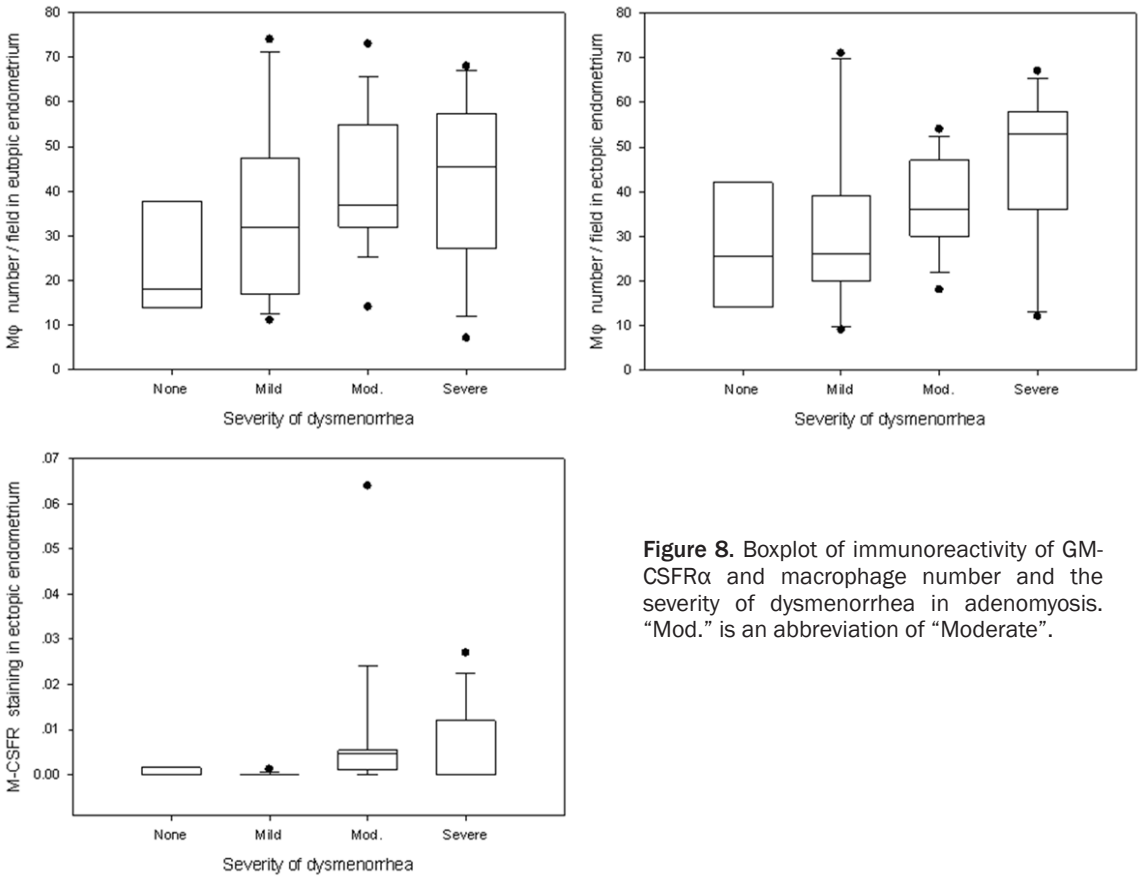

Figure 8. Boxplot of immunoreactivity of GM-CSFRα and macrophage number and the severity of dysmenorrhea in adenomyosis. "Mod." is an abbreviation of "Moderate".

dictors of the symptoms and signs in adenomyosis remains [28].

It was reported that endometriotic epithelial cells express the macrophage colony stimulating factor receptor (M-CSFR) which was found in nearly 70% of endometriosis patients compared with a statistically significant lower percentage in normal endometrium [36]. The ligand M-CSF was primarily produced by the monocyte-macrophages system, which also secreted GM-CSF [18, 19]. It was reported in vivo that GM-CSFR were functionally expressed on afferent nerve fibers and its blockage led to reduced tumor growth and pain abrogation [17]. Our study shows higher GM-CSFR expression in ectopic endometrium than in control endometrium and, interestingly, GM-CSFR expression in ectopic endometrium were positively correlated with the severity of dysmenorrheal, suggesting this receptor to be a predictor of treatment targets for adenomyosis-associated heavy bleeding and dysmenorrhea.

It was reported that the expression of CD68 as well as other biomarkers was significantly higher in peritoneal fluid macrophages obtained from women with endometriosis than in controls [37]. Report showed that a significant increase in macrophage cell numbers was shown in eutopic endometrium in women with endometriosis during all stages of the proliferative phase compared with normal controls [20]. There was a significant elevation of macrophages in both normal peritoneum and peritoneal lesions from women with endometriosis compared with normal peritoneum from women without endometriosis, suggesting these cells may well play roles in the growth and development of endometriotic lesions and in the generation of pain through interaction [38]. Interestingly, our study also points to that direction that a higher number of macrophage was seen in eutopic and ectopic endometrium than in control endometrium and can offer as a predictor for dysmenorrhea severity. The close linkage between biomarker like CD68 and angiogenesis and inflammatory process is intriguing.

There has been report that the VWF-positive micro-vessel density was significantly decrea-

sed in the endometria of women with endometriosis, adenomyosis and uterine myoma in the GnRHa-treated group when compared with that in the non-treated group [21]. Marked decreases in inflammatory and angiogenic responses were observed in lesions and myometrium of these diseases. Given the relatively objectivity in the measurement of menses, our finding that VWF-positive micro-vessel number and heavier menses are positively associated offers a strong clue and important addition to the possible set of biomarkers for symptomatic adenomyosis.

In conclusion, we had the unique luxury of using the triad of CD68, GM-CSFR and vWF as possible biomarkers to probe further into the pathogenesis of adenomyosis and confirmed their participation through the intricate process of inflammation and angiogenesis. The finding that inflammation and angiogenesis are entangled with dysmenorrhea and menorrhagia is phenomenal as they can be future treatment targets offering a brand new set of treatment modality.

Acknowledgements

This research was supported by grant 8157-1416 (Jichan Nie) from the National Science Foundation of China. We thank Sun-Wei Guo for his helpful comments on conceiving and designing the entire study.

Disclosure of conflict of interest

None.

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