Human Reproduction, Vol.00, No.0, pp. 1–16, 2021
doi:10.1093/humrep/deab090

human reproduction

ORIGINAL ARTICLE ***Reproductive biology***

An axonemal alteration in apical endometria of human adenomyosis

Khaleque N. Khan [1,*], Akira Fujishita[2], Takashi Suematsu[3], Kanae Ogawa[1], Akemi Koshiba[1], Taisuke Mori[1], Kyoko Itoh[4], Satoshi Teramukai[5], Katsuya Matsuda[6], Masahiro Nakashima [6], and Jo Kitawaki[1]

[1]Department of Obstetrics and Gynecology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan [2]Department of Gynecology, Saiseikai Nagasaki Hospital, Nagasaki, Japan [3]Central Electron Microscopy Laboratory, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan [4]Department of Pathology and Applied Neurobiology, Kyoto Prefectural University of Medicine, Kyoto, Japan [5]Department of Biostatistics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan [6]Department of Molecular and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan

*Correspondence address. Department of Obstetrics and Gynecology, The Clinical and Translational Research Center, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan. Tel: +81-75-251-5560; Fax: +81-75-212-1265; E-mail: nemokhan@koto.kpu-m.ac.jp https://orcid.org/0000-0002-9493-3340

Submitted on October 23, 2020; resubmitted on March 19, 2021; editorial decision on March 24, 2021

STUDY QUESTION: Is there any change in the distribution of microvilli and microtubules in the apical endometria of women with adenomyosis?

SUMMARY ANSWER: We observed microvilli damage in the apical endometria and an axonemal alteration characterized by abnormal distribution of longitudinal bundles of microtubules within microvilli in women with adenomyosis.

WHAT IS KNOWN ALREADY: Human adenomyosis has a negative impact on female fertility. Abnormal utero-tubal sperm transport, tissue inflammation and toxic effect of chemical mediators have been proposed as contributing factors. Inflammation-induced damage of mucosal cilia in the Fallopian tube has been reported. However, information on inflammation-induced damage of microvilli on the apical endometrial cells and its core bundles of microtubules in adenomyosis remains unknown.

STUDY DESIGN, SIZE, DURATION: This is a prospective cohort study with subjects undergoing laparoscopic surgery or hysterectomy for clinical indication and evaluations of endometrial biopsy samples in two academic university hospitals. During the period between March 2015 and December 2018, endometrial biopsy samples were prospectively collected from 15 control women and 45 women with adenomyosis for immunohistochemical analysis and a separate cohort of 10 control women with cervical intraepithelial neoplasia Grade 3 (CIN3) and 20 women with adenomyosis for analysis by immunohistochemistry and transmission electron microscopy (TEM).

PARTICIPANTS/MATERIALS, SETTING, METHODS: For immunohistochemical study, endometrial biopsy samples were prospectively collected from 15 control women with fibroids, 25 women with focal adenomyosis and 20 women with diffuse adenomyosis after surgery. The diagnosis of fibroid and adenomyosis was made clinically by transvaginal ultrasonography and magnetic resonance imaging and confirmed by histology. Immunohistochemical analysis was performed retrospectively using antibody against CD68 (marker of macrophages) in endometrial biopsy specimens of women with and without adenomyosis. TEM was performed with the apical endometria collected from a separate cohort of 10 control women with CIN3 and 20 women with focal and diffuse adenomyosis for the identification of any change in the distribution of microvilli and longitudinal bundles of microtubules within microvilli.

MAIN RESULTS AND ROLE OF CHANCE: Comparing to control endometria and contralateral side, tissue infiltration of macrophages (Mφ) in the endometria was significantly higher on the ipsilateral side of focal adenomyosis ($P = 0.02$ and $P = 0.03$, respectively) and anterior/posterior walls of diffuse adenomyosis ($P = 0.01$ for both). In a subgroup analysis of patients with focal adenomyosis with and without symptoms, the endometria of symptomatic women displayed a tendency of higher Mφ infiltration on the ipsilateral side than in asymptomatic women ($P = 0.07$). Comparing to contralateral side endometria of symptomatic women, Mφ infiltration was significantly higher in the endometria of symptomatic women collected from the ipsilateral side of focal adenomyosis ($P = 0.03$). We found a significantly less tissue infiltration of Mφ in the endometria of women with CIN3 than that in endometria of women with focal adenomyosis. TEM analysis showed that number of microvilli in the endometria was significantly decreased on the ipsilateral side ($P = 0.003$) comparing to that on the

contralateral side of focal adenomyosis. The Chi-squared test indicated that cases with abnormal (disruption in the normal arrangement of 9 peripheral pairs + 1 central pair) microtubules (MT) were significantly higher in women with adenomyosis than in cases with normal patterns ($P = 0.0016$). While contralateral side displayed significantly less abnormal MT ($P = 0.0002$), ipsilateral side of focal adenomyosis showed significantly higher abnormal MT ($P = 0.0164$) comparing to normal patterns. Cases with symptomatic adenomyosis showed significantly higher abnormal MT than normal MT ($P = 0.0004$). An axonemal alteration characterized by abnormal structural distribution of microtubules within microvilli in the apical endometria in response to endometrial inflammation may be involved in adverse reproductive outcome in women with adenomyosis.

LIMITATIONS, REASONS FOR CAUTION: The average age of women in this study was high that may be associated with overall decline in fertility regardless of the presence or absence of adenomyosis or endometriosis. We collected endometrial biopsy samples from two completely separate cohorts of women for analysis by immunohiostochemistry and TEM. We need future follow-up study with increased sample size and from the same patients to precisely clarify the mechanistic link between axonemal alteration and negative fertility outcome.

WIDER IMPLICATIONS OF THE FINDINGS: Our current findings may have some biological implication to better understand the endometrial epithelial biology and pathology in women with adenomyosis and may open the avenue for future study in other reproductive diseases. The ultra-structural abnormalities of microvilli and microtubules in the apical endometria in response to tissue inflammatory reaction may clarify the possible association between negative fertility outcome and adenomyosis. Our findings may be clinically useful during counseling with symptomatic patients with adenomyosis desiring pregnancy.

STUDY FUNDING/COMPETING INTEREST (S): This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Culture, Science and Technology of Japan. There is no conflict of interest related to this study.

TRIAL REGISTRATION NUMBER: N/A.

Key words: focal adenomyosis / diffuse adenomyosis / endometrium / macrophages / symptomatic/ asymptomatic / microvilli / normal microtubules / abnormal microtubules

Introduction

Adenomyosis is an estrogen-dependent chronic inflammatory condition and is considered as a heterogeneous disease. Human adenomyosis is characterized by the presence of endometrial glands and stroma within the myometrium causing enlargement of the uterus as a result of reactive hyperplastic and/or hypertrophic change of the surrounding myometrium (Ferenczy, 1998; Bergeron *et al.*, 2006). Different theories have been proposed to explain the pathogenesis of adenomyosis. Among them, a down-growth and invagination of the basalis endometrium into the myometrium is the most widely accepted opinion (Ferenczy, 1998; Khan *et al.*, 2015, 2016). Recently, epithelial–mesenchymal transition (EMT) has been reported to be involved in the development of adenomyosis (Chen *et al.*, 2010; Oh *et al.*, 2013; Khan *et al.*, 2015). Although adenomyosis is commonly believed to occur during the fourth and fifth decades of life and after the completion of childbearing activity, recent imaging modalities such as trans-vaginal ultrasonography and magnetic resonance imaging (MRI) have indicated that adenomyosis may occur in women of younger ages (Bergeron *et al.*, 2006; Exacoustos *et al.*, 2014). Women suffering from adenomyosis manifest different problems such as decreased quality of life due to severe painful symptoms/abnormal uterine bleeding and occurrence of a state of subfertility/infertility demanding proper treatment (Wang *et al.*, 2009; Li *et al.*, 2014).

Different forms of adenomyosis can be observed clinically in the myometrium: diffuse, focal and rare cases of cystic adenomyoma and these are better detected by MRI (Bergeron *et al.*, 2006; Gordts *et al.*, 2008; Pistofidis *et al.*, 2014). In radiological images, while diffuse adenomyosis is recognized by the diffuse and disperse presence of numerous foci of endometrial glands and stroma within the myometrium, focal adenomyosis appears as circumscribed nodular aggregates on either anterior or posterior wall of the uterus (Van den Bosch *et al.*, 2015). A wide range of adenomyosis prevalence rates has been demonstrated. Analysis of histological specimens obtained after hysterectomy revealed that the prevalence of adenomyosis varied between 5% and 70% due to the difference in diagnostic criteria used (Azziz, 1989).

Recently adenomyosis has been diagnosed with increasing frequency in infertile patients because these women delay their first pregnancy until they are aged in their late 30s or early 40s (Harada *et al.*, 2016). Adenomyosis is associated with infertility but the exact mechanism behind this relationship is still unclear. A number of factors have been proposed as follows: (i) abnormal utero-tubal sperm transport secondary to intrauterine abnormalities and increased uterine peristalsis; (ii) altered endometrial function and receptivity due to abnormal endometrial steroid metabolism, increased inflammatory response, defective intra-uterine oxidative stress environment, and/or impairment of implantation (Kunz *et al.*, 2000; Brosens *et al.*, 2004; Lessey *et al.*, 2006; Tremellen and Russell, 2012; Harada *et al.*, 2016). Achieving a physiologically optimized environment for fertilization and early embryonic development leading to successful spontaneous conception requires normal function of endometrium and Fallopian tube. This provides a conduit for the gametes to convene and for the embryo to reach the uterine cavity (Ezzati *et al.*, 2014). Efficient microtubule-mediated movement of microvilli in the apical surface of endometrium contributes to successful capture and/or migration of sperm and embryo. If there is any structural damage or alteration in the arrangement of microvilli/microtubules in response to endometrial inflammation, successful fertilization and implantation could be impaired. Although inflammation-induced damage of mucosal cilia in the Fallopian tube has been described in women with ectopic pregnancy and salpingitis (Ezzati *et al.*, 2014), information on the distribution pattern of

microvilli/microtubules in the apical mucosal cells of endometria in response to inflammation in endometriosis or adenomyosis is unknown.

The concept of axoneme as identified by electron microscopy in apical endometria is complex. A longitudinal bundle of microtubules is encased at the core of the microvilli ultra-structure and is known as the axoneme. These microtubules are similar in arrangement to those in the Fallopian tube. Normal microtubules are arranged in a 9 + 2 pattern in which nine peripheral microtubule doublets surround a core of two central single microtubules (Kamiya, 1999; Satir, 1980, 1989, 1992). There are two components in each doublet microtubule such as an A and a B component. The linkage, extending from each A microtubule to the B microtubule of the adjacent doublet, is called a dynein arm. Depending on whether they anchor to the inner or outer side of the A microtubule, these dynein arms are called inner dynein arm (IDA) or outer dynein arm (ODA), respectively. An optimal amount of energy is required for efficient movement of microvilli or cilia in the apical mucosal cells of endometrium or Fallopian tube. The energy required for microvilli or cilia movement is derived from ATP hydrolysis through ATPs activity of ODA causing transformation of chemical energy from ATP into a mechanical movement of the single microvillus (Sale and Satir, 1977; Ezzati *et al.*, 2014). A different ultrastructural component, called radial spokes, plays a key role in transmitting the sliding movements between peripheral doublets to the central part of the axoneme (central microtubules), resulting in microvilli bending (Sale and Satir, 1977). It can be noted that any abnormality in the 9 + 2 arrangement of microtubules in the microvilli or its core component may impair ciliary/microvilli movement of Fallopian tube or endometrium.

There are contradictions within the available evidence that impede a clear understanding of the impact of adenomyosis on ART outcome. While some groups report that adenomyosis negatively impacts outcomes of IVF (Ballester *et al.*, 2012; Tomassetti *et al.*, 2013), others have not found this negative association. Several reports indicated that adenomyosis has been associated with a higher prevalence of miscarriage (Martínez-Conejero *et al.*, 2011) and with generally worse perinatal outcomes (Mochimaru *et al.*, 2014). The coexistence of endometriosis and adenomyosis is common and presence of adenomyosis among endometriosis patients could be a contributing factor to infertility (Kunz *et al.*, 2005). An interesting Italian study demonstrated that clinical pregnancy rate, implantation rate and live birth rate are not impaired in asymptomatic women with adenomyosis comparing to groups of women without adenomyosis in IVF cycles (Benaglia *et al.*, 2014). On the other hand, a systemic review and meta-analysis targeting IVF outcome suggested that women with symptomatic adenomyosis have a 28% reduction in the likelihood of clinical pregnancy and 2-fold increase in the risk of miscarriage (Vercellini *et al.*, 2014). This clinical information indicates that a variable degree of endometrial inflammation in these women might be involved in the adverse reproductive outcome. However, the pattern of tissue inflammatory reaction in the endometria between symptomatic and asymptomatic women with adenomyosis is yet to be determined. We previously demonstrated an increased tissue infiltration of macrophages in endometria and myometria derived from women with endometriosis, adenomyosis and uterine myoma (Khan *et al.*, 2004, 2010). Macrophages, in addition to their phagocytic activity, have the capacity to produce different pro-inflammatory cytokines (TNF-α, IL-1, IL-6) as well as reactive oxygen species that can be toxic to embryos culminating in adverse reproductive outcome (Agarwal *et al.*, 2005; Tremellen and Russell, 2012). However, the pattern of tissue inflammatory reaction in the endometria of different types of adenomyosis is not well described.

Therefore, first, we aimed to investigate tissue infiltration of CD68-stained macrophages in the endometria and myometria collected from control women, women with focal adenomyosis and diffuse adenomyosis. Second, we examined the pattern of tissue inflammatory reaction in the endometria collected from symptomatic and asymptomatic women with adenomyosis. Third, with the hypothesis in mind that endometrial inflammation may affect normal function of microvilli and/or microtubules, we performed a transmission electron microscopic (TEM) study to confirm the distribution of microvilli in the apical epithelial cells of endometria and to examine any change in the pattern of normal arrangement of microtubules within microvilli collected from different anatomical sites of endometria derived from hysterectomy specimens of women with and without adenomyosis.

Materials and methods

Patients

The subjects in this study were women within their reproductive age as shown in Tables I and II. We collected endometrial biopsy samples from two completely separate cohorts of control women and women with adenomyosis for immunohistochemical analysis and TEM study.

Collection of biopsy samples for immunohistochemistry

During the period between March 2015 and December 2017, full thickness (from the endometrium to the myometrium) biopsy specimens were collected after hysterectomy from 25 women with focal adenomyosis and 20 women with diffuse adenomyosis. The collected uteri were transported to the laboratory in DMEM/F12 media (GIBCO, Grand Island, NY, USA) on ice under sterile conditions. To avoid the bias of an induced strong inflammatory reaction in submucosal myoma (Miura *et al.*, 2006), we selected 10 patients with intramural myoma and five patients with subserosal myoma for our control group who underwent either laparoscopic myomectomy or hysterectomy. Diffuse types of adenomyosis had lesions on both uterine walls and focal types had lesions on either anterior (n = 9) or posterior uterine wall (n = 16). Biopsy specimens were collected from the anterior wall and posterior wall for the cases with diffuse adenomyosis and from contralateral side (side opposite the lesion) and ipsilateral side (lesion side) for the cases with focal adenomyosis after hysterectomy. All collected biopsy specimens were prepared for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical study. The diagnosis of uterine myoma and adenomyosis was made clinically by transvaginal ultrasonography and magnetic resonance image (MRI) and confirmed by histology of biopsy specimens obtained after hysterectomy or myomectomy. We excluded cases with cystic adenomyomas and circumscribed nodular aggregates on either anterior or posterior wall of the uterus were considered as focal adenomyosis.

Focal adenomyosis was defined by the presence of circumscribed adenomyotic lesion in either of outer, middle or inner myometrium

Table I Clinical profiles of patients in the immunohistochemical study.

		Adenomyosis		
	Control (n = 15)	Focal (n = 25)	Diffuse (n = 20)	P value
Age in years (mean ± SD)	41.3 ± 6.6	43.6 ± 4.3	44.1 ± 5.4	0.075
Range in age (years)	29-52	35-50 3	3-52	–
Parity (mean ± SD)	1.4 ± 1.2	1.3 ± 1.2	1.2 ± 0.8	0.905
Range in parity (number)	0–4	0–3	0–3	–
Uterine myoma: IMM/SSM	10/5	2/3	2/6	0.164
Menstrual cycle: P/S/M/A	3/7/0/5	7/10/3/5	4/11/0/5	0.628
GnRHa-treated/-untreated	5/10	5/20	5/15	0.646
Symptoms: dysm/LAP/ menorrhagia/metrorrhagia/none	0/3/6/5/1	8/3/5/3/6	6/6//5/3/0	0.026
Site of adenomyosis lesion:				
AW/PW	0/0	9/16	20/20	–
Coexisting diseases:				
endometriosis: yes/no	3/12	5/20	6/14	0.735
uterine myoma: yes/no	15/0	5/20	8/12	<0.001
adenomyosis: yes/no	0/15	25/0	20/0	–
Surgery:				
LM/TAH/LAVH/TLH	10/2/3/0	0/6/15/4	0/4/10/6	<0.001

Continuous variables were compared by the Kruskal–Wallis test. Categorical variables were compared by Fisher's exact test. IMM, intramural myoma; SSM, subserosal myoma; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; GnRHa, gonadotropin releasing hormone agonist; dysm, dysmenorrhea; LAP, lower abdominal pain; AW, anterior wall; PW, posterior wall; LM, laparoscopic myomectomy; TAH, total abdominal hysterectomy; LAVH, laparoscopy-assisted vaginal hysterectomy; TLH, total laparoscopic hysterectomy.

Table II Clinical profiles of patients in the transmission electron microscopy (TEM) study.

	Controls (CIN3) (n = 10)	Adenomyosis (n = 20)	*P* value
Age in years (mean ± SD)	41.9 ± 7.8	44.3 ± 5.1	0.279
Range in age (years)	28–54	31–52	–
Parity (mean ± SD)	2.0 ± 1.2	1.6 ± 0.9	0.356
Range in parity (number)	0–4	0–3	–
Menstrual cycle: P/S/M/A	4/3/0/3	5/11/0/4	0.419
*Symptoms: dysm/menorrhagia/none	0/0/10	7/9/4	–
Type of adenomyosis: focal/diffuse	0/0	17/3	–
Location of focal adenomyosis: AW/PW	0/0	4/13	–
Coexisting diseases:			
endometriosis: yes/no	0/10	6/14	0.074
uterine myoma: yes/no	1/9	3/17	1.000
adenomyosis: yes/no	0/10	20/0	–
Surgery: TAH/LAVH/TLH	0/1/9	1/2/17	1.000

Continuous variables were compared by Wilcoxon rank-sum test. Categorical variables were compared by Fisher's exact test. TEM, transmission electron microscopy; CIN3, cervical intraepithelial neoplasia grade 3; P, proliferative phase, S, secretory phase, M, menstrual phase, A, amenorrhea; dysm, dysmenorrhea; AW, anterior wall, PW, posterior wall; TAH, total abdominal hysterectomy, LAVH, laparoscopy-assisted vaginal hysterectomy, TLH, total laparoscopic hysterectomy.
*Five of nine women with adenomyosis complaining of menorrhagia had coexistent dysmenorrhea.

involving anterior or posterior wall of the uterus as previously described by MRI (Gordts *et al.*, 2008; Kishi *et al.*, 2012). Diffuse adenomyosis was defined by the following criteria on MRI: (a) maximum thickness of the Junctional Zone (JZ_{max}) of at least 12 mm as a result of hyperplastic change or distortion of JZ as a result of scattered invasion of basalis glands into the myometrium (Kunz *et al.*, 2005; Khan *et al.*, 2016); (b) JZ_{max} to myometrial thickness ratio of >40% (Bazot *et al.*, 2001). We excluded all cases with adenomyoma and cystic lesions in the uterine musculature from our current study. The representative MRI images before surgery and morphological appearances of focal adenomyosis and diffuse adenomyosis in the hysterectomy specimens are shown elsewhere (Khan *et al.*, 2019).

Collection of biopsy samples for TEM

The subjects in this study were women of reproductive age. During the period between March 2017 and December 2018, we collected endometrial biopsy samples from hystecrectomy specimens of 36 control women with cervical intraepithelial neoplasia Grade 3 (CIN3) and 48 women with adenomyosis for TEM study. Considering the appropriate sample size, apical alignment of surface epithelial cells of the collected endometria, and quality of proper tissue fixation, finally we selected 10 control women with CIN3 and 20 women with adenomyosis for the ultra-structural investigation of endometria by TEM. We collected apical endometrial biopsy samples from five different locations in each subject and embedded them separately. We analyzed 20 microtubules in the cross-section of 20 different microvilli in each apical endometrial biopsy samples. If structure of all 20 microtubules was found normal in arrangement (9 + 1 pair arrangement), we designated them as a case with 'normal microtubule'. If more than 10 microtubules (>50%) was found as structurally abnormal (disruption of 9 + 1 pair arrangement), we designated them as a case with 'abnormal microtubule'. In addition to TEM, we retrieved tissue blocks from the hysterectomy specimens of women with CIN3 and adenomyosis for immunohistochemical study.

This is a prospective non-randomized observational study with prospective collection of endometrial biopsy samples from women after surgery and their evaluation. The phases of the menstrual cycle in women without hormonal therapy were determined by histological dating of eutopic endometria. All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board of our University (IRB No. 16005). A written informed consent was obtained from all women.

Antibodies used

We performed immunohistochemical studies to investigate the immunoreaction of CD68 for macrophages (Mφ) in intact tissues. CD68 (KP1), a mouse monoclonal antibody was derived from Dako, Denmark. A 1:50 dilution was used. CD68 antigen (clone KP1), which we used for our current study as a marker of matured and activated Mφ, is a glycosylated trans-membrane glycoprotein that is mainly located in lysosomes. It belongs to a family of lysosomal granules (Holness and Simmons, 1993). Non-immune immunoglobulin (Ig) G1 (1:50), a mouse monoclonal antibody from Dako, Denmark, was used as a negative control.

Immunohistochemistry

The details of immunohistochemical staining procedures are described elsewhere (Khan *et al.*, 2004, 2010). Briefly, 5-μm thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After immersion in 0.3% H_2O_2/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 anti-CD68 antibody. The slides were subsequently incubated with biotinylated second antibody for 10 min, followed by incubation with avidin–peroxidase for 10 min and visualized with diaminobenzidine. Finally, the tissue sections were counterstained with Mayer's hematoxylin, dehydrated with serial alcohols, cleared in xylene, and mounted. We had at least three slides per biopsy for immunohistochemical analysis. The immunoreactive CD68 spots were counted in five different fields of one section (×200 magnification) by light microscopy and expressed as the mean Mφ number per field in one specimen.

Procedure of TEM study

The detailed procedure of TEM is described elsewhere (Kitamura *et al.*, 1998; Algarroba *et al.*, 2020). Briefly, fresh biopsy samples (1 × 1 × 1 mm) were obtained from three anatomical sites (anterior wall, posterior wall and fundus) of endometria derived from each of control women and women with focal adenomyosis and diffuse adenomyosis. We confirmed respective site of apical endometria by low magnification electron microscopy for further evaluation by TEM (Fig. 1). Samples for TEM were fixed in 2.5% glutaraldehyde solution (w/v) buffered to pH 7.4 with 0.1 M phosphate buffer for 4 h at 4 °C. Post-fixation was performed with 1% osmium tetroxide solution buffered to pH 7.4 with the same buffer for 2 h at 4 °C. They were then dehydrated in graded series of ethanol and embedded in Epon 812. Ultrathin sections (60–70 nm) were cut with an ultra-microtome (Ultracut S, Leica, Austria) with diamond knife and doubly stained with uranyl acetate and lead nitrate. All ultrathin sections were observed with TEM (JEM-1200EX, JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV and photographed. Three ultrathin sections from each endometrial biopsy samples were prepared and observed. We counted five different fields of each ultrathin section and mean of five fields was considered as the number of microvilli in the apical endometria per patient. The pattern of microtubule arrangement in cross section image of 20 different microvilli was observed and analyzed.

Statistical analysis

All results are expressed as mean ± SD, mean ± SEM, as appropriate, or median and interquartile ranges. The clinical characteristics of the subjects between groups were analyzed by one-way analysis of variance (ANOVA). Any difference in the number of macrophages or microvilli between groups was analyzed by the Mann–Whitney *U* test. Continuous variables and categorical variables were analyzed by the Kruskal–Wallis test/Wilcoxon rank-sum test and Fisher's exact test, respectively. Multiple regression analysis with different variables was done to identify independent risk factor to cause endometrial inflammation. The distribution of macrophages according to groups was expressed using the box and whisker plots with the medians and inter-quartile range (IQR). The case distribution between normal and

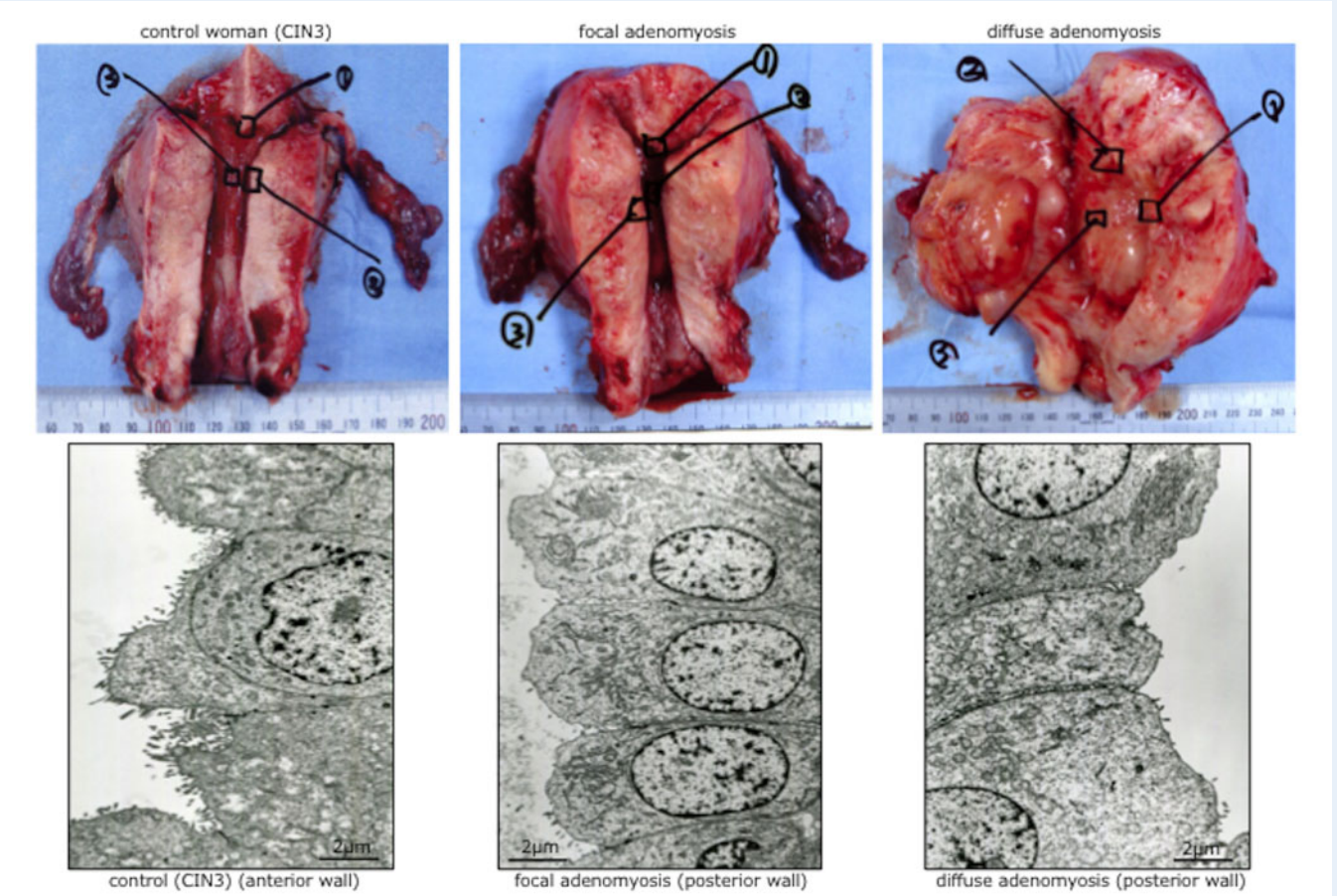

Figure 1. Anatomical sites for collection of endometrial biopsy samples and transmission electron microscopic images of apical endometria. Biopsy samples were collected from different anatomical sites of hysterectomy specimens derived from control women with CIN3, women with focal adenomyosis and diffuse adenomyosis, Numbers in each hysterectomy specimen indicate the site of each biopsy specimen such as anterior wall, posterior wall, and fundus (upper row). Respective site of apical endometria by low magnification electron microscopy was confirmed for further evaluation by transmission electron microscopy (lower row). Each number denotes the following anatomical site: for control women (CIN3), 1 (fundus), 2 (anterior wall), 3 (posterior wall); for focal adenomyosis, 1 (fundus), 2 (posterior wall), 3 (anterior wall); for diffuse adenomyosis, 1 (anterior wall), 2 (fundus), and 3 (posterior wall).

abnormal microtubules in different groups was compared using the Chi-squared test. A value of $P < 0.05$ was considered statistically significant. Data analysis was conducted using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

The clinical characteristics of patients with and without adenomyosis from whom endometrial biopsy samples were collected for the analysis by immunohistochemistry are shown in Table I. Five women in each group underwent treatment with GnRHa for a variable period of 3–6 months before surgery. While all women in diffuse adenomyosis group were symptomatic, 19 women with focal adenomyosis were symptomatic and six women were asymptomatic. The clinical profiles of the patients were compared among the three groups using the Kruskal–Wallis test for continuous variables and Fisher's exact test for categorical variables. There were statistically significant differences among control women, women with focal adenomyosis and women with diffuse adenomyosis in symptoms ($P = 0.026$), myoma of coexisting diseases ($P < 0.001$) and surgery ($P < 0.001$) (Table I).

CD68-positive Mφ infiltration in the endometria and myometria of women with adenomyosis

Immunohistochemistry was performed with endometrial biopsy samples collected from GnRHa-untreated 10 control women, 20 women with focal adenomyosis and 15 women with diffuse adenomyosis. Macrophage (Mφ) infiltration, as shown by CD68-positivity brown spots, is shown in the endometria (upper row) and myometria (lower row) derived from control women (extreme left column) contralateral/ipsilateral side of focal adenomyosis (left two columns in box) and anterior/posterior wall of diffuse adenomyosis (right two columns in box)

(Fig. 2A). The mean Mφ number per field in the respective endometria (left panel) and myometria (right panel) of control women and women with adenomyosis are shown in Fig. 2B. We analyzed tissue infiltration of Mφ in each endometrial biopsy sample collected from GnRHa-untreated women. Comparing to contralateral side, Mφ infiltration in the endometria was significantly higher on the ipsilateral side of focal adenomyosis ($P < 0.05$). Comparing to control endometria, Mφ infiltration was significantly higher in the endometria derived from each of ipsilateral side of focal adenomyosis, anterior wall and posterior wall of diffuse adenomyosis ($P < 0.05$ for each) (Fig. 2B, left panel).

Non-parametric analysis revealed no significant difference in mean Mφ numbers between the proliferative phase and secretory phase of menstrual cycle in control women and women with focal and diffuse adenomyosis. Although we found a modest increase in Mφ number in endometria derived from focal adenomyosis during the menstrual phase, the Kruskal–Wallis test indicated no significant difference in Mφ infiltration across the phases of menstrual cycle in these women. Therefore, we represented our data regardless of phases of the menstrual cycle.

Comparing to myometria of control women and contralateral/ipsilateral side of focal adenomyosis, tissue infiltration of Mφ was significantly higher in the myometria collected from anterior wall and posterior wall of diffuse adenomyosis ($P < 0.05$ for each) (Fig. 2B, right panel).

CD68-positive Mφ infiltration in the endometria of symptomatic and asymptomatic women with adenomyosis

Since all women with diffuse adenomyosis were symptomatic, we analyzed tissue infiltration of Mφ in the endometria and myometria

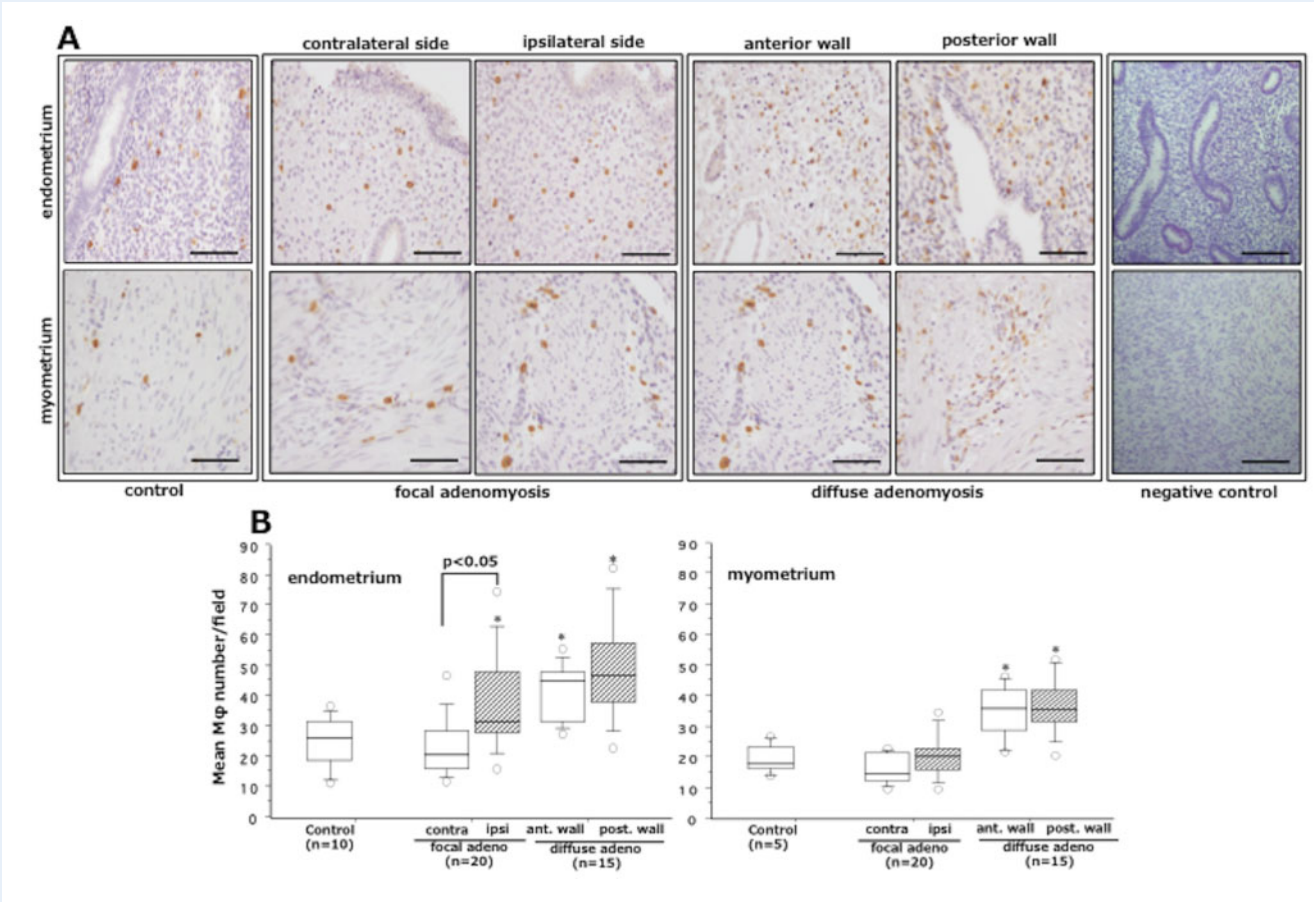

Figure 2. Macrophage infiltration in endometria and myometria. (**A**) Immunohistochemical image and tissue infiltration of macrophages. Immunohistochemical analysis of CD68-stained macrophages (Mφ) in the endometria (upper row) and myometria (lower row) collected from control women with fibroids (extreme left column), the contralateral/ipsilateral side of focal adenomyosis (left two columns in box) and anterior/posterior walls of diffuse adenomyosis (right two columns in box) An image of negative control is shown on the extreme right column. (**B**) Tissue infiltration of Mφ in the endometria was significantly higher on the ipsilateral side comparing to contralateral side of focal adenomyosis ($P < 0.05$). Compared with control endometria, mean Mφ numbers were also significantly higher on the ipsilateral side of focal adenomyosis and anterior/posterior walls of diffuse adenomyosis (*$P < 0.05$ for each) (left panel). While no obvious difference in mean Mφ number was observed between contralateral and ipsilateral side, a significantly increased issue infiltration of Mφ was found in the myometria collected from anterior/posterior walls of diffuse adenomyosis than in focal adenomyosis and control women (*$P < 0.05$ for each) (right panel). The boxes represent the interquartile ranges and horizontal lines in the boxes represent median values. Scale bar = 100 μm for negative controls and scale bar = 50 μm for other slides.

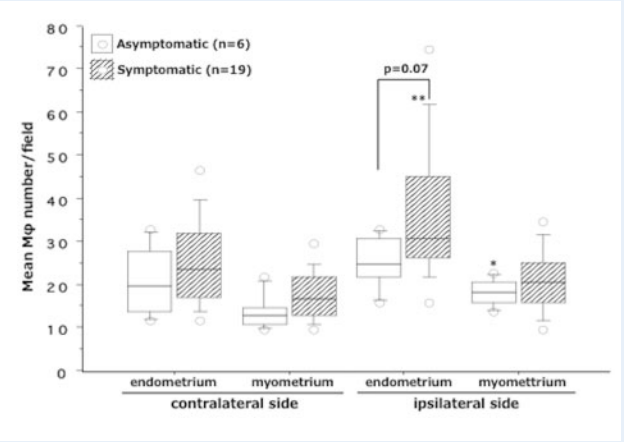

Figure 3. Tissue infiltration of macrophages based on symptoms of patients. Tissue infiltration of CD68-stained macrophages (Mφ) in the endometria and myometria derived from symptomatic (hatched box) and asymptomatic (white box) women with focal adenomyosis as measured by immunohistochemistry. Mean Mφ numbers had a tendency to significantly increase in the endometria of ipsilateral side among symptomatic women comparing to asymptomatic women ($P = 0.07$). Comparing to endometria of contralateral side, mean Mφ numbers were significantly increased on the ipsilateral side of symptomatic focal adenomyosis (**$P < 0.05$). Even in asymptomatic women, tissue infiltration of Mφ was significantly higher in the myometria of ipsilateral side comparing to that of contralateral side (*$P < 0.05$). The boxes represent the interquartile ranges and horizontal lines in the boxes represent median values.

collected from 19 symptomatic women and six asymptomatic women with focal adenomyosis (Fig. 3). The endometria of symptomatic women displayed a modest increase in Mφ infiltration on the ipsilateral side than in asymptomatic women ($P = 0.07$). In contrast, comparing to contralateral side endometria of symptomatic women, Mφ infiltration was significantly higher in the endometria of symptomatic women collected from the ipsilateral side ($P < 0.05$) (Fig. 3). Even in asymptomatic women, myometria on the ipsilateral side harbor significantly higher number of Mφ comparing to that of contralateral side of focal adenomyosis ($P < 0.05$).

Distribution of microvilli on the apical endometrial cells collected from women with and without adenomyosis

We collected fresh fragments of apical endometrial samples from different anatomical sites of 10 control women with CIN3 and 20 women with adenomyosis who underwent hysterectomy. The clinical profiles of these two groups of women are shown in Table II. Amongst 20 adenomyosis women, 17 were diagnosed with focal adenomyosis and the remaining three with diffuse adenomyosis. While all control women were asymptomatic, 16 women in the adenomyosis group were symptomatic and four women were asymptomatic. The clinical profiles of the patients were compared between the two groups using the Wilcoxon rank-sum test for continuous variables and Fisher's exact test for categorical variables. There was no statistically significant difference in any of the variables between the two groups (Table II).

Distribution of microvilli was visualized by electron microscopy in the different anatomical sites of endometria collected from control women with CIN3 (upper row)), focal adenomyosis (middle row) and diffuse adenomyosis (lower row) (Fig. 4A). Since of number of cases with diffuse adenomyosis was small, we analyzed the number of microvilli in each anatomical sites of control women and women with focal adenomyosis (Fig. 4B). We counted microvilli from both longitudinal section and cross section image of microvilli. The number of microvilli in control women appears to be higher in all anatomical sites. Comparing to contralateral side, number of microvilli was significantly decreased in the endometria on the ipsilateral side ($P = 0.003$) and a marginal significance in their decrease was observed on the fundus ($P = 0.05$) of focal adenomyosis (Fig. 4B). Microvilli were blunted/discrete and less in number in the anterior wall, posterior wall and fundus of diffuse adenomyosis. Although the data are not shown, symptomatic women with focal adenomyosis showed a decreased number of microvilli in endometria comparing to asymptomatic women. We did not find any difference in the number of microvilli between proliferative phase and secretory phase of the menstrual cycle of control women and women with focal adenomyosis (Fig. 4C).

CD68-positive Mφ infiltration in the endometria of women with CIN3 and adenomyosis

In an attempt to confirm the link between endometrial inflammation and microvilli malformation, we retrospectively retrieved all tissue blocks from the same cohort of 10 women with CIN3 and 20 women with adenomyosis to investigate tissue infiltration of Mφ in respective endometria. An immunohistochemical image of CD68 staining in endometria collected from different anatomical sites is shown in Fig. 5A. We found a remarkably less tissue infiltration of Mφ in the endometria of women with CIN3 comparing to that in the endometria of focal or diffuse adenomyosis (Fig. 5B). Due to small sample size of diffuse adenomyosis, we analyzed Mφ results in endometria between CIN3 and focal adenomyosis. The endometrial infiltration of Mφ was significantly higher in either contralateral side ($P = 0.008$) or ipsilateral side ($P = 0.0002$) of focal adenomyosis than in anterior wall or posterior wall of uterus harboring CIN3 lesions. Endometria collected from ipsilateral side displayed a higher tissue inflammatory reaction comparing to that from contralateral side of focal adenomyosis ($P = 0.001$, Fig. 5B).

Multiple regression analysis with different variables such as age of the patient, parity, menstrual cycle, symptoms and coexisting diseases indicated that dysmenorrhea ($P = 0.024$) and menorrhagia ($P = 0.014$) were significant risk factors associated with higher tissue infiltration of Mφ in endometria of focal adenomyosis.

Distribution of normal and abnormal microtubules by TEM

We collected apical endometrial biopsy samples from five different locations of each anatomical site and alteration of microtubules was observed in three apical biopsy samples (60%) in each subject. TEM study was performed to identify the distribution of normal

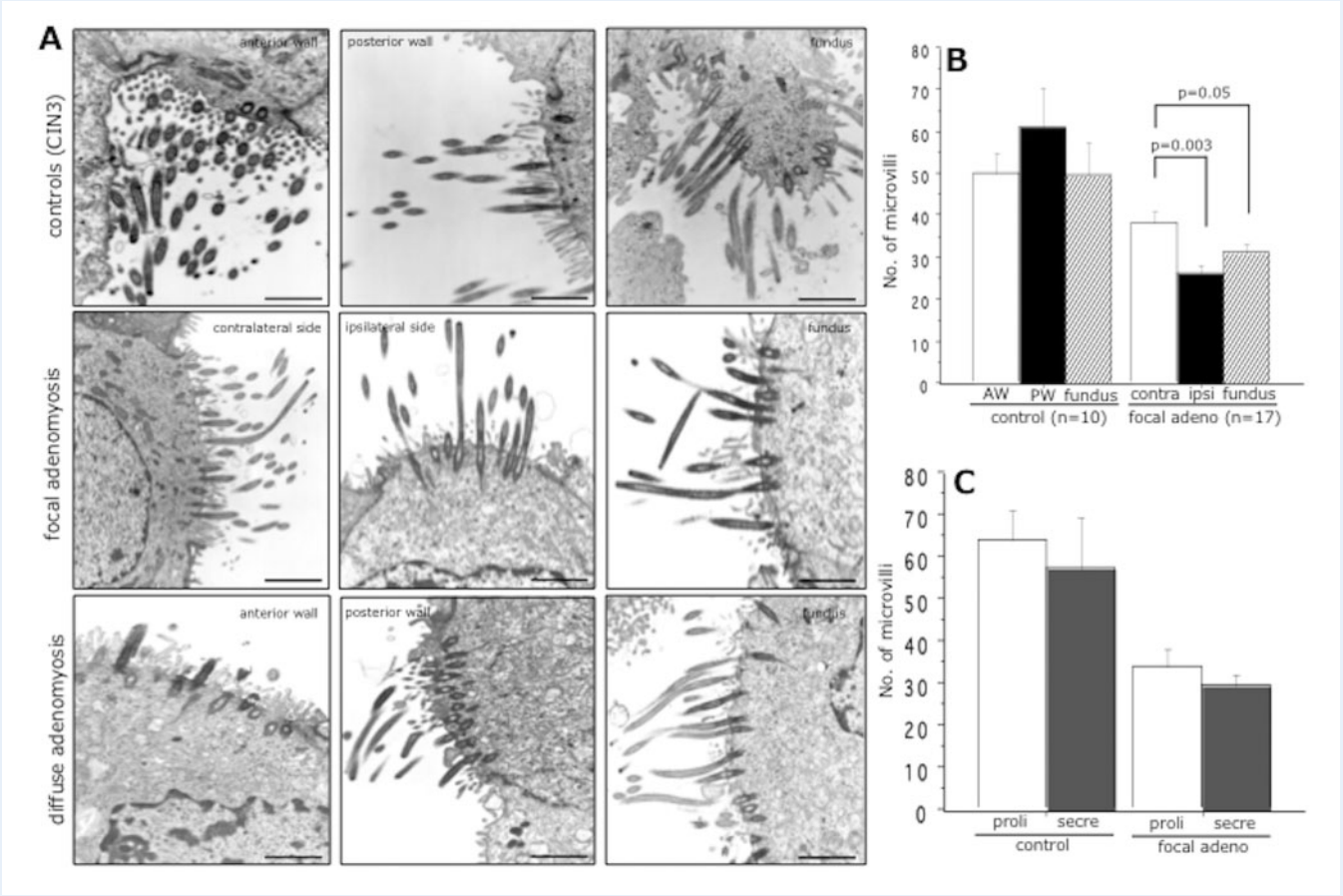

Figure 4. Microvilli in apical endometria. (**A**) TEM analysis of microvilli in apical endometria shows distribution of microvilli in the different anatomical sites of endometria collected from control women with CIN3 (upper row), focal adenomyosis (middle row), and diffuse adenomyosis (lower row). (**B**) Number of microvilli in each anatomical sites of control women and women with focal adenomyosis. Number of microvilli in control women appears to be higher in all anatomical sites. Comparing to contralateral side, number of microvilli was significantly decreased in the endometria on the ipsilateral side ($P = 0.003$) and a marginal significance in their decrease on the fundus ($P = 0.05$) of focal adenomyosis. (**C**) Distribution of microvilli based on phases of the menstrual cycle of women with CIN3 and focal adenomyosis. There was no significant difference in the number of microvili between proliferative phase and secretory phase of these two groups of women. Scale bar = 1 μm for each slide (**A**). The results are expressed as mean ± SEM.

(9 peripheral pairs + 1 separated central pair) and abnormal arrangement of microtubules (MT) in the cross-sectional images of microvilli collected from the apical endometrial cells of control women and women with adenomyosis (Fig. 6). A diagrammatic representation of the normal arrangement and different components of microtubules (axoneme) is shown in Fig. 6A (Kamiya, 1999; Ezzati *et al.*, 2014). While a normal distribution (9 + 1 pair arrangement) of microtubule was observed in the apical endometrial microvilli of control women (Fig. 6B), an abnormal distribution was found with disappearance of central pair of MT in the microvilli collected from diffuse adenomyosis (Fig. 6C). Ten different abnormal patterns of peripheral (P) and central (C) microtubules in the microvilli collected from apical endometrial cells of adenomyosis are shown in Fig. 7. These are described as follows: (1) P-MT-intact, C-MT-disappears; (2) P-MT-intact, C-MT, one arm disappears; (3) P-MT-intact, C-MT-damaged; (4) P-MT-alignment distorted, C-MT-disappears; (5) P-MT-8 pairs, C-MT-one arm disappears; (6) P-MT-7 pairs, C-MT-disappears; (7) P-MT-alignment distorted with damaged A/B component in one doublet, C-MT-intact; (8) P-MT-alignment distorted with damaged A or B component in two doublets, C-MT-disappears; (9) P-MT-intact, C-MT-more than 2; (10) P-MT-alignment distorted, C-MT-many (Fig. 7).

Distribution of microtubules in different cases by TEM

TEM was performed to visualize the distribution of microtubules (MT) in the microvilli of apical endometria collected from three representative cases of control women (CIN3) (upper row), contralateral side (middle row) and ipsilateral side (lower row) of focal adenomyosis (Fig. 8). All three cases from control women (a, b, c) showed normal distribution of MT (9 peripheral pairs + 1 separated central pair). Two cases from contralateral side showed normal distribution of MT (d, e) and one case shows abnormal distribution (distorted alignment of peripheral MT with disappearance of central MT (f). All three cases from

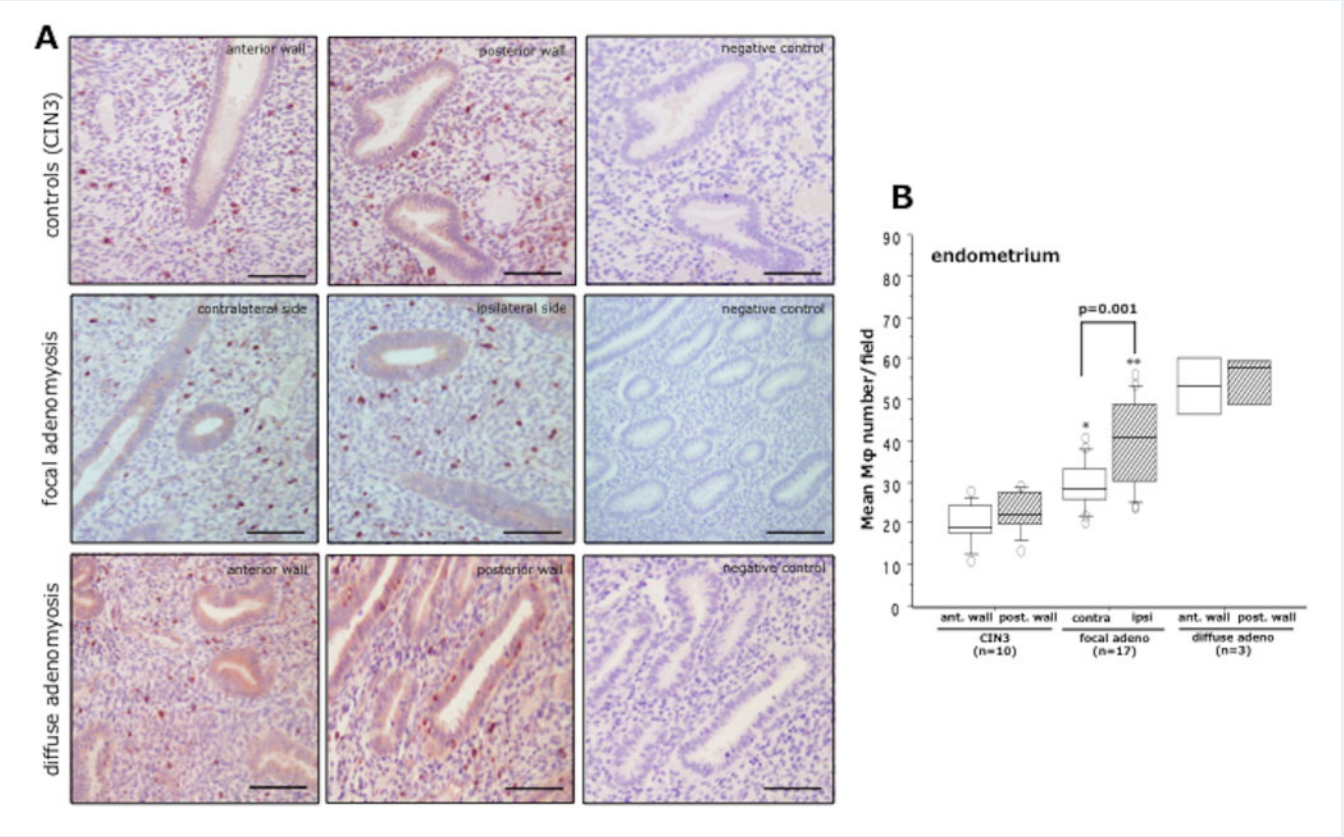

Figure 5. Infiltration of macrophages in endometria collected from women in the transmission electron microscopy study. (**A**) Immunohistochemical image of macrophages in endometrium. Immunohistochemical analysis of CD68-stained macrophages (Mφ) in the endometria collected from different anatomical sites of control women with CIN3 (upper row), women with focal adenomyosis (middle row) and diffuse adenomyosis (lower row). The corresponding images of negative controls are shown on the right column. (**B**) Immunohistochemical analysis of macrophages in endometria of women with CIN3 and focal adenomyosis. Tissue infiltration of Mφ in the endometria was significantly higher on the ipsilateral side comparing to contralateral side of focal adenomyosis ($P = 0.001$). Comparing to anterior wall and posterior wall of control endometria, mean Mφ numbers were also significantly higher on the contralateral side ($^{*}P = 0.008$) and ipsilateral side ($^{**}P = 0.0002$) of focal adenomyosis. There was no difference in tissue accumulation of Mφ in the endometria between anterior wall and posterior wall of diffuse adenomyosis. The boxes represent the interquartile ranges and horizontal lines in the boxes represent median values. Scale bar = 100μm for each slide (**A**).

ipsilateral side showed abnormal distribution of MT (g, alignment distorted in peripheral MT; h, peripheral MT-intact but one arm of central MT disappears; i, peripheral MT-intact but central MT is damaged) (Fig. 8).

The detail case distributions of normal and abnormal microtubules (MT) in control women and in women with focal and diffuse adenomyosis are shown in Table III. The criteria for the designation of normal and abnormal MT are described in section Methods. The distribution of MT in all control cases was found to be normal as visualized by TEM. The cases with abnormal MT were significantly higher in women with adenomyosis (75%) than in cases with normal MT (25%) ($P = 0.0016$). While the contralateral side showed significantly less cases with abnormal MT (18%) ($P = 0.0002$), the cases of abnormal MT were significantly higher on the ipsilateral side of focal adenomyosis (71%) ($P = 0.0164$) comparing to cases with normal MT on each side. All three cases with diffuse adenomyosis showed abnormal distribution of MT on either anterior wall or posterior wall. Thirteen cases with symptomatic adenomyosis (81%) displayed a significantly higher abnormal MT arrangement comparing to three cases with normal MTs ($P = 0.0004$) (Table III).

Discussion

In an attempt to find an association between endometrial inflammation and an axonemal alteration in the apical endometria, we performed a prospective cohort study with endometria derived from women with and without adenomyosis. We found that endometria of women with focal and diffuse adenomyosis displayed strong tissue inflammatory reaction as measured by abundant tissue infiltration of macrophages (Mφ) comparing to that of control women with both fibroids and CIN3. Endometria collected from symptomatic women with focal adenomyosis also showed significantly increased tissue inflammatory reaction comparing to that of asymptomatic women. To confirm the consequence of this tissue inflammatory reaction in endometria, we performed TEM and found that number of microvilli on the apical

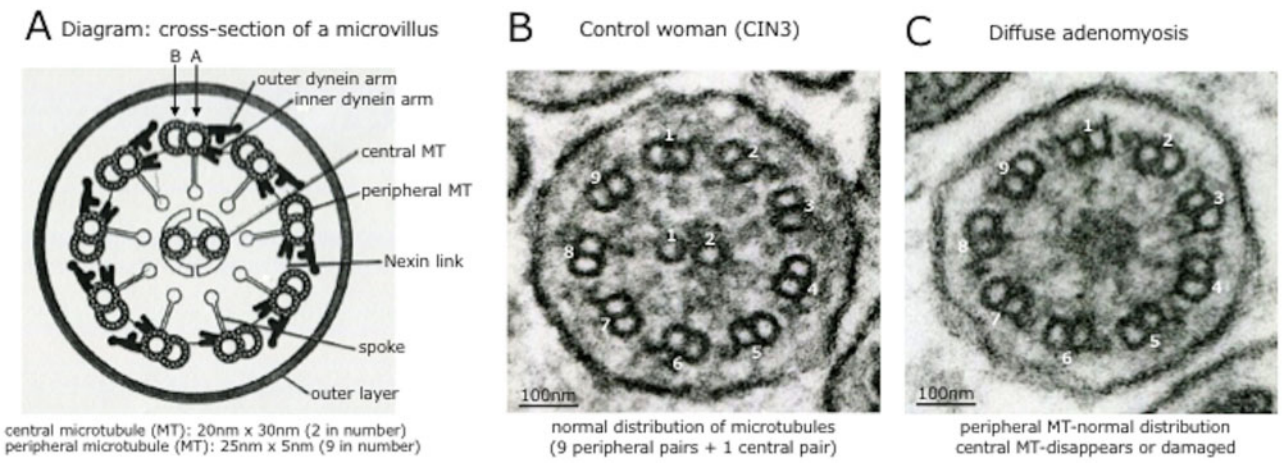

Figure 6. TEM shows distribution of microtubules (MT) after cross section of a single microvillus. (**A**) Diagrammatic representation of normal arrangement of microtubules (9 pairs of peripheral microtubules + 1 separated central pair). Each pair of peripheral MT has A and B component and is connected to adjacent pair with outer dynein arm and inner dynein arm. Nexin link mediates the function of two adjacent peripheral microtubules. All nine pairs of peripheral MT are connected to central pair of MT by nine individual spokes and are encased by outer membranous layer forming a composite structure of an axoneme (Kamiya, 1999; Ezzati et al., 2014). (**B**) Distribution of microtubules in endometria of control women. Normal distribution of nine pairs of peripheral MT and one pair of central MT (marked by numbers) in a microvillus of endometria collected from control women (CIN3). (**C**) Distribution of microtubules in endometria of adenomyosis. Abnormal distribution of MT in a microvillus collected from the endometria of diffuse adenomyosis showing that central pair of MT is either disappeared or damaged.

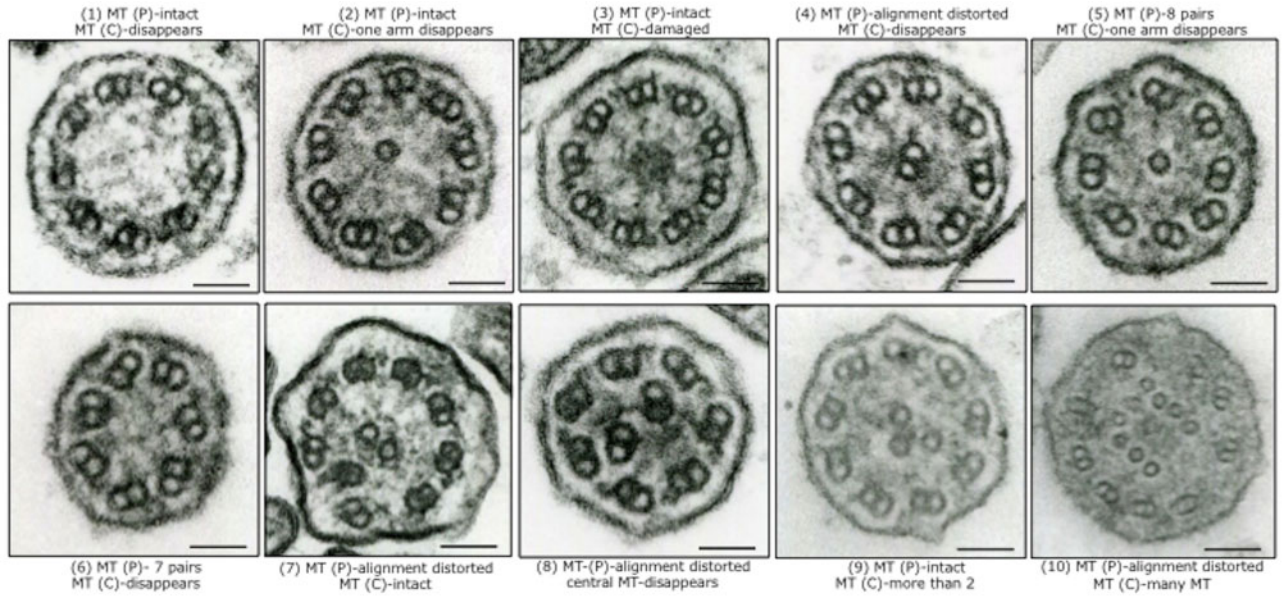

Figure 7. Different patterns of abnormal microtubules in adenomyosis. Ten different patterns of abnormal peripheral (P) and central (C) microtubules (MT) that were identified by TEM in different microvilli collected from the endometria of either focal adenomyosis and diffuse adenomyosis. Pattern numbers [(1)–(5), upper row)], [(6)–(10)], lower row) and their short description against each pattern are shown in this figure. The detailed description is mentioned in method section. Scale bar = 100nm for each slide.

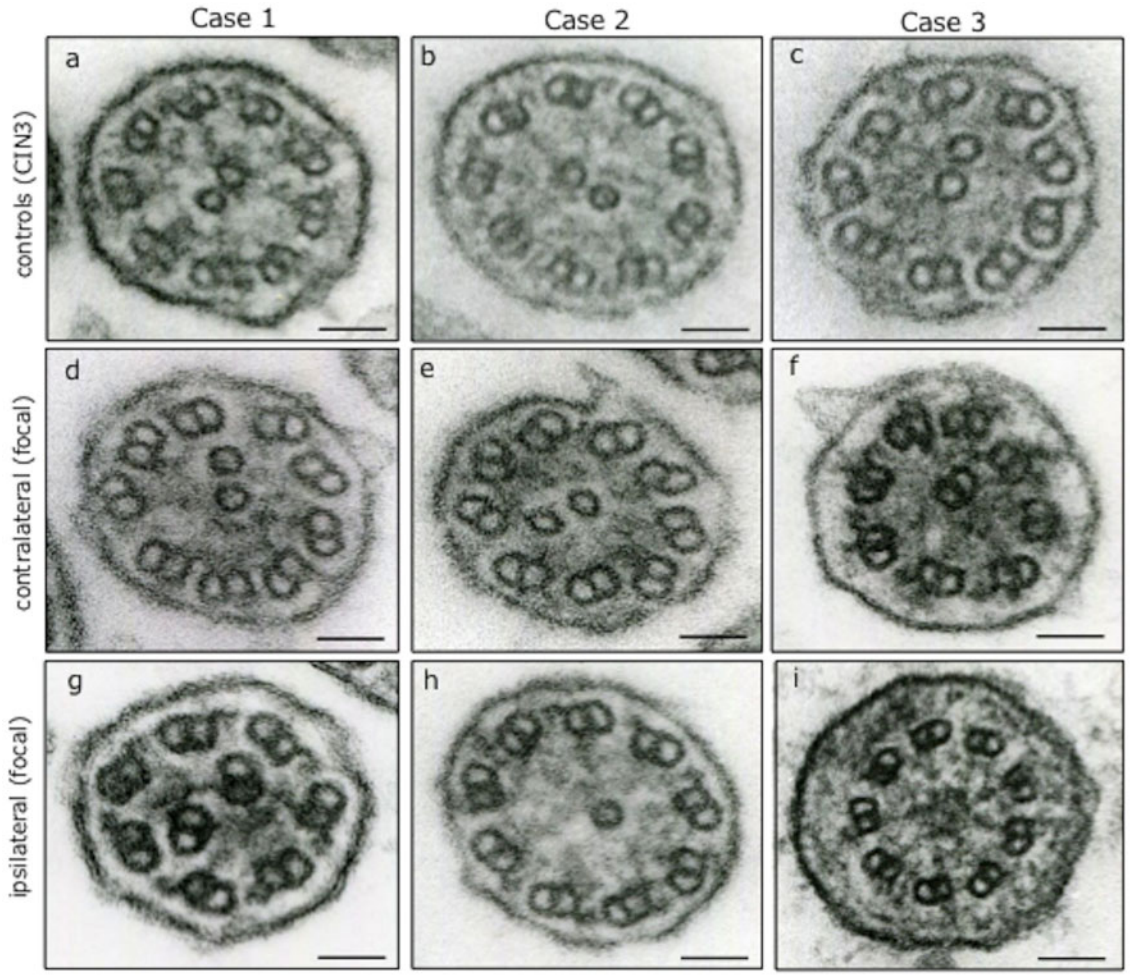

Figure 8 Distribution of microtubules in different cases with CIN3 and adenomyosis. TEM shows distribution of microtubules (MT) in the microvilli of endometria collected from three different cases of control women (CIN3) (upper row), contralateral side (middle row) and ipsilateral side (lower row) of focal adenomyosis. All 3 cases from control women (a, b, c) showed normal distribution of MT (9 peripheral pairs + 1 separated central pair). Two cases from contralateral side showed normal distribution of MT (d, e) and one case (f) shows abnormal distribution (distorted alignment of peripheral MT and central MT disappears). All three cases from ipsilateral side showed abnormal distribution of MT (g, alignment distorted in peripheral MT; h, peripheral MT-intact but one arm of central MT disappears; i, peripheral MT-intact but central MT is disappeared or damaged). Scale bar = 100nm for each slide.

epithelial cells of endometria collected from women with focal and diffuse adenomyosis was significantly decreased resulting in marked abnormality in their axonemal arrangement. We postulate that these biological and ultra-structural abnormal findings in the endometria can be linked to the negative fertility outcome in women with adenomyosis. These findings were more frequently observed in women with symptomatic adenomyosis than in asymptomatic women.

Several lines of evidence demonstrated that endometria and myometria of women with adenomyosis harbored abundant Mφ and showed increased cellular apoptosis. These unwanted endometrial inflammatory reactions were significantly decreased after treatment with GnRHa (Khan *et al.*, 2004, 2010). In addition to direct phagocytic activity, Mφ retain the capacity to produce different pro-inflammatory cytokines (TNF-α, IL-1, IL-6) as well as reactive oxygen species that can be toxic to embryos culminating in adverse reproductive outcome (Tremellen and Russell, 2012; Agarwal *et al.*, 2005). Our current finding is an extended piece of evidence that a strong tissue inflammatory reaction occurs in the endometria of women with both focal and diffuse adenomyosis. The local tissue inflammatory reaction in the endometria and/or myometria may be involved in uterine hyperperistasis, abnormal uterine transport of sperms/gamete, and altered endometrial function and receptivity resulting in the impairment of successful implantation. Although we did not perform experiments on the association between macrophage infiltration and abnormal markers of uterine receptivity, there are some published reports on this issue in women with endometriosis (Yin *et al.*, 2012; Lessey and Kim, 2017; Vallvé-Juanico *et al.*, 2019). Further studies are needed to clarify this issue in women with adenomyosis.

Table III Distribution of normal and abnormal microtubules.

	Normal Microtubules	Abnormal microtubules	*P* value
Controls (CIN3) (n = 10), n (%)	10 (100)	0 (0)	0.0000
Adenomyosis (n = 20), n (%)	5 (25)	15 (75)	0.0016
Focal adenomyosis (n = 17):			
Contralateral side, n (%)	14 (82)	3 (18)	0.0002
Ipsilateral side, n (%)	5 (29)	12 (71)	0.0164
Diffuse adenomyosis (n = 3)			
Anterior wall, n (%)	0 (0)	3 (100)	0.1336
Posterior wall, n (%)	0 (0)	3 (100)	0.1336
Symptomatic (n = 16), n (%)	3 (19)	13 (81)	0.0004
Asymptomatic (n = 4), n (%)	3 (75)	1 (25)	0.1573

Data were analyzed by the Chi-squared test. CIN3, cervical intraepithelial neoplasia Grade 3.
We analyzed 20 microtubules in the cross-section of 20 different microvilli in the apical endometrial biopsy samples in each subject. If structure of all 20 microtubules was found normal in arrangement (9 + 1 arrangement), we designated them as a case with normal microtubule. If more than 10 microtubules was found as structurally abnormal (disruption of 9 + 1 arrangement), we designated them as a case with 'abnormal microtubule'.

The biological evidence of increased tissue inflammatory reaction in the endometria of symptomatic women with adenomyosis supports the findings of reproductive outcome in IVF/ICSI cycles (Benaglia *et al.*, 2014; Vercellini *et al.*, 2014). The significantly less tissue inflammatory reaction in the endometria of asymptomatic women with adenomyosis may be associated with the negative impact of adenomyosis in IVF outcome (Benaglia *et al.*, 2014). In contrast, women with symptomatic adenomyosis with associated increased tissue inflammatory reaction in their endometria may be linked to the increased occurrence of miscarriage and decreased clinical pregnancy rate in IVF/ICSI cycles (Vercellini *et al.*, 2014). A concurrent damage of microvilli and axonemal alteration in response to tissue inflammatory reaction in the endometria may further support the link between adenomyosis and its detrimental effect on natural conception or on IVF/ICSI reproductive outcome.

Numerous studies in the past decade have shown that biochemical signature of peritoneal fluid (PF) in patients with endometriosis is more pro-inflammatory compared to healthy women (Halme *et al.*, 1983; Lyons *et al.*, 2002). Different macromolecules in PF of women with endometriosis impose a significant inhibitory effect on ciliary beat frequency (CBF) (Lyons *et al.*, 2002). Another possible mechanism linking endometriosis to infertility is defective interactions between the sperm and the Fallopian tube epithelium in women with endometriosis (Reeve *et al.*, 2005). In addition to strong endometrial inflammation in women with adenomyosis as we reported here, the role of pro-inflammatory cytokines, their receptors and NK-kB in adenomyosis has been demonstrated in a recent review article (Harada *et al.*, 2016).

To successfully achieve a spontaneous or acquired pregnancy, a complex system of endometrial and tubal transport must be operational to allow a timely interaction between sperm and oocyte for the developing embryo to reach the uterus. Although physiological regulation of ciliary motion in the Fallopian tube and the pathological states than can potentially cause altered or impaired ciliary activity have been demonstrated (Ezzati *et al.*, 2014), to our knowledge, the ultra-structural findings and functional activity of endometrial microvilli in women with endometriosis or adenomyosis are still unknown. Here we report an abnormal pattern of microvilli distribution and axonemal arrangement in the apical epithelial cells of endometria derived from women with and without adenomyosis using TEM.

With the speculation that tissue inflammatory reaction in endometria could impair functional activity or organization of apical endometrial microvilli, we could detect abnormal distribution of microvilli in the endometria of women with both focal and diffuse adenomyosis. Since normal axonemal arrangement (9 pairs of peripheral microtubules + 1 pair central microtubules) in each microvillus contributes to effective movement of endometrial microvilli, we investigated distribution of normal and abnormal axonema and could find 10 different patterns of abnormal axonema in the endometrial microvilli of women with adenomyosis in our current study using TEM (Fig. 7). While all endometrial biopsy samples collected from any anatomical site of control women (CIN3) showed normal axonemal arrangement, most of the endometrial biopsy samples collected from either ipsilateral side of focal adenomyosis or anterior/posterior walls of diffuse adenomyosis displayed more than one of 10 different abnormal patterns of axonema within their microvilli. These findings may indicate that an endometrial inflammation-induced microvilli damage and consequent abnormal arrangement of microtubules might exhibit impaired movement of microvilli that are required to maintain endometrial function and to achieve a successful pregnancy.

In fact, similar to wave-like movement of paddies towards the direction of wind as observed in an agricultural field, a cervico-fundal movement of all these microvilli around ovulation is responsible for the capture of sperms and their ascending migration to the tube for successful fertilization, transmigration of embryo to the uterine cavity and subsequent implantation (Vannuccini *et al.*, 2016; Bhurke *et al.*, 2016; Zhang et al., 2013). Therefore, any damage of microvilli or alteration of axonemal arrangement in response to tissue inflammatory reaction in the endometria may exhibit a detrimental effect on fertility outcome.

There are some biological and clinical significance of our current findings. (1) Occurrence of a variable tissue inflammatory reaction in the endometria of women with focal and diffuse adenomyosis particularly in women with symptomatic adenomyosis may contribute to microvilli malformation and disruption of axonemal arrangement in the apical endometria of these women. (2) The currently used diagnostic tests in infertility clinic such as hysterosalpingography (HSG) or laparoscopic chromo-pertubation are not capable to evaluate endometrial or tubal physiology except understanding the state of tubal patency and/or endometrial pathology. The functional integrity of apical endometrial cells is required for the establishment of a successful pregnancy. Our current findings can deliver some message to the patients with symptomatic adenomyosis who are planning to conceive naturally or by ART. In fact, clinical symptoms in women with adenomyosis were the independent risk factors to cause strong endometrial inflammation in current study. (3) Damage or restricted movement of apical endometrial microvilli in response to endometrial inflammation may result in defective sperm capture/migration, fertilization and consequent successful implantation.

There are some potential limitations in this study: (1) we collected endometrial biopsy samples from two completely separate cohorts of control women and women with adenomyosis for analysis by immunohiostochemistry and TEM. Initially we had a different study design with cohorts who underwent immunohistochemistry. When we changed our study plan to perform TEM, all hysterectomy specimens were already immersed in neutral formalin. Therefore, we decided to collect fresh apical endometrial biopsy samples to study TEM with a separate cohort of women. (2) Sample size in each group of control women and women with adenomyosis was small. (3) The average age of women in the study (all groups) was high that may be associated with overall decline in fertility regardless of the presence or absence of adenomyosis or endometriosis. Our current biological findings may at least postulate some outcome evaluation in response to endometrial inflammation. Further study is warranted with large sample size and collection of endometrial biopsy samples from the same cohort of women to address the true association between tissue inflammation and axonemal alteration in apical endometria of women with adenomyosis or endometriosis.

In conclusion, we demonstrated for the first time an alteration in the distribution of microvilli and arrangement of microtubules in the apical epithelial cells of endometria collected from women with focal and diffuse adenomyosis. These ultra-structural abnormalities in response to tissue inflammatory reaction in the endometria may be involved in negative fertility outcome in women with adenomyosis. Our findings may be clinically useful during counseling with patients with symptomatic adenomyosis who desire for successful pregnancy. Further studies are needed to strengthen the validity of our current findings and to explore their link with fertility outcome.

Data availability

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Acknowledgments

This work was partly supported by the Program of the Network-type Joint Usage/Research Center for Radiation Disaster Medical Science. The authors thank all doctors of Saiseikai Nagasaki Hospital and Atomic Bomb Disease Institute both in Nagasaki for their kind assistance in collecting biopsy samples and sample preparation for this study. The authors also thank Mr. Mieno Masatoshi of Nagasaki University and Dr. Go Horiguchi/Ms. Miyuki Mori of Kyoto Prefectural University of Medicine for their excellent technical assistance in data analysis and handling of endometrial biopsy samples.

Authors' roles

K.N.K. was involved in original concept, study design, experiments, interpretation, and manuscript writing/editing; A.F., K.O., A.K., T.M. and K.M. contributed to sample collection/preparation and experiments; T.S. was involved in sample preparation, T.E.M. experiment and image interpretation; S.T. contributed to data monitoring and statistical analysis; K.I. and M.N. equally contributed to imaging/data analysis and interpretation; J.K. was involved in draft editing and discussion.

Funding

This work was supported in part by Grants-in-aid for Scientific Research (Grant nos. 24592474, 15K10675 and 18K09268 to K.N.K.) from the Japan Society for the Promotion of Science (JSPS).

Conflict of interest

The authors declare that they have no financial or other conflict of interest.

References

Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005;**3**:28.

Algarroba GN, Rekawek P, Vahanian SA, Khullar P, Palaia T, Peltier MR, Chavez MR, Vintzileos AM. Visualization of severe acute respiratory syndrome coronavirus 2 invading the human placenta using electron microscopy. *Am J Obstet Gynecol* 2020;**223**:275–278.

Azziz R. Adenomyosis: current perspectives. *Obstet Gynecol Clin North Am* 1989;**16**:221–235.

Bazot M, Cortez A, Darai E, Rouger J, Chopier J, Antoine JM, Uzan S. Ultrasonography compared with magnetic resonance imaging for the diagnosis of adenomyosis: correlation with histopathology. *Hum Reprod* 2001;**16**:2427–2433.

Ballester M, d'Argent EM, Morcel K, Belaisch-Allart J, Nisolle M, Daraï E. Cumulative pregnancy rates after ICSI-IVF in patients with colorectal endometriosis: results of a multicenter study. *Hum Reprod* 2012;**27**:1043–1049.

Benaglia L, Cardellicchio L, Leonardi M, Faulisi S, Vercellini P, Paffoni A, Somigliana E, Fedele L. Asymptomatic adenomyosis and embryo implantation in IVF cycles. *Reprod Biomed Online* 2014;**29**:606–611.

Bergeron C, Amant F, Ferenczy A. Pathology and pathophysiology of adenomyosis. *Best Pract Res Clin Obstet Gynaecol* 2006;**20**:511–521.

Bhurke AS, Bagchi IC, Bagchi MK. Progesterone-regulated endometrial factors controlling implantation. *Am J Reprod Immunol* 2016;**75**:237–245.

Brosens J, Verhoeven H, Campo R, Gianaroli L, Gordts S, Hazekamp J, Hagglund L, Mardesic T, Varila E, Zech J. *et al.* High endometrial aromatase P450 mRNA expression is associated with poor IVF outcome. *Hum Reprod* 2004;**19**:352–356.

Chen YJ, Li HY, Huang CH, Twu NF, Yen MS, Wang PH, Chou TY, Liu YN, Chao KC, Yang MH. Estrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis. *J Pathol* 2010;**222**:261–270.

Exacoustos C, Manganaro L, Zupi E. Imaging for the evaluation of endometriosis and adenomyosis. *Best Pract Res Clin Obstet Gynaecol* 2014;**28**:655–681.

Ezzati M, Djahanbakhch O, Arian S, Carr BR. Tubal transport of gametes and embryos: in review of physiology and pathophysiology. *J Assist Reprod Genet* 2014;**31**:1337–1347.

Ferenczy A. Pathophysiology of adenomyosis. *Hum Reprod Update* 1998;**4**:312–322.

Gordts S, Brosens JJ, Fusi L, Benagiano G, Brosens I. Uterine adenomyosis: a need for uniform terminology and consensus classification. *Reprod Biomed Online* 2008;**17**:244–248.

Harada T, Khine YM, Kaponis A, Nikellis T, Decavalas G, Taniguchi F. The impact of adenomyosis on women's fertility. *Obstet Gynecol Surv* 2016;**71**:557–568.

Halme J, Becker S, Hammond MG, Raj MH, Raj S. Increased activation of pelvic macrophages in infertile women with mild endometriosis. *Am J Obstet Gynecol* 1983;**145**:333–337.

Holness CI, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood* 1993; **81**:1607–1613.

Kamiya R. Microtubules. In: H Ishikawa, H Kondo, Y Shibata (eds). *Standard Cell Biology*, 1st edn. Tokyo, Japan: Igaku Shoin Publishing Co., 1999, 137–151 (in Japanese).

Kitamura S, Miyazaki T, Iwata S, Akaboshi K, Osawa Y, Yoshimura Y. Ultra-structural evaluation following catheterization of the Fallopian tube with a hysteroscopic catheter. *J Assist Reprod Genet* 1998;**15**:411–417.

Kishi Y, Suginami H, Kumamori R, Yabuta M, Suginami R, Taniguchi F. Four suptypes of adenomyosis assessed by magnetic resonance imaging and their specification. *Am J Obstet Gynecol* 2012;**207**: e1–e7.

Khan KN, Fujishita A, Kitajima M, Masuzaki H, Nakashima M, Kitawaki J. Biological differences between functionalis and basalis endometria in women with and without adenomyosis. *Eur J Obstet Gynecol Reprod Biol* 2016;**203**:49–55.

Khan KN, Fujishita A, Koshiba A, Mori T, Kuroboshi H, Ogi H, Itoh K, Nakashima M, Kitawaki J. Biological differences between focal and diffuse adenomyosis and response to hormonal treatment. *Reprod Biomed Online* 2019;**38**:634–646.

Khan KN, Kitajima M, Hiraki K, Fujishita A, Nakashima M, Masuzaki H. Involvement of hepatocyte growth factor-induced epithelial–mesenchymal transition in human adenomyosis. *Biol Reprod* 2015; **92**:351–311.

Khan KN, Kitajima M, Hiraki K, Fujishita A, Sekine I, Ishimaru T, Masuzaki H. Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy. *Hum Reprod* 2010;**25**:642–653.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T. Differential macrophage infiltration in early and advanced endometriosis and adjacent peritoneum. *Fertil Steril* 2004;**81**:652–661.

Kunz G, Beil D, Huppert P, Leyendecker G. Structural abnormalities of the uterine wall in women with endometriosis and infertility visualized by vaginal sonography and magnetic resonance imaging. *Hum Reprod* 2000;**15**:76–82.

Kunz G, Beil D, Huppert P, Noe M, Kissler S, Leyendecker G. Adenomyosis in endometriosis-prevalence and impact on fertility. Evidence from magnetic resonance imaging. *Hum Reprod* 2005;**20**: 2309–2316.

Lessey BA, Kim JJ. Endometrial receptivity in eutopic endometrium of women with endometriosis it is affected, let me show you why. *Fertil Steril* 2017;**108**:19–27.

Lessey BA, Palomino WA, Apparao KBC, Young SL, Lininger RA. Estrogen receptor-alpha (ER-alpha) and defects in uterine receptivity in women. *Reprod Biol Endocrinol* 2006;**4**:S9.

Li X, Liu X, Guo SW. Clinical profiles of 710 pre-menopausal women with adenomyosis who underwent hysterectomy. *J Obstet Gynaecol Res* 2014;**40**:485–494.

Lyons RA, Djahanbakhch O, Saridogan E, Naftalin AA, Mahmood T, Weekes A, Chenoy R. Peritoneal fluid, endometriosis, and ciliary beat frequency in the human fallopian tube. *Lancet* 2002;**360**: 1221–1222.

Martínez-Conejero JA, Morgan M, Montesinos M, Fortuño S, Meseguer M, Simón C, Horcajadas JA, Pellicer A. Adenomyosis does not affect implantation but is associated with miscarriage in patients undergoing oocyte donation. *Fertil Steril* 2011;**96**:943–950.

Miura S, Khan KN, Kitajima M, Hiraki M, Moriyama S, Masuzaki H, Samejima T, Fujishita A, Ishimaru T. Differential infiltration of macrophages and prostaglandin production by different uterine leiomyomas. *Hum Reprod* 2006;**21**:2545–2554.

Mochimaru A, Aoki S, Oba MS, Kurasawa K, Takahashi T, Hirahara F. Adverse pregnancy outcomes associated with adenomyosis with uterine enlargement. *J Obstet Gynecol Res* 2014;**3**:1–5.

Oh SJ, Shin JH, Kim TH, Lee HS, Yoo JY, Ahn JY, Broaddus RR, Taketo MM, Lydon JP, Leach RE. *et al.* β-Catenin activation contributes to the pathogenesis of adenomyosis through epithelial-mesenchymal transition. *J Pathol* 2013;**231**:210–222.

Pistofidis G, Makrakis E, Koukoura O, Bardis N, Balinakos P, Anaf V. Distinct types of uterine adenomyosis based on laparoscopic and histologic criteria. *Clin Exp Obstet Gynecol* 2014;**41**:113–118.

Reeve L, Lashen H, Pacey AA. Endometriosis affects sperm-endosalpingeal interactions. *Hum Reprod* 2005;**20**:448–451.

Satir P. Mechanisms of ciliary movement: contributions from electron microscopy. *Scanning Microsc* 1992;**6**:573–579.

Satir P. The role of axonemal components in ciliary motility. *Comp Biochem Physiol A Comp Physiol* 1989;**94**:351–357.

Satir P. Structural basis of ciliary movement. *Environ Health Perspect* 1980;**35**:77–82.

Sale WS, Satir P. Detection of active sliding of microtubules in Tetrahymena cilia. *Pros Natl Acad Sci U S A* 1977;**74**:2045–2049.

Tomassetti C, Meuleman C, Timmerman D, D'Hooghe T. Adenomyosis and subfertility: evidence of association and causation. *Semin Reprod Med* 2013;**31**:101–108.

Tremellen KP, Russell P. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure II: adenomyosis and macrophages. *J Reprod Immunol* 2012; **93**:58–63.

Van den Bosch T, Dueholm M, Leone FPG, Valentin L, Rasmussen CK, Votino A, Van Schoubroeck D, Landolfo C, Installé AJF, Guerriero S. *et al.* Terms, definitions and measurements to describe sonographic features of myometrium and uterine masses: a consensus opinion from the Morphological Uterus Sonographic Assessment (MUSA) group. *Ultrasound Obstet Gynecol* 2015;**46**: 284–298.

Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update* 2019;**25**:565–592.

Vannuccini S, Clifton VL, Fraser IS, Taylor HS, Critchley H, Giudice LC, Petraglia F. Infertility and reproductive disorders: impact of hormonal and inflammatory mechanisms on pregnancy outcome. *Hum Reprod Update* 2016;**22**:104–115.

Vercellini P, Consonni D, Dridi D, Bracco B, Frattaruolo MP, Somigliana E. Uterine adenomyosis and in vitro fertilization outcome: a systematic review and meta-analysis. *Hum Reprod* 2014;**29**:964–977.

Wang PH, Fuh JL, Chao HT, Liu WM, Cheng MH, Chao KC. Is the surgical approach beneficial to subfertile women with symptomatic extensive adenomyosis? *J Obstet Gynaecol Res* 2009;**35**:495–502.

Yin X, Pavone ME, Lu Z, Wei J, Kim JJ. Increased activation of PI3K/AKT pathway compromises decidualization of stromal cells from endometriosis. *J Clin Endocrinol Metab* 2012;**97**:E35–E43.

Zhang S, Lin H, Kong S, Wang S, Wang H, Wang H, Armant DR. Physiological and molecular determinants of embryo implantation. *Mol Aspects Med* 2013;**34**:939–980.