Antibiotic therapy with metronidazole reduces endometriosis disease progression in mice: a potential role for gut microbiota

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STUDY QUESTION: Does altering gut microbiota with antibiotic treatment have any impact on endometriosis progression?

SUMMARY ANSWER: Antibiotic therapy reduces endometriosis progression in mice, possibly by reducing specific gut bacteria.

WHAT IS KNOWN ALREADY: Endometriosis, a chronic condition causing abdominal pain and infertility, affects up to 10% of women between the ages of 25 and 40, ~5 million women in the USA. Current treatment strategies, including hormone therapy and surgery, have significant side effects and do not prevent recurrences. We have little understanding of why some women develop endometriosis and others do not.

STUDY DESIGN, SIZE, DURATION: Mice were treated with broad-spectrum antibiotics or metronidazole, subjected to surgically-induced endometriosis and assayed after 21 days.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The volumes and weights of endometriotic lesions and histological signatures were analysed. Proliferation and inflammation in lesions were assessed by counting cells that were positive for the proliferation marker Ki-67 and the macrophage marker Iba1, respectively. Differences in faecal bacterial composition were assessed in mice with and without endometriosis, and faecal microbiota transfer studies were performed.

MAIN RESULTS AND THE ROLE OF CHANCE: In mice treated with broad-spectrum antibiotics (vancomycin, neomycin, metronidazole and ampicillin), endometriotic lesions were significantly smaller (~ 5-fold; P < 0.01) with fewer proliferating cells (P < 0.001) than those in mice treated with vehicle. Additionally, inflammatory responses, as measured by the macrophage marker Iba1 in lesions and IL-1β, TNF-α, IL-6 and TGF-β1 in peritoneal fluid, were significantly reduced in mice treated with broad-spectrum antibiotics (P < 0.05). In mice treated with metronidazole only, but not in those treated with neomycin, ectopic lesions were significantly (P < 0.001) smaller in volume than those from vehicle-treated mice. Finally, oral gavage of faeces from mice with endometriosis restored the endometriotic lesion growth and inflammation (P < 0.05 and P < 0.01, respectively) in metronidazole-treated mice.

LARGE-SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: These findings are from a mouse model of surgically-induced endometriosis. Further studies are needed to determine the mechanism by which gut bacteria promote inflammation, identify bacterial genera or species that promote disease progression and assess the translatability of these findings to humans.
Introduction
Endometriosis causes pain in the pelvis and lower abdomen and affects up to 10% of women between the ages of 25 and 40. ~5 million women in the USA. Nearly, half of these women experience chronic pelvic pain that significantly diminishes their quality of life (Giudice, 2010). Factors implicated in establishment and expansion of endometriotic lesions include hormonal imbalance, immune dysfunction, epigenetic modifications triggered by environmental toxicants (Rier and Foster, 2003; Hsiao et al., 2017) and unopposed estrogen action coupled with progesterone resistance. The current treatments for endometriosis, principally hormone therapy and surgery, have negative side effects and do not prevent recurrences. Therefore, a new approach is needed to combat this disease (Falcke and Flackt, 2018).

A well-accepted theory is that endometriosis is caused by endometrial tissue which enters the peritoneal cavity via retrograde menstruation and implants onto pelvic organs and peritoneal surfaces. However, up to 90% of women experience retrograde menstruation, yet only 10% of women develop endometriosis. This suggests that other factors contribute to the onset of endometriosis onset (Sourial et al., 2014). It is thought that the immune system usually clears the cells that enter the peritoneal cavity during retrograde menstruation, but when it is unable to do so, the lesions spread as a result of inflammation brought about by macrophages releasing pro-inflammatory cytokines and growth factors into the peritoneal cavity (Ahn et al., 2015b). This hypothesis is supported by findings in mouse models of endometriosis (Lin et al., 2006; Han et al., 2015). For example, macrophages drive lesion growth and vascularisation (Lin et al., 2006; Bacci et al., 2009; Capobianco et al., 2011) and enhance IL-1β signalling in response to inflammatory activation, which also promotes endometriotic angiogenesis (Lebovic et al., 2000; Bullon and Navarro, 2017).

Endometriosis may also be influenced by the microbiome. Distinct microbial communities have been identified in the reproductive tracts of reproductive-age women (Moreno et al., 2016; Chen et al., 2017), and some microbial compositions appear to correlate with reproductive pathologies such as preterm birth and infertility (Parnell et al., 2017b). Additionally, Cregger et al. (3–6) identified differences in the cervical and uterine microbiome communities between women with and without endometriosis (Cregger et al., 2017). Here, we tested the hypothesis that the gut microbiome, which encodes 150 times more genes than the host genome (O’Hara and Shanahan, 2006; Ursell et al., 2014), influences endometriosis disease progression. We demonstrate that treating mice with broad-spectrum antibiotics greatly curtails the early growth and progression of endometriotic lesions. Whereas metronidazole treatment reduced endometriotic lesion growth, oral gavage of bacteria from mice with endometriosis restored endometriotic lesion growth and associated inflammatory responses. These results suggest that gut bacteria promote endometriosis disease progression and have implications for microbiota-based therapies to combat this painful disease.

Materials and Methods

Study approval
Animal studies were performed according to a protocol (number 20160227) approved by the Washington University School of Medicine Institutional Animal Care and Use Committee.

Mouse surgical endometriosis model
We used a well-established endometriosis model in which uterine tissue from estrus-stage mice is autologously transplanted onto the peritoneal wall. After 3 weeks, the resulting endometriotic lesions are composed of a single cyst (Cummings and Metcalff, 1995; Pelch et al., 2012) and resemble those observed in human endometriosis (Fainaru et al., 2008; Umezawa et al., 2009; Korbel et al., 2010). Briefly, one uterine horn from 10-week-old, estrus-stage mice (C57BL/6, Taconic, n = 4 to 15 per group) was excised and cut longitudinally. Next, a dermal biopsy punch was used to isolate a 3-mm endometriotic fragment, which was sutured to the peritoneal wall in the same mouse through a midline incision (Fainaru et al., 2008; Schreinemacher et al., 2012; Machado et al., 2016; Kiani et al., 2018). For the sham surgery, a similar procedure was performed except that a thread was sutured onto the peritoneal wall without an endometrial fragment.

Antibiotic treatment
Twenty-four hours after endometriosis-induction surgery, mice were provided drinking water containing 0.5 g/l vancomycin, 1 g/l neomycin, 1 g/l metronidazole and 1 g/l ampicillin (VNMA) for 21 days as described previously (Rakoff-Nahoum et al., 2004). To mask the taste of the antibiotics, 2 g/l aspartame was added to the VNMA-containing water. Control mice received drinking water containing aspartame alone (Huang et al., 2015). In other experiments, mice received water containing only 1 g/l metronidazole or 1 g/l neomycin plus aspartame, or water containing only aspartame. Then, mice were euthanised, faecal samples were collected and eutopic endometrium and endometriotic lesions were isolated. Peritoneal fluid was collected by washing the peritoneum with 1 ml sterile PBS. Lesions were weighed (mg), and lesion volumes (mm³) were measured with a Vernier Calliper (VCB001, United scientific Supplies Waukegan, IL, USA) by an investigator blinded to treatment groups.
Faecal pellets were immediately frozen at −80°C as reported previously (Hintze et al., 2014). Faecal pellets were resuspended in phosphate-buffered saline (PBS) (one faecal pellet/0.1 ml of PBS), and 200 μl of the suspension was given by oral gavage to each mouse (Wong et al., 2017a) as indicated in Fig. 5A. The numbers of mice were as follows: endometridazole + non-endo faeces, n = 4 and endo-metronidazole + endo faeces, n = 4.

**Results**

**Treatment with broad-spectrum antibiotics reduces endometriosis progression, proliferation and inflammation**

To determine whether antibiotics affect endometriosis progression, we treated mice with the broad-spectrum antibiotics VNMA in drinking water containing aspartame to mask the antibiotics taste. Control mice received drinking water containing aspartame alone. We then performed endometriosis-induction surgery (Fig. 1A). Mice that consumed VNMA (VNMA-endo) had smaller endometriosis lesions than those that consumed vehicle alone (vehicle-endo) (Fig. 1B–D). In a second experiment aimed at assessing progression of established endometriosis lesions, we treated mice with antibiotics after endometriosis surgery (Fig. 1E). Lesions were smaller in mice that consumed VNMA (endo-VNMA) than in those that consumed vehicle (endo-vehicle) (Fig. 1F–H). These two experiments indicated that antibiotic treatment reduced both early growth and progression of endometriosis lesions.

To begin to uncover the mechanism by which antibiotics affected endometriosis progression, we treated mice with antibiotics immediately after endometriosis surgery and performed a series of analyses (Fig. 2). First, we confirmed that neither surgery nor antibiotic treatment had any effect on body weight (Supplementary Figure S1). Second, hematoxylin and eosin staining revealed that whereas lesions from endo-vehicle mice had typical endometriosis-like structures, including a thick epithelial layer and glandular areas, lesions from endo-VNMA mice had thinner epithelial areas and no glands (Fig. 2E). Additionally, consistent with reports that stromal cell volume correlates with lesion growth (Korbel et al., 2010), lesions from endo-VNMA mice had smaller stromal areas than lesions from endo-vehicle mice (Fig. 2E). Importantly, the eutopic uteri had similar epithelial, glandular and stromal areas in both endo-vehicle and endo-VNMA mice (Fig. 2E). Third, we stained the lesions with an antibody specific to estrogen receptor alpha (ERα), which is thought to promote proliferation and inflammation and thus drive endometriosis lesion growth and expansion (Huhtinen et al., 2012). However, ERα expression was similar between lesions from endo-vehicle and endo-VNMA mice (Figure S2). Furthermore, consistent with a report that stage of estrous had no impact on lesion growth (Fainaru et al., 2008; Schreinemacher et al., 2012; Machado et al., 2016; Kiani et al., 2018), lesion volumes did not appear to correlate with the stage of estrous at sacrifice (data not shown).

Fourth, we assessed epithelial proliferation, which is a hallmark of endometriosis in women and is widely used to assess disease progression in rodent models of endometriosis (Wu et al., 2006; Celik et al., 2008; Burney and Giudice, 2012; Han et al., 2012, 2015; Ozer et al., 2013; Song et al., 2014; Zhao et al., 2015). Consistent with their larger size, lesions from endo-vehicle mice had significantly more epithelial cells that were positive for the proliferation marker Ki-67 than did lesions from endo-VNMA mice (Fig. 2F). Fifth, we examined macrophage infiltration in lesions because macrophages drive lesion growth...
and vascularisation in a mouse model of endometriosis. As illustrated by the macrophage marker Iba1 (Lin et al., 2006; Bacci et al., 2009; Capobianco et al., 2011), lesions from endo-vehicle mice contained significantly more macrophages than did lesions from endo-VNMA mice (Fig. 2G). Finally, we measured peritoneal concentrations of IL-1β, as this cytokine is elevated in the peritoneal fluid and peritoneal macrophages of women with endometriosis (Mori et al., 1992; Lebovic et al., 2000). Endo-vehicle mice had higher peritoneal IL-1β than did endo-VNMA mice (Fig. 2H left panel). Similarly, endo-vehicle mice had higher peritoneal concentrations of TNF-α, IL-6 and TGF-β1 than did endo-VNMA mice (Fig. 2H). Together, these data indicate that treatment with broad-spectrum antibiotics reduces endometriotic lesion proliferation and peritoneal inflammation.

**Composition of the gut microbiota is altered in mice with endometriotic lesions**

To determine the effect of broad-spectrum antibiotics on gut microbial composition, we performed 16S rRNA gene sequencing of DNA isolated from faecal samples from endo-vehicle and endo-VNMA mice. Additionally, we included mice that did not undergo endometriosis-inducing surgery (non-endo). As shown in Supplementary Figure S3A,
microbial diversity (alpha, or Shannon, Diversity) was higher in faeces from non-endo and endo-vehicle mice than in faeces from endo-VNMA mice. MDS analysis uniquely clustered each group, suggesting distinct bacterial community profiles in non-endo, endo-vehicle and endo-VNMA faecal samples (Supplementary Figure S3B). We calculated three metrics of between-group diversity (beta diversity) and noted the greatest microbial diversity in endo-vehicle mice and lowest diversity in endo-VNMA mice (Supplementary Figure S3C).
Furthermore, the faecal bacterial composition of endo-VNMA mice was broadly dissimilar from that of either non-endo or endo-vehicle mice (Supplementary Figure S3B–C). This analysis demonstrated that antibiotic treatment altered the enteric bacterial diversity.

To determine whether the unique enteric bacterial profiles were attributed to specific taxa, we profiled the phyla across samples in each group. Faecal samples from endo-vehicle mice contained a higher abundance of Bacteroidetes and lower abundance of Firmicutes than samples from non-endo mice (Fig. 3A). In contrast, faecal samples from endo-VNMA mice contained negligible abundance of Bacteroidetes and Firmicutes but had increased abundance of Proteobacteria (Fig. 3A).

We confirmed these findings by analysing the 10 most abundant OTUs in the datasets (Fig. 3B). We next examined bacteria at the genus level and detected Bacteroides genera in the endo-vehicle mice but not in non-endo or endo-VNMA mice (Fig. 3C–D). The Bacteroides genus are gram-negative, non-spore-forming, anaerobic bacteria that are part of the endogenous microbiota of humans and other mammals (Brook, 1989). Finally, to assess whether surgery altered faecal microbial composition, we performed sham surgery on a group of mice. After 3 weeks, the abundances of Bacteroidetes and Firmicutes in these mice were similar to those in non-endo mice (Supplementary Figure S4A–B), indicating that surgery had no effect on gut bacteria composition. We conclude that the gut microbial composition was altered in mice with endometriosis.

**Metronidazole-sensitive gut bacteria may promote endometriotic lesion growth**

Because members of the Bacteroides genus are highly susceptible to metronidazole and are resistant to neomycin (Ingham et al., 1968;
Sutter et al., 1973; Yehya et al., 2013), we examined the effects of metronidazole and neomycin individually on endometriotic lesion growth. Mice treated with metronidazole alone (endo-metronidazole) developed ectopic lesions that were significantly smaller in volume and mass than those that developed in endo-vehicle mice (Fig. 4A–C). In contrast, mice treated with neomycin alone (endo-neomycin) developed similarly sized ectopic lesions as endo-vehicle mice (Fig. 4A–C). Histological analysis revealed that lesions from endo-metronidazole mice lacked the typical endometriosis-like appearance (e.g. glands and thick epithelial layer) seen in lesions from endo-vehicle and endo-neomycin mice (Fig. 4D). Consistent with endometriotic lesion growth, metronidazole-treated mice had fewer macrophages in lesions and less IL-1β in the peritoneal fluid than vehicle- or neomycin-treated mice (Fig. 4E–F). Together, these data indicate that metronidazole suppresses endometriotic lesion growth in mice, possibly by reducing Bacteroides growth.

Faeces from endometriotic mice promotes endometriotic lesion progression

Given our observation that faeces from endo-vehicle mice contained more Bacteroides than faeces from non-endo mice, we wondered whether this altered gut bacteria in the faeces from mice with endometriosis was sufficient to drive endometriosis progression. To address this possibility, we performed endometriosis-induction surgery on Day 0, provided mice with metronidazole in drinking water on
Days 1 through 5, orally gavaged the mice with PBS containing faeces from mice with or without endometriosis on Days 7 and 14, and examined lesions on Day 28 (illustrated in Fig. 5A). Endo-metronidazole mice gavaged with faeces from mice with endometriosis (endo-faeces) developed endometriotic lesions that were similar in mass and volume to those in endo-vehicle mice. In contrast, endo-metronidazole mice gavaged with faeces from mice without endometriosis (non-endo faeces) developed significantly smaller lesions (Fig. 5B–D). As a control, we examined endometriotic lesion growth in mice that were not gavaged with faeces but were allowed to recover from metronidazole until Day 28. As expected, endometriotic lesions were significantly smaller in these mice than in those that did not receive metronidazole (Supplementary Figure S5). We observed typical endometriosis-like histology (presence of glands and thick epithelial layer) in lesions from endo-metronidazole mice gavaged with faeces from endo-mice (Fig. 5E).

In contrast, lesions from endo-metronidazole mice gavaged with faeces from non-endo mice lacked glands and had a thin epithelial layer (Fig. 5E). Furthermore, endo-metronidazole mice that received endo-faeces contained more macrophages in lesions and more IL-1β in the peritoneal fluid than endo-metronidazole mice that received non-endo faeces (Fig. 5F–G). Taken together, these findings suggest a role for gut microbiota in endometriosis disease progression.

**Discussion**

Given that their ability to influence systemic and peritoneal inflammation and estrogen regulation, gut microbiota could contribute to endometriosis. Here, we showed that antibiotic treatment reduced...
endometriotic lesions in a mouse model of endometriosis. Additionally, mice with endometriosis had more Bacteroidetes and less Firmicutes in their guts than mice without endometriosis. Finally, metronidazole, which targets Bacteroides genus, reduced endometriotic lesion growth, but lesion growth was restored in mice gavaged with faeces from mice with endometriosis, suggesting that gut bacteria promote endometriotic lesion progression.

Once an initial endometriotic lesion is established, pro-inflammatory cytokines and growth factors are released into the peritoneal cavity, and the resulting inflammation promotes lesion spread (Ahn et al., 2015a,b). Additionally, macrophages drive lesion growth and vascularisation in a mouse model of endometriosis (Lin et al., 2006; Bacci et al., 2009; Capobianco et al., 2011). Inflammmasomes and IL-1β also contribute to endometriotic lesion growth (Snider et al., 2010; Goncalves et al., 2017). We showed that mice treated with VNMA or metronidazole alone had fewer macrophages in their lesions and lower peritoneal IL-1β concentration than vehicle-treated mice. Additionally, metronidazole-treated mice that were orally gavaged with faeces from mice with endometriosis had a similar number of lesion macrophages and peritoneal IL-1β concentration as vehicle-treated mice. Gut bacteria can modulate systemic inflammatory responses (Borody and Khoruts, 2011; Ellekleide et al., 2014; Rose et al., 2015), and release of bacterial products into the peritoneal cavity promotes auto-immunity (Luckey et al., 2013). Thus, we suggest that gut bacteria promote endometriosis by promoting inflammation. Future work should further test this model and define the mechanism by which this occurs.

We found that microbial diversity was altered in faeces from mice with endometriotic lesions and that mice with endometriosis had a higher abundance of Bacteroidetes and lower abundance of Firmicutes in their guts than mice without endometriosis. Our results differ somewhat from those of Yuan et al. (2018), who reported that, along with changes in Firmicutes and Bacteroidetes, Bifidobacterium was altered in mice with endometriosis. This difference perhaps reflects the origin of the mice and differences in diet.

In summary, our findings suggest that gut bacteria promote endometriosis disease progression in mice. If our findings are translated to humans, they may lead to new diagnostic strategies and microbiota-based therapies to treat this debilitating disease.

**Supplementary data**

Supplementary data are available at Human Reproduction online.

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**Authors’ roles**

SBC and RK designed experiments, conducted most of the studies, and analysed the data. MC assisted with animal surgeries, collected tissues and fluids, and generated some of the reagents. LAP, YY, and AS analysed metagenomics data. IUM analysed some data, provided reagents, and reviewed the final draft of the manuscript. RK conceived the project, supervised the work, and wrote the manuscript.

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**Conflict of interest**

The authors have declared that no conflict of interest exists.

**References**


A role for gut microbiota in endometriosis


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