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ORIGINAL ARTICLE ***Reproductive biology***

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, afflicts 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.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings suggest that gut bacteria promote endometriosis progression in mice. This finding if translated to humans, could aid in the development of improved diagnostic tools and personalised treatment strategies.

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Key words: endometriosis / inflammation / microbiome / gut bacteria / metronidazole

Introduction

Endometriosis causes pain in the pelvis and lower abdomen and afflicts 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 (Falcone and Flyckt, 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 inflammasome 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 curtailes 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 Metcalf, 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 endometrial 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 contatining 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 1ml sterile PBS. Lesions were weighed (mg), and lesion volumes (mm^3) were measured with a Vernier Calliper (VCB001, United scientific Supplies Waukegan, IL, USA) by an investigator blinded to treatment groups.

Faecal transplantation

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., 2017) as indicated in Fig. 5A. The numbers of mice were as follows: endo-metronidazole + non-endo faeces, $n = 4$ and endo-metronidazole + endo faeces, $n = 4$.

Bacterial 16S rRNA gene sequencing and diversity analysis

DNA was extracted from faecal pellets (0.1 gm) by using the QIAmp Power Faecal DNA Kit (12850-50, Qiagen) as per the manufacturer's protocol. The numbers of mice were as follows: non-endo, $n = 5$; endo-vehicle, $n = 5$; and endo-VNMA, $n = 4$. Amplicon generation and sequencing were performed by the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine as described previously (Parnell et al., 2017a). Fastq sequences were uploaded to the NCBI Sequence Read Archive (SUB3753571). Quantitative Insights Into Microbial Ecology analysis of variable region four was used to remove operational taxonomic units (OTUs) that were only observed once and rarefy tables to 600 OTUs. This dataset was used for downstream alpha and beta diversity analysis, and identification of the top 10 OTUs was as described previously (Parnell et al., 2017a).

Histological analysis

Tissues were fixed in 4% paraformaldehyde, and sections were immunostained ($n = 4$–5 per group) as described previously (Kommagani et al., 2016). Briefly, tissue sections were blocked with 5% goat serum and then incubated overnight at 4°C in 2% goat serum containing the following primary antibodies from Abcam: Rabbit anti-ER-α (ab75635), anti-Ki-67 (ab16667) or anti-Iba1 (ab178847). Sections were then incubated with biotinylated secondary antibody and counter-stained with hematoxylin. Finally, sections were dehydrated and mounted in Permount histological mounting medium (Fisher Scientific Inc.). Ki-67- and Iba1-positive cells were counted manually in images taken at 400X magnification by two independent investigators blinded to treatment groups. Cells were counted in at least five different areas in ectopic lesions and plotted as percent positive cells relative to total number of cells as described previously (Kommagani et al., 2013). For hematoxylin and eosin staining, tissue sections were fixed, processed, embedded, deparaffinized and stained as described previously (Kommagani et al., 2016).

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) kits were used to measure the peritoneal concentrations of IL-1β (KMC0011, Invitrogen, Life Technologies), TNF-α (ab208348, Abcam), IL-6 (ab100712, Abcam), IL-10 (ab108870, Abcam) and TGF-β1 (ab119557, Abcam), according to the manufacturer's instructions ($n = 10$ per group). The intra- and inter-assay coefficients of variation for the IL-1β ELISA were 8.4% and 4.9%, respectively. Peritoneal concentrations were deduced from standard curves, and the final concentration was calculated by normalising with the total protein concentration in peritoneal fluid.

Statistics

A two-tailed paired Student's *t*-test was used for statistical significance testing for all data except the 16 S sequencing data, which did not follow a normal distribution. The non-parametric Kruskal–Wallis test was used to compare the Shannon diversities of microbiota from non-endo, endo-vehicle and endo-VNMA groups and the Mann–Whitney test was used to compare Shannon diversities between groups. For multidimentional scaling (MDS) plots, the R 'Phyloseq' package was used to perform permutation analysis of variance. $P < 0.05$ was considered significant.

Results

Treatment with broad-spectrum antibiotics reduces endometriotic lesion growth, proliferation and inflammation

To determine whether antibiotics affect early endometriotic lesion growth, 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 endometriotic lesions than those that consumed vehicle alone (vehicle-endo) (Fig. 1B–D). In a second experiment aimed at assessing progression of established endometriotic 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 endometriotic lesions.

To begin to uncover the mechanism by which antibiotics affected endometriotic lesion 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 water consumption or 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 tissue 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 endometriotic 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

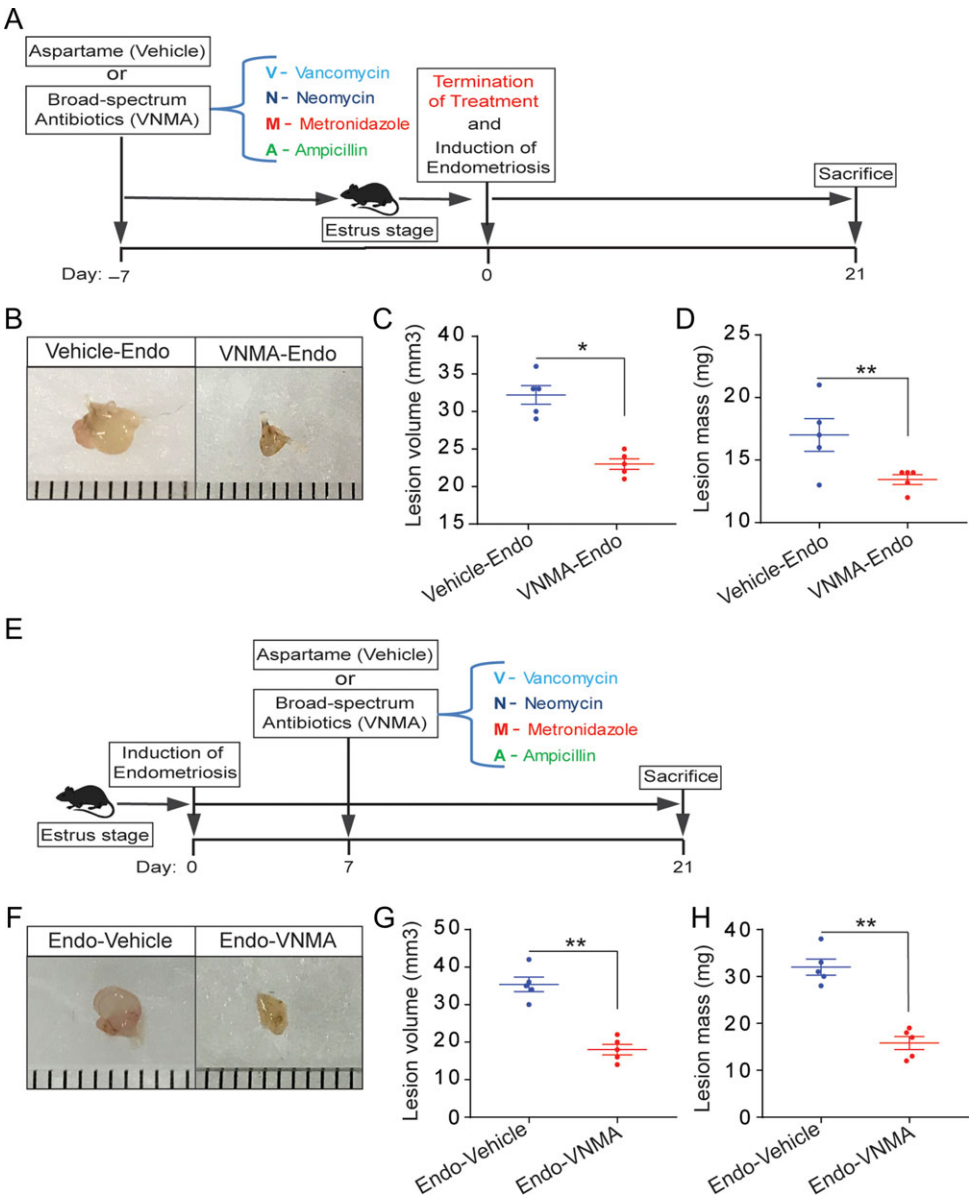

Figure 1 Treatment with broad-spectrum antibiotics prevents early endometriotic lesion growth and progression. (**A** and **E**) Schematic of experimental timeline and procedures. (**B–D** and **F–H**) Representative gross images (**B** and **F**), volumes (**C** and **G**), and masses (**D** and **H**) of ectopic endometriotic lesions from the indicated treatment groups 21 days after surgical induction of endometriosis. Data are presented as mean ± SE($n = 5$) *$P < 0.05$, **$P < 0.01$.

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 16 S 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,

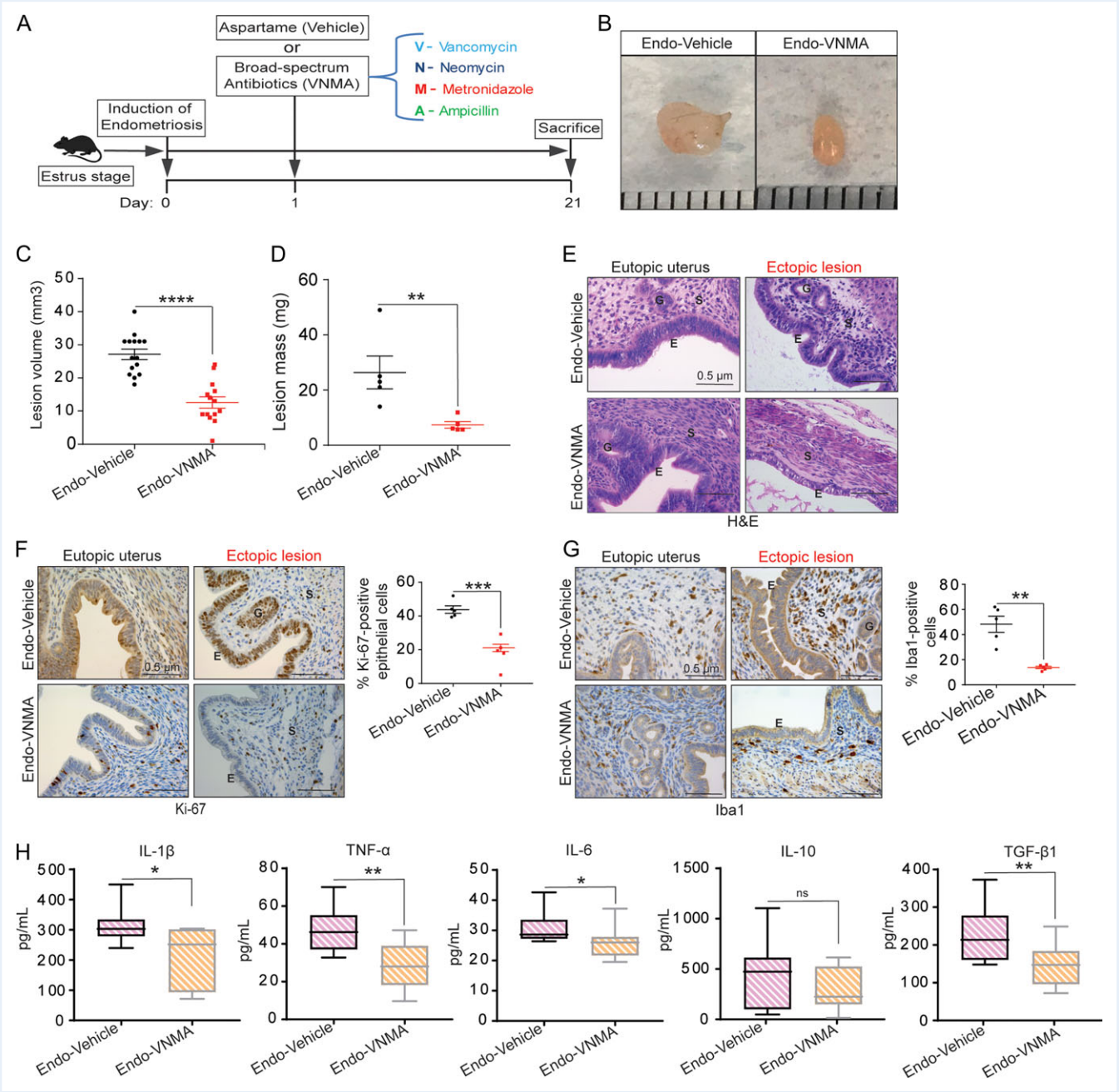

Figure 2 Treatment with broad-spectrum antibiotics reduces endometriotic lesion proliferation and inflammation. (**A**) Schematic of experimental timeline and procedures. (**B**–**D**) Representative gross images (**B**), volumes (**C**) and masses (**D**) of ectopic endometriotic lesions from the indicated treatment groups 21 days after surgical induction of endometriosis. Data are presented as mean ± SE; endo-vehicle ($n = 15$) and endo-VNMA ($n = 14$). (**E**) Representative Hematoxylin and Eosin-stained cross-section images of the eutopic uteri and ectopic lesions from the indicated treatment groups. The scale bar (0.5 µm) applies to all images; $n = 5$. (**F**–**G**) Representative cross-sectional images (left) of the eutopic uteri and ectopic lesions stained for Ki-67 (**F**) and Iba1 (**G**); respective graphs on the right show positively stained cells counted in at least five different areas in ectopic lesions and plotted as percent positive cells relative to total cells. The scale bar (0.5 µm) applies to all images; $n = 5$. 'E', 'G' and 'S' denote epithelia, glands and stroma, respectively. (**H**) ELISA-based quantification of IL-1β, TNF-α, IL-6, IL-10 and TGF-β1 levels in peritoneal fluid from the indicated treatment groups. Data are presented as mean ± SE ($n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ and ns, non-significant.

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).

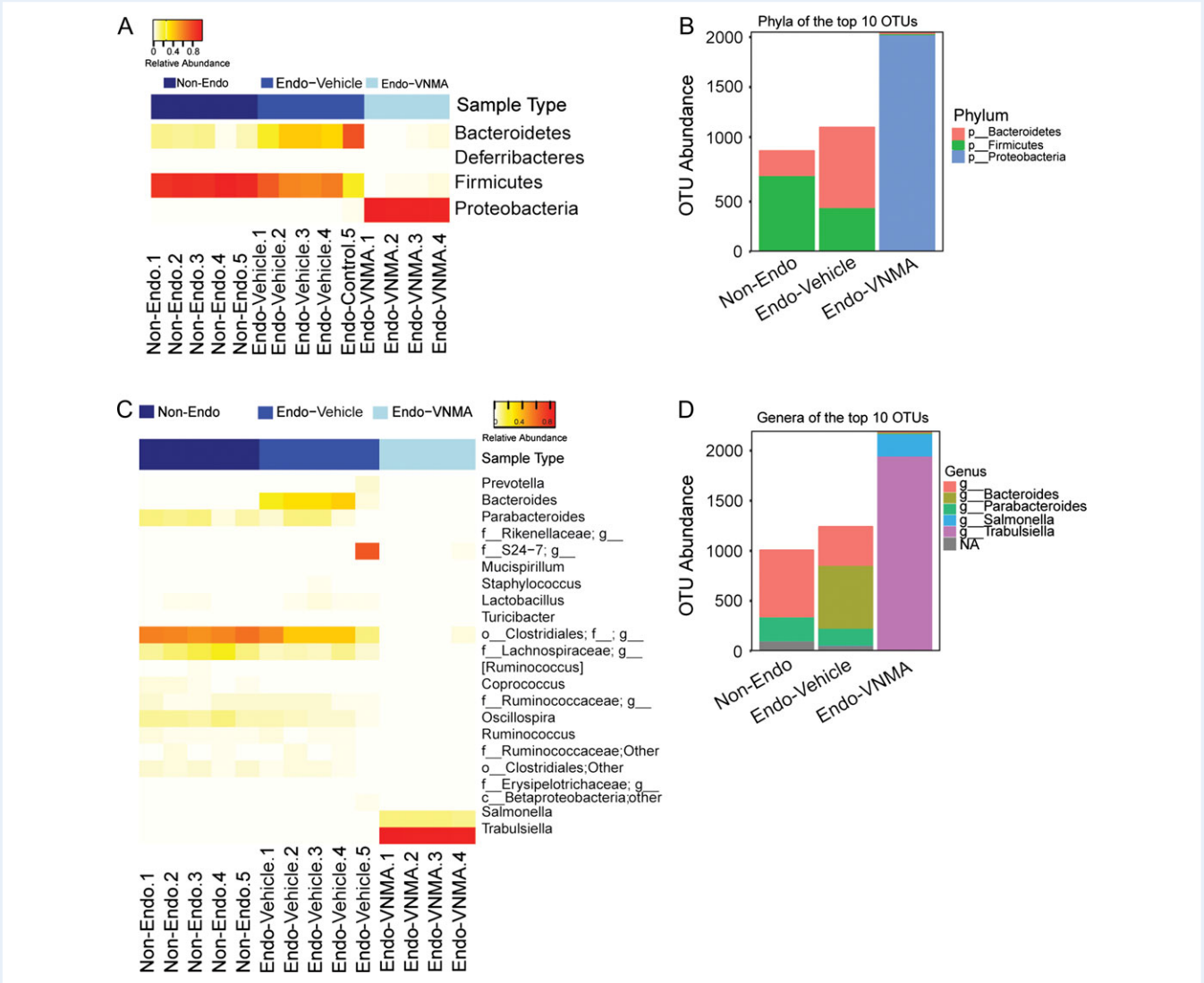

Figure 3 ***Bacteroides*** **are enriched in faeces from mice with endometriotic lesions.** (**A**) Heat map representation of relative abundances of the phyla in faecal samples from non-endo ($n = 5$), endo-vehicle ($n = 5$) and endo-VNMA ($n = 4$) mice. (**B**) Stacked bar plots of the phyla belonging to the 10 most abundant OTUs. (**C**) Heat map depiction of the relative abundances of the genera in each faecal sample. (**D**) The genera belonging to the 10 most abundant OTUs across each group are shown as stacked bar plots.

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. 3 C–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;

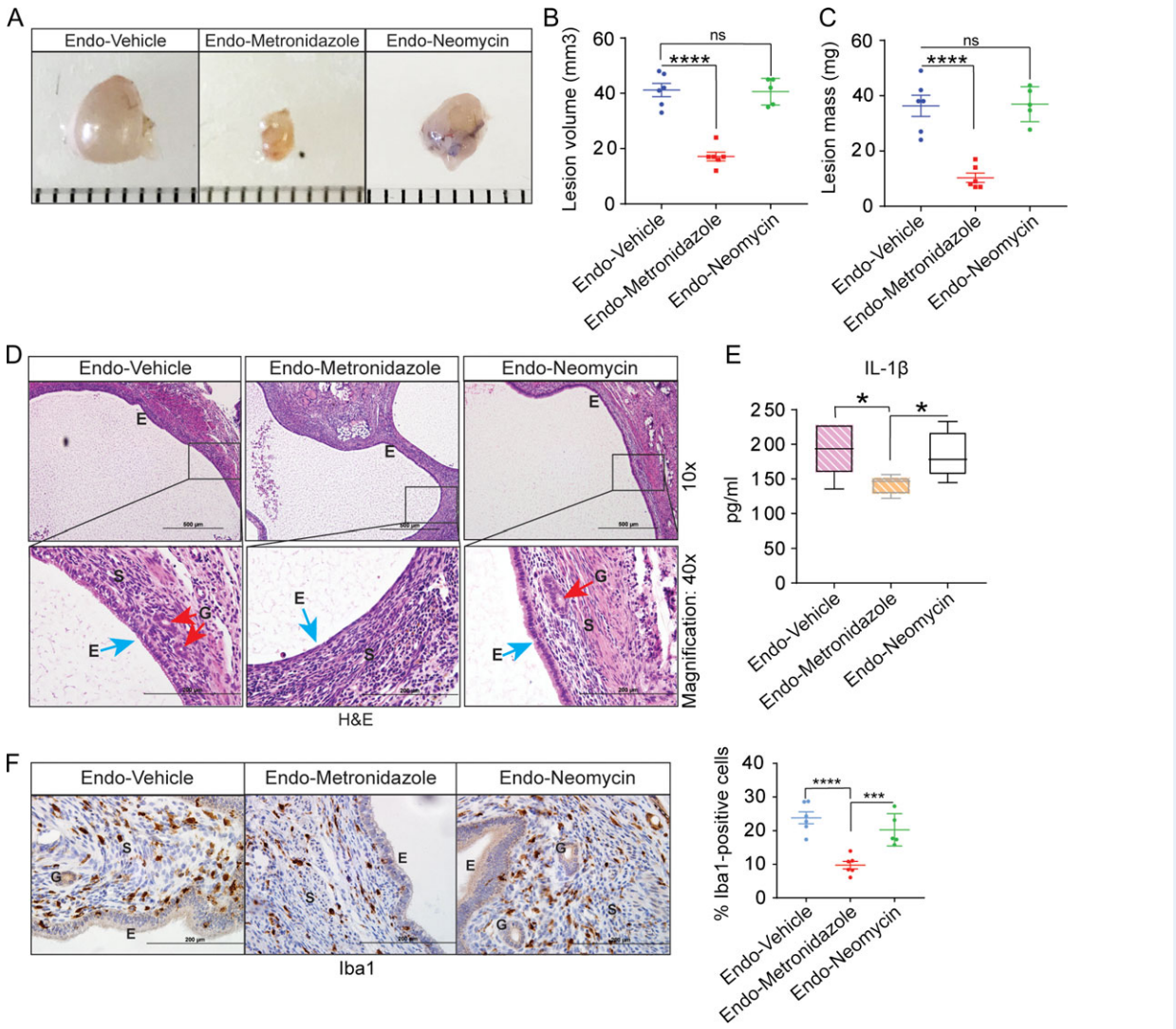

Figure 4 Metronidazole treatment reduces endometriotic lesion growth. (**A**–**C**) Representative gross images (**A**), volumes (**B**) and masses (**C**) of ectopic endometriotic lesions from the indicated treatment groups; ($n = 5$). (**D**) Representative Hematoxylin and Eosin-stained cross-section images of ectopic lesions from the indicated treatment groups; $n = 5$. Scale bars represent 200 μm (upper panel) or 500 μm (lower panel). (**E**) Quantification of IL-1β concentration in peritoneal fluid from the indicated treatment groups. (**F**) Representative cross-sectional images of ectopic lesions stained for Iba1; graph on the right shows the number of positively stained cells counted in at least five different areas in ectopic lesions and plotted as percent positive cells relative to total cells. 'E', 'G' and 'S' denote epithelia, glands and stroma, respectively. Data are presented as mean ± SE; ($n = 5$). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, and ns, non-significant.

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

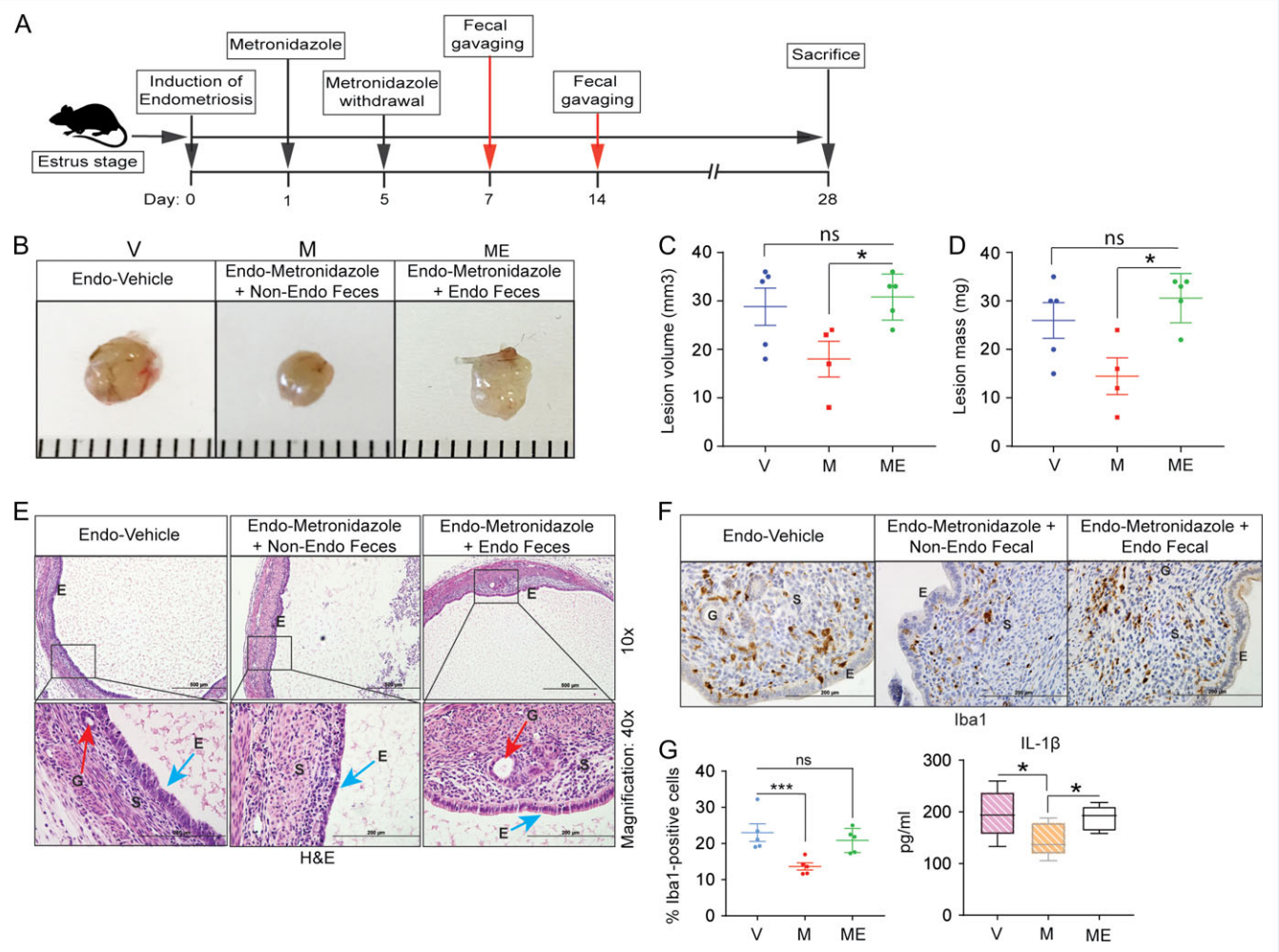

Figure 5 Oral gavage of faeces from endometriotic mice promotes endometriotic lesion growth in antibiotic-treated mice. (**A**) Schematic of experimental timeline and procedures. (**B**–**D**) Representative gross images (**B**), volumes (**C**) and masses (**D**) of ectopic endometriotic lesions from the indicated treatment groups 28 days after surgical induction of endometriosis; 'V', 'M' and 'ME' denote endo-vehicle, endo-metronidazole + non-endo faeces, and endo-metronidazole + endo faeces, respectively; $n = 4$–5. (**E**) Representative Hematoxylin and Eosin-stained cross-section images of ectopic lesions from the indicated treatment groups; $n = 4$–5. Scale bars represent 200 μm (upper panel) and 500 μm (lower panel). (**F**) Representative cross-sectional images of ectopic lesions stained for Iba1. (**G**) Quantification of Iba1-positive cells counted in at least five different areas in ectopic lesions and plotted as percent positive cells relative to total cells (left panel) and quantification of IL-1β concentration in peritoneal fluid from the indicated treatment groups (right panel). Data are presented as mean ± SE ($n = 4$–5). 'E', 'G' and 'S' denote epithelia, glands and stroma, respectively. *$P < 0.05$, ***$P < 0.001$, and ns, non-significant.

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). Inflammasomes 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; Ellekilde *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.

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