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ORIGINAL ARTICLE

Effects of gut microbiota and their metabolites on benign diseases of the female reproductive system: A Mendelian randomization study

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ABSTRACT

Background: Alterations in gut microbiota, including the composition of the flora, the abundance of specific species, and bacterial metabolites are related to the pathogenesis of benign diseases of the female reproductive system. Because of potential biases, such as confounding or reverse causation, the causal relationship between diseases of the female reproductive system and alterations in gut microbiota remains unclear. We applied two-sample Mendelian randomization (MR) to investigate the effect of alterations in gut microbiota on 18 benign diseases of the female reproductive system. **Methods:** Instrumental variables for different classification levels of the gut microbiota including phylum, class, order, family, and genus, and several bacterial metabolites were selected. MR analysis was performed using inverse-variance weighted (IVW), MR-Egger, weighted median, MR-Robust Adjusted Profile Scoring (MR-RAPS), and MR pleiotropy residual sum and outlier tests. Cochran's IVW Q statistics were used to detect any potential heterogeneity.**Results:** Notably, 49 genera of the gut microbiota exhibited a causal relationship with the benign diseases of the female reproductive system, with more than half of the genera exhibiting a role in two or more diseases. Six metabolites of the gut microbiota can be considered as indicators to detect the presence of the disease.**Conclusions:** Alterations in a total of four phyla, five classes, four orders, ten families, and 49 genera of gut microbiota and six kinds of bacterial metabolites exhibited positive or negative effects on the female reproductive system. Our findings provide insights into potential novel biomarkers and therapy targets for many disease of the female reproductive system.

Keywords: gut microbiota, bacterial metabolites, female reproductive system, Mendelian randomization study, single-nucleotide polymorphisms

INTRODUCTION

The critical role of the benign diseases of the female reproductive system on the physiological health of women and pregnancy outcomes, including menstrual abnormalities, menopausal syndromes, polycystic ovary syndrome (PCOS), and endometriosis, is attracting increasing interest.[1] Despite this, major clinical investigations on the diseases of the female reproductive system still tend to focus on systemic metabolism. In addition, such investigations may adversely impact the endocrine, immunological, and even psychological health of the affected women. The etiology of these diseases remains poorly elusive.[2,3] Hence, to reduce the burden of female reproductive system diseases on women and their families, well-designed studies are needed to establish a causal crosstalk between modifiable risk factors and these diseases, such as alterations in the gut microbiota.

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The current evidence shows that both the composition and metabolites of the gut microbiota play a crucial role in regulating the metabolic status of the whole body.[4] Various studies have highlighted the endocrine effects of gut microbiota as essential factors contributing to the pathogenesis of disorders related to the female reproductive endocrine system. The gut microbiota is closely involved in both the normal and pathological state of each stage of female reproduction, that is maturation of follicles, regulation of menstrual cycles, fertilization, the entire pregnancy period, embryo development, and even parturition.[5] Alterations in gut microbiota, including the composition of the flora, the abundance of specific species, and bacterial metabolites, lead to pathogenic events of the diseases of the female reproductive system, such as female infertility, uterine fibroids, and localized inflammation.[2,6–8] However, because of potential biases such as confounding or reverse causation, the causal relationship between the benign diseases of the female reproductive system and alterations in the gut microbiome has remained unclear. Hence, systematic efforts are needed to clearly demonstrate a causal relationship.

Mendelian randomization (MR) analysis can be used to identify causality between exposures and outcomes. As an alternative to randomized controlled trials (RCTs), MR is an indispensable tool in avoiding confounding or reverse causation by integrating specific instrument variant indexing to infer the causality of risk factors with the disease of interest.[9]

We applied a two-sample Mendelian randomization (TSMR) analysis using summary statistics from large-scale genome-wide association studies (GWAS) to investigate the causal effect of gut microbiota on benign diseases of the female reproductive system. The TSMR may help in determining the causal relationship between the gut microbiota and benign diseases of the female reproductive system to prevent, diagnose, and identify therapeutic targets for these diseases.

METHODS

Study design

The TSMR was used to examine the causal influence of 131 gut microbiota genera on 18 benign diseases of the female reproductive system *via* GWAS summary statistics (Figure 1).

GWAS summary data of exposure

The data on gut microbiota were harvested from the largest genome-wide meta-analysis from the MiBioGen consortium (https: //mibiogen.gcc.rug.nl/).[10] This prodigious analysis encompassed 24 cohorts, belonging to diverse regions and ethnicities, comprised of 18, 340 women. Among these cohorts, 13, 266 women were of European descent, comprising 72.3% of the study population. In addition, 22 cohorts consisted of adults and adolescents, while the remaining two had children. To analyze the microbial composition, three distinct variable regions (V4, V3–V4, and V1–V2) were employed to target the 16S ribosomal RNA (rRNA) gene.

The data on gut microbial metabolites, including beta-hydroxybutyric acid, betaine, trimethylamine N-oxide (TMAO), carnitine, choline, glutamate, kynurenine, phenylalanine, propionic acid, serotonin, tryptophan, and tyrosine, were obtained from a cohort of 2, 076 women that participated in the Framingham Heart Study.[11] With an average age of 55 years and a female representation of 51%, a fastidious metabolite analysis of plasma samples was carried out.

GWAS summary data of outcome

The summary statistics utilized for analyzing the 18 benign diseases of the female reproductive system were obtained from the seventh version of the FinnGen database (https://r7.finngen.fi/). The FinnGen project was initiated in 2017. Its R7 version was updated in June 2022, ensuring remarkable timeliness. Notably, a vast majority of Finnish biobanks have acquired consent explicitly in conjunction with hospital appointments, resulting in a significantly greater patient count. For detailed information regarding the specific features of the outcome indicators, (Supplementary Tables 1-18).

Selection of genetic instruments

Given that GWAS-identified gene loci for both gut microbiota and their metabolites rarely achieve genome-wide significance levels ($P < 5\times10^{-8}$), single-nucleotide polymorphisms (SNPs) with suggestive genome-wide significance thresholds ($P < 1\times10^{-5}$) were selected as instrumental variables (IVs). To obtain independent IVs, we established a linkage disequilibrium (LD) threshold at an r^2 value of < 0.001 and implemented a clumping window size of 10, 000 kb. The European sample data obtained from the 1000 Genomes Project was utilized as a point of reference to evaluate the LD between SNPs. Additionally, we systematically eliminated palindromic SNPs and ensured that the effects of SNPs on exposure corresponded with their effects on the outcome. We ensure that the IVs used in our MR analyses satisfy three fundamental assumptions: (i) the selected IVs should be firmly representative for the exposure we mentioned ($P < 1\times10^{-5}$); (ii) all the effects of the selected IVs on the outcomes only depend on the presence of the exposure; (iii) the selected IVs are independent of additional risk factors or potential confounders.

Statistical analysis

The inverse variance weighted (IVW) method is widely

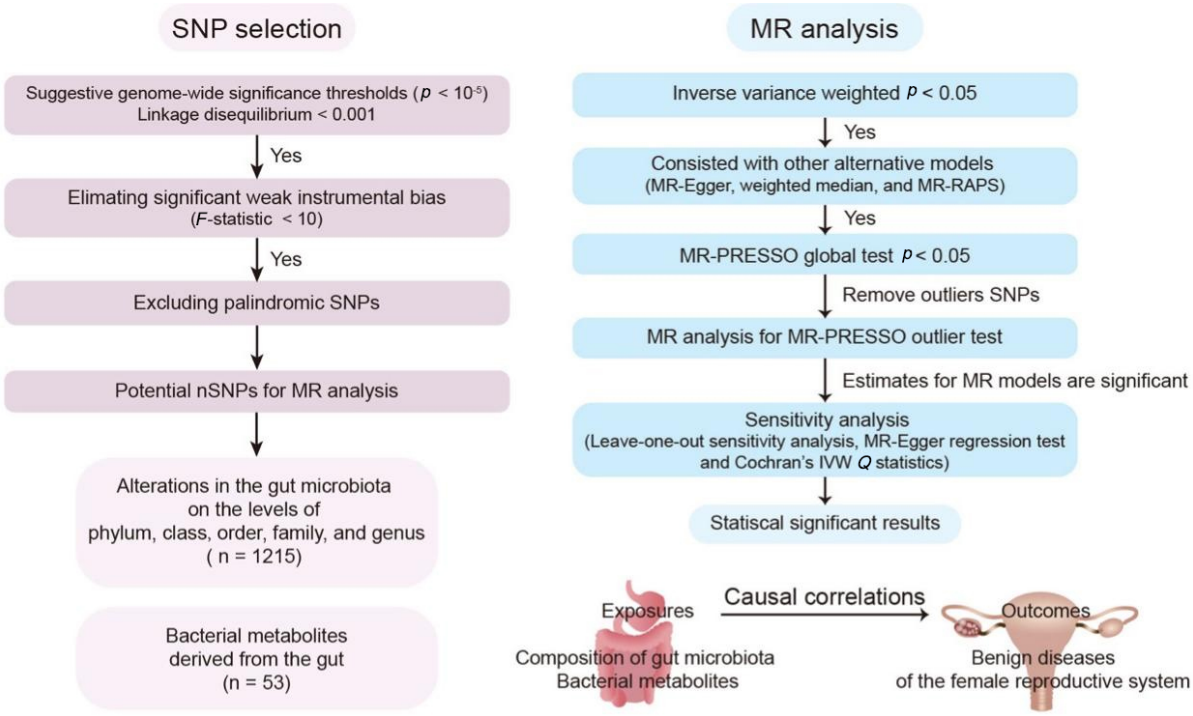

Figure 1. Study design overview. SNP, single-nucleotide polymorphism; MR, Mendelian randomization; IVW, inverse-variance weighted; MR-RAPS, MR-Robust Adjusted Profile Scoring.

used in MR because of its high efficiency and precision in estimating causal effects. We used the IVW method to perform the primary analysis. To further investigate the robustness of our findings, we conducted sensitivity analyses using alternative methods, namely MR-Egger, weighted median, and MR-Robust Adjusted Profile Scoring (MR-RAPS). The MR-Egger approach enables MR analyses to account for the presence of directional pleiotropy,[12] a phenomenon that can lead to biased causal estimates. Similarly, the weighted median approach offers reliable estimates even in situations where up to half of the genetic instruments are deemed invalid, making it an essential tool for researchers seeking to ensure the accuracy of their results.[13] Finally, the MR-RAPS method is specifically tailored to address issues related to weak instrument bias,[14] providing researchers with a valuable means for improving the robustness of their findings.

Our analysis deemed results as positive only if they fulfilled two essential criteria: first, the *P*-value of the IVW method should be < 0.05, and second, the direction of the sensitivity analyses should align with that of the IVW method. This approach can help in mitigating the impact of potential biases and increase confidence in the validity of our causal estimates. Overall, our study used a rigorous analytical approach to investigate the causal effects of the exposure variable on the outcome variable.

We employed the MR-PRESSO method to identify outliers.[15] To determine whether a particular variant drove the association between the exposure and the outcome variable, we conducted a leave-one-out sensitivity analysis by omitting a single SNP each time. Additionally, we performed the MR-Egger regression test to investigate horizontal pleiotropy in MR analysis, especially if the intercept term had statistical significance.[16] Finally, we calculated Cochran's IVW Q statistics to detect any potential heterogeneity.[17]

The effectiveness of IVs was evaluated using the F-statistic, which was determined using the following equation:

$$F = \beta_{\text{exposure}}^2 / SE_{\text{exposure}}^2.$$

F exceeding 10 indicated the absence of any significantly weak instrumental bias.

The statistical significance threshold used in this study was set at $P = 0.05$. We performed all statistical analyses using R, Version 4.2.2, and utilized the two-sample MR and MR-PRESSO packages.

Ethics

Our investigation utilized GWAS summary statistics that are available to the public. No novel data were gathered, and thus, there was no need for additional ethical

approval.

RESULTS

We used 1268 SNPs as IVs for 131 bacterial genera and their metabolites. The IVW method was used for dominant analysis to assess causality. We determined the form of multiple exposures and multiple outcomes using large-scale data. Our results showed that alterations in four phyla, five classes, four orders, ten families, and 49 genera of gut microbiota, as well as six kinds of bacterial metabolites exhibited either positive or negative effects on the female reproductive system.

Endometriosis and adenomyosis

Families Prevotellaceae and Peptostreptococcaceae, and three genera—*Anaerotruncus*, *Holdemania*, and *Unknown genus* (i.d. 1000000073) —were causally related to endometriosis. Phylum Lentisphaeria, order Victivallales, family Prevotellaceae, and four genera (*Anaerofilum*, *Anaerostipes*, *Unknown genus* [i.d. 1000000073], and *Unknown genus* [i.d. 2755]) were responsible for the development of adenomyosis. Additionally, propionic acid released by the gut microbiota had a positive impact on both endometriosis (OR = 0.913, 95% CI: 0.854–0.975, $P = 0.007$) and adenomyosis (OR = 0.846, 95% CI: 0.750–0.955, $P = 0.007$).

High exposure to Peptostreptococcaceae (OR = 1.174, 95% CI: 1.043–1.322, $P = 0.008$) and Prevotellaceae (OR = 1.219, 95% CI: 1.057–1.405, $P = 0.006$) and genus *Anaerotruncus* (OR = 1.277, 95% CI: 1.078–1.512, $P = 0.005$) appeared to be closely associated with the pathogenesis of endometriosis. Importantly, after excluding the outliers by MR-PRESSO, family Prevotellaceae remains a risk factor for the development of endometriosis. The *P*-value obtained by the IVW method changed from 0.006 to 0.009 (OR = 1.219, 95% CI: 1.057–1.405, $P = 0.009$), as shown in Supplementary Table 18. Genera *Holdemania* (OR = 0.872, 95% CI: 0.779–0.976, $P = 0.017$) and *Unknown genus (id. 1000000073)* (OR = 0.862, 95% CI: 0.776–0.957, $P = 0.005$) showed a promising therapeutic effect, as shown in Figure 2A. Raw data for the original MR analysis are shown in Supplementary Table 1.

In the case of adenomyosis, an increase in the number of microbiota belonging to family Prevotellaceae (OR = 1.290, 95% CI: 1.054–1.579, $P = 0.013$) was observed. In contrast, the microbiota of class Lentisphaeria (OR = 0.859, 95% CI: 0.748–0.988, $P = 0.033$), order Victivallales (OR = 0.859, 95% CI: 0.748–0.988, $P = 0.033$), and four genera (*Anaerofilum* (OR = 0.828, 95% CI: 0.705–0.973, $P = 0.022$), *Anaerostipes* (OR = 0.744, 95% CI: 0.567–0.976, $P = 0.033$), *Unknown genu*s (id. 1000000073)(OR= 0.825, 95% CI: 0.684–0.996, $P = 0.045$), and *Unknown genus* (id. 2755) (OR = 0.736, 95% CI: 0.618–0.876, $P = 0.001$) decreased, as shown in Figure 2B and Supplementary Table 2.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect the underlying heterogeneity or pleiotropy. MR-PRESSO was used to exclude outliers. The leave-one-out test was used to make sure that no potentially influential SNPs were excluded. The causal effects remain significant even after all sensitivity analyses had been considered.

Inflammatory diseases of the cervix or uterus

Gut microbiota of order Pasteurellales, family Pasteurellaceae, and five genera (*Blautia*, *Haemophilus*, *Lachnospiraceae* [FCS020 group], *Lachnospiraceae* [ND3007 group], and *Prevotella-7*) exhibited a causal relationship with the inflammatory diseases of the cervix. Phylum Proteobacteria and five genera, namely *Eubacterium* (fissicatena group), *Eubacterium* (rectale group), *Desulfovibrio*, *Ruminiclostridium 6*, and *Unknown genus* (i.d. 2001), and beta-hydroxybutyric acid derived from the gut were found to be related to inflammatory diseases of the uterus.

The IVW estimates suggested a protective correlation between the inflammatory diseases of the cervix resulting from order Pasteurellales (OR = 0.701, 95% CI: 0.538–0.914, $P = 0.009$), family Pasteurellaceae (OR = 0.701, 95% CI: 0.538–0.914, $P = 0.009$), and three genera, namely *Haemophilus* (OR = 0.610, 95% CI: 0.445–0.838, $P = 0.002$), *Lachnospiraceae* (FCS020 group) (OR= 0.630, 95% CI: 0.399–0.996, $P = 0.048$), and *Prevotella 7* (OR = 0.799, 95% CI: 0.649–0.983, $P = 0.034$). The increasing frequency of genera *Blautia* (OR = 1.689, 95% CI: 1.043–2.669, $P = 0.033$) and *Lachnospiraceae* (ND3007 group) (OR = 2.680, 95% CI: 1.196–6.008, $P = 0.017$) appeared to have a negative effect on the development of inflammatory diseases of the cervix, as shown in Figure 3A and Supplementary Ta ble 3.

Phylum Proteobacteria (OR = 0.713, 95% CI: 0.546–0.932, $P = 0.013$) decreased the likelihood of the pathogenesis of inflammatory diseases of the uterus; however, genus *Ruminiclostridium 6* (OR = 1.280, 95% CI: 1.010–1.623, $P = 0.041$), belonging to Proteobacteria, might increase the risks for inflammatory diseases of the uterus. *Eubacterium* (fissicatena group) (OR = 1.290, 95% CI: 1.098–1.516, $P = 0.002$), *Desulfovibrio* (OR = 1.268, 95% CI: 1.009–1.595, $P = 0.042$), and *Unknown genus* (i.d. 2001) (OR = 1.265, 95% CI: 1.014–1.577, $P = 0.037$) exhibited a negative relationship with the inflammatory diseases of the uterus. The MR findings of *Eubacterium* (rectale group) (OR = 0.683, 95% CI: 0.489–0.953, $P = 0.025$) revealed a protective correlation with the occurrence. Beta-hydroxybutyric acid (OR = 1.274, 95% CI: 1.086–1.495, $P = 0.003$) might lead to an adverse effect and aggravate the inflammatory responses within the

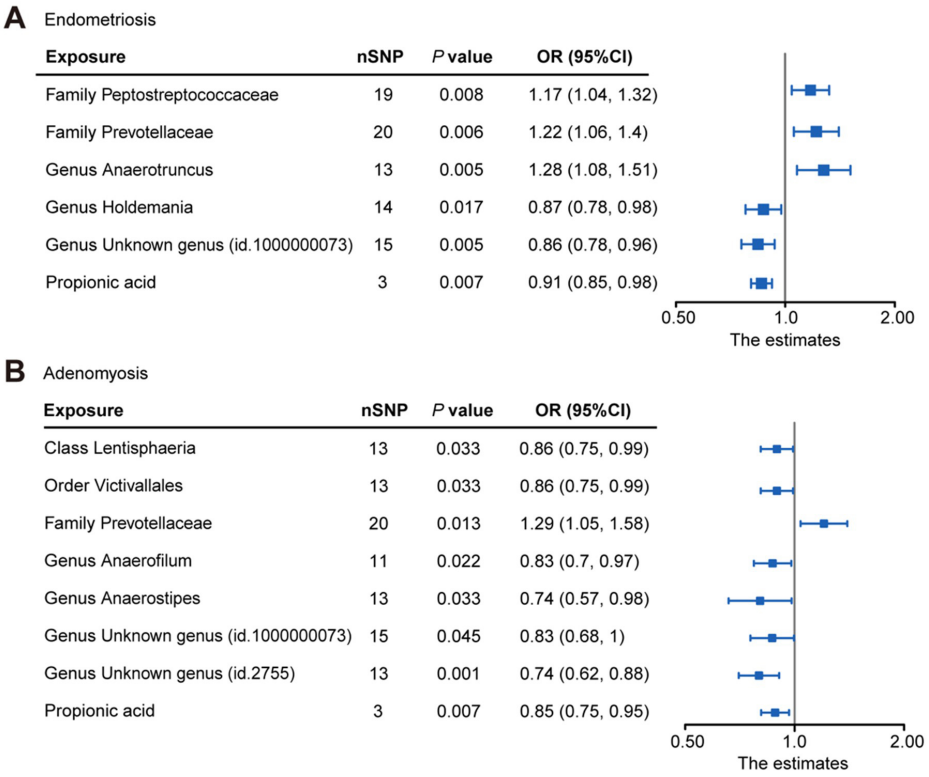

Figure 2. Forest plot of IVW estimates in endometriosis and adenomyosis. (A) Endometriosis. (B) Adenomyosis. nSNP, number of single-nucleotide polymorphism; OR, odd ratio; 95% CI, 95% confidence interval; IVW, inverse-variance weighted.

uterus, as shown in Figure 3B and Supplementary Table 4.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect underlying heterogeneity or pleiotropy. MR-PRESSO was used to exclude outliers. Furthermore, the leave-one-out test was used to exclude any potentially influential SNP. The causal effects remain significant after all sensitivity analyses were considered.

Female infertility

As shown in Figure 4, the gut microbiota belonging to five genera, namely *Coprococcus 2*, *Desulfovibrio*, *Faecalibacterium*, *Family XIII* (AD3011 group), and *Holdemania*, were found to be causally related to female infertility. According to the IVW estimates, *Coprococcus 2* (OR = 1.993, 95% CI: 1.009–1.426, $P = 0.040$) and *Family XIII* (AD3011 group) (OR = 1.234, 95% CI: 1.041–1.464, $P = 0.015$) were considered as risk factors for female infertility. Meanwhile, the positive effect of *Desulfovibrio* (OR = 0.851, 95% CI: 0.741–0.977, $P = 0.022$), *Family XIII* (AD3011 group) (OR = 0.820, 95% CI: 0.707–0.951, $P = 0.009$), and *Holdemania* (OR = 0.882, 95% CI: 0.780–0.996, $P = 0.046$) on female infertility was observed.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect any underlying heterogeneity or pleiotropy, while MR-PRESSO was used to exclude any outliers. Furthermore, the leave-one-out test was used to exclude any potentially influential SNPs. The causal effects remain significant even after all sensitivity analyses were considered, as shown in Supplementary Ta ble 5.

Although assisted reproductive technology (ART) provides women suffering from infertility a certain opportunity to conceive successfully, the abnormal abundance of these bacterial populations could be associated with the failure or adverse outcome of ART. This topic needs to be investigated in detail.

Dysplasia of the female genital tract

This section discusses the female genital tract dysplasia, as well as prolapse and hernia of the ovary and fallopian tube. The MR method showed that gut microbiota of class Betaproteobacteria, family Lactobacillaceae, and four genera, namely *Eubacterium* (ruminantium group), *Eubacterium* (ventriosum group), *Methanobrevibacter*, and *Senegalimassilia*, are involved in the dysplasia of the female genital tract. Four genera, *Holdemania*, *Parasutterella*, *Unknown genus* (i.d. 1868), and *Unknown genus* (i.d. 2071), had a causal relationship with the

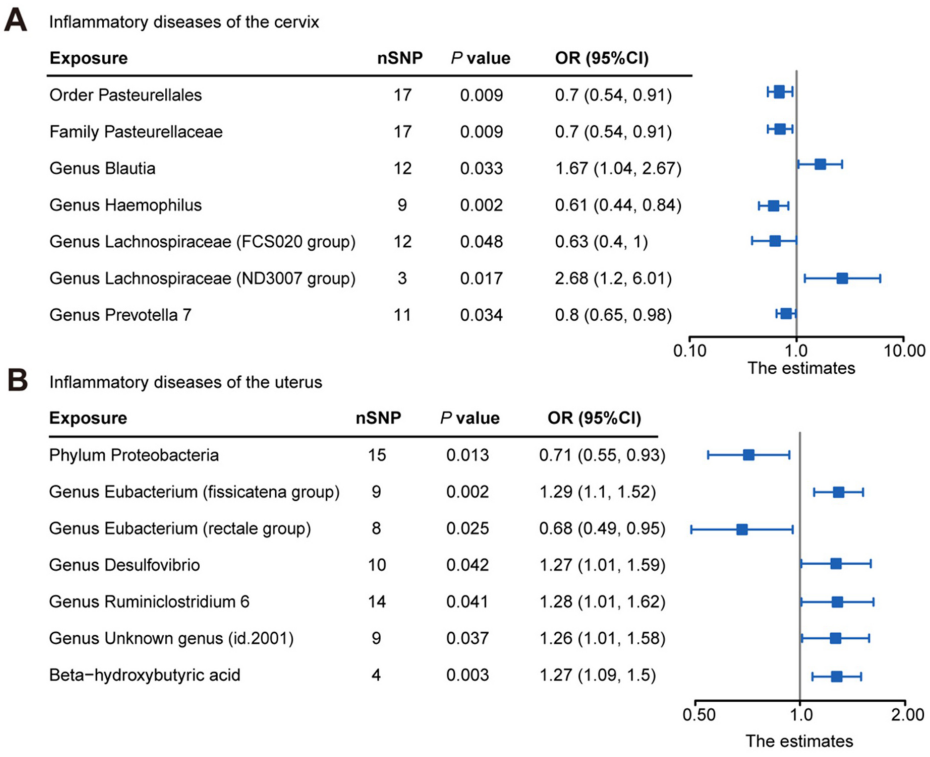

Figure 3. Forest plot of IVW estimates in inflammatory diseases of the cervix and the uterus. (A) Inflammatory diseases of the cervix. (B) Inflammatory diseases of the uterus.

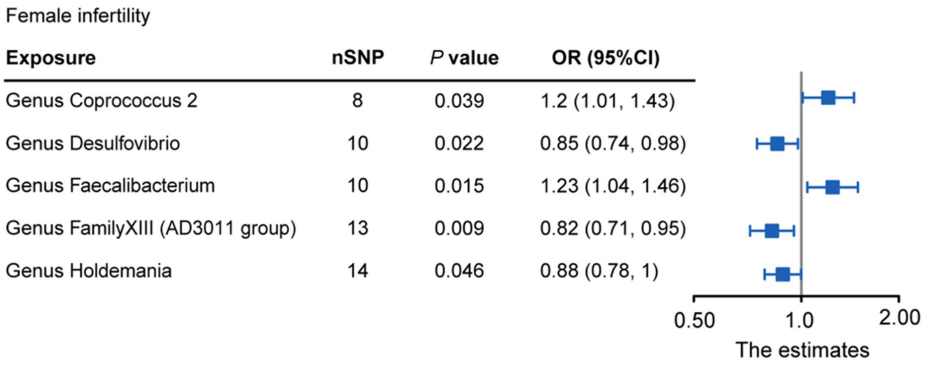

Figure 4. Forest plot of IVW estimates in female infertility. nSNP, number of single-nucleotide polymorphism; OR, odd ratio; 95% CI, 95% confidence interval; IVW, inverse-variance weighted.

prolapse and hernia of the ovary and fallopian tube. Furthermore, the tyrosine supplement appeared to have a protective factor against the prolapse and hernia of the ovary and fallopian tube (OR = 0.943, 95% CI: 0.892–0.996, $P = 0.037$).

According to our results, an increasing abundance of microbiota belonging to class Betaproteobacteria (OR = 1.303, 95% CI: 1.040–1.634, $P = 0.022$), family Lactobacillaceae (OR = 1.180, 95% CI: 1.011–1.377, $P = 0.035$), and genera *Eubacterium* (ventriosum group) (OR = 1.261, 95% CI: 1.042–1.525, $P = 0.017$) was a predictor for cervical dysplasia. A beneficial effect of the elevated population of gut microbiota of genera *Senegalimassilia* (OR = 0.716, 95% CI: 0.517–0.991, $P= 0.044$), *Eubacterium* (ruminantium group) (OR = 0.882, 95% CI: 0.796–0.977, $P = 0.016$), and *Methanobrevibacter* (OR = 0.870, 95% CI: 0.758–0.999, $P = 0.048$) on the dysplasia of the female genital tract has also been reported. *Senegalimassilia* (OR = 0.684, 95% CI: 0.502–0.931, $P = 0.016$) showed a significant causality with cervical dysplasia, as shown in Figure 5 and Supplementary Tables 6 and 7.

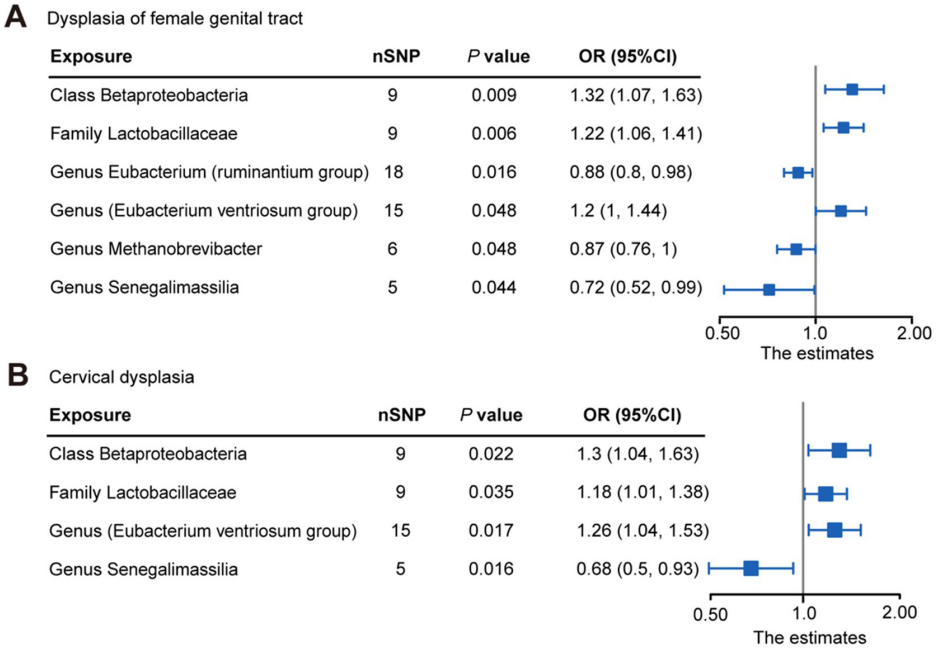

Figure 5. Forest plot of IVW estimates in the dysplasia of the female genital tract.

In terms of the prolapse and hernia of the ovary and fallopian tube, accumulation of gut microbiota belonging to genus *Parasutterella* (OR = 1.499, 95% CI: 1.001–2.244, $P = 0.049$) might increase the occurrence of female reproductive diseases, while those belonging to genera *Holdemania* (OR = 0.668, 95% CI: 0.472–0.946, $P = 0.023$), *Unknown Genus* (i.d. 1868) (OR = 0.602, 95% CI: 0.386–0.940, $P = 0.025$), and *Unknown Genus* (i.d. 2071) (OR = 0.631, 95% CI: 0.423–0.939, $P = 0.023$) decrease the prevalence of the disease, as shown in Figure 6 and Supplementary Table 8.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect any underlying heterogeneity or pleiotropy. MR-PRESSO was used to exclude outliers. Furthermore, the leave-one-out test was used to exclude any potentially influential SNPs. The causal effects remain significant even after all sensitivity analyses were considered.

Proliferative lesion and benign leiomyoma

This section discusses polyps of the uterus or female genital tract, and benign leiomyoma of the uterus. The MR estimates showed that alterations in class Delta-proteobacteria, order Enterobacteriales, families Enterobacteriaceae and Streptococcaceae, and genus *Ruminococcus* (torques group) were involved in the occurrence of polyps of the female tract. Genus *Roseburia* was particularly correlated with uterine polyps. Family Prophyromonadaceae and six genera, namely *Bacteroides*, *Enterorhabdus*, *Gordonibacter*, *Howardella*, *Lachnospiraceae UCG 008*, and *Turicibacter*, showed a causal relationship with the pathogenesis of benign leiomyoma.

The increase in the number of gut microbiota belonging to class Delta-proteobacteria (OR = 1.226, 95% CI: 1.001–1.501, $P = 0.049$) had a negative impact on the prevalence of the polyp of the female tract, while the positive correlation between order Enterobacteriales (OR = 0.783, 95% CI: 0.659–0.930, $P = 0.005$), families Enterobacteriaceae (OR = 0.783, 95% CI: 0.659–0.930, $P = 0.005$) and Streptococcaceae (OR = 0.824, 95% CI: 0.689–0.984, $P = 0.032$), and genus *Ruminococcus* (torques group) (OR = 0.719, 95% CI: 0.573–0.903, $P = 0.005$) and the disease was observed. Interestingly, although genus *Roseburia* (OR = 1.417, 95% CI: 1.024–1.962, $P = 0.036$) did not exhibit a significant relationship with the occurrence of the polyp of the female genetic tract, it played a negative role in the pathogenesis of the polyp of the uterus, as shown in Figure 7A and Supplementary Ta ble 9.

Upregulation of family Prophyromonadaceae (OR = 1.119, 95% CI: 1.003–1.248, $P = 0.044$) and genera *Bacteroides* (OR = 1.183, 95% CI: 1.023–1.369, $P = 0.023$), *Gordonibacter* (OR = 1.072, 95% CI: 1.003–1.147, $P = 0.041$), *Lachnospiraceae UCG 008* (OR = 1.089, 95% CI: 1.006–1.180, $P = 0.036$), and *Turicibacter* (OR= 1.125, 95% CI: 1.020–1.240, $P = 0.018$) might increase the risks for uterine fibroids, while genera *Enterorhabdus* (OR = 0.853, 95% CI: 0.767–0.949, $P = 0.003$) and *Howardella* (OR = 0.941, 95% CI: 0.890–0.996, $P = 0.036$) reversed the adverse impact, as shown in Figure 7B and Supplementary Table 10.

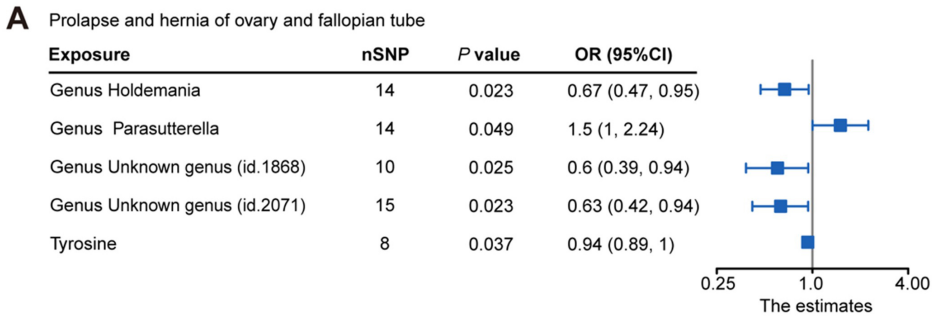

Figure 6. Forest plot of IVW estimates in prolapse and hernia of the ovary and fallopian tube. nSNP, number of single-nucleotide polymorphism; OR, odd ratio; 95% CI, 95% confidence interval; IVW, inverse-variance weighted.

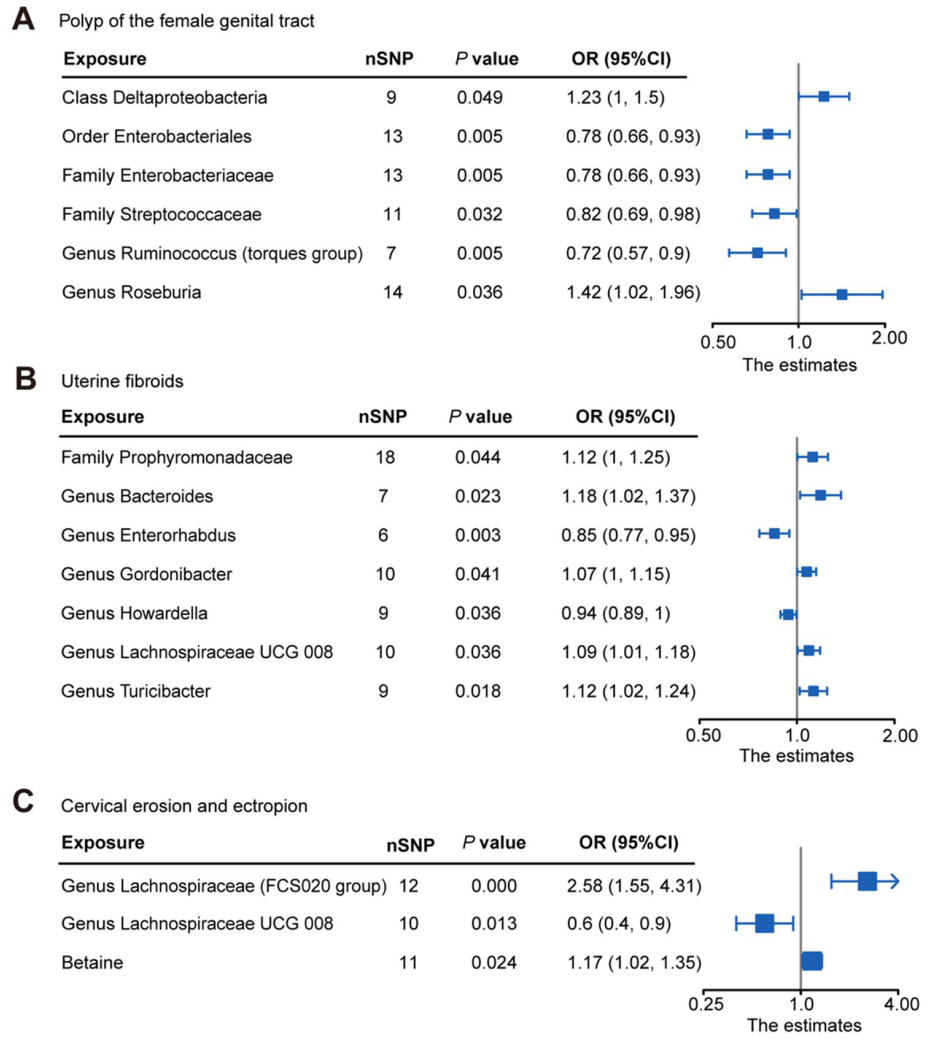

Figure 7. Forest plot of IVW estimates in proliferative lesions and cervical erosion and ectropion. (A) Polyp of the female genital tract. (B) Uterine fibroids. (C) Cervical erosion and ectropion. nSNP, number of single-nucleotide polymorphism; OR, odd ratio; 95% CI, 95% confidence interval; IVW, inverse-variance weighted.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect underlying heterogeneity or pleiotropy. MR-PRESSO was used to exclude outliers. Furthermore, the leave-one-out test was used to exclude any potentially influential SNPs. The causal effects remain significant after all sensitivity analyses had been

considered.

Cervical erosion or ectropion

Genera *Lachnospiraceae* (FCS020 group) and *Lachnospiraceae UCG 008*, as well as betaine derived from the gut, indicated a causal relationship between alterations of the abundance and erosion or ectropion of the cervix. The IVW estimate showed that both *Lachnospiraceae* (FCS020 group) and *Lachnospiraceae UCG 008* belong to class Clostridia that exhibited an opposite trend on the erosion or ectropion of the cervix. An increase in the frequency of *Lachnospiraceae* (FCS020 group) was considered a risk factor for the erosion or ectropion of the cervix (OR = 2.581, 95% CI: 1.545–4.313, $P < 0.001$), while *Lachnospiraceae UCG 008* indicated a beneficial effect (OR = 0.587, 95% CI: 0.399–0.897, $P = 0.013$). Additionally, betaine derived from the gut microbiota exhibited an adverse impact on the diseases (OR = 1.174, 95% CI: 1.022–1.348, $P = 0.024$), as shown in Figure 7C and Supplementary Table 11.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect any underlying heterogeneity or pleiotropy. MR-PRESSO was used to exclude outliers. Furthermore, the leave-one-out test was used to exclude any potentially influential SNPs. The causal effects remained significant after all sensitivity analyses were considered.

Endocrine disorders

This section discusses the relationship between gut microbiota and amenorrhea, oligomenorrhoea, irregular or excessive menstruation, PCOS, primary ovary failure, and precocious puberty. Gut microbiota belonging to three phyla (Bacteroidetes, Lentisphaerae, and Verrucomicrobia), three classes (Bacteroidia, Betaproteobacteria, and Clostridia), two orders (Bacteroidales and Enterobacteriales), four families (Enterobacteriaceae, FamilyXIII, Streptococcaceae, and Bacteroidales [S24.7 group]), and 24 genera (*Anaerotruncus*, *Bilophila*, *Butyricimonas*, *Clostridium sensustricto 1*, *Coprobacter*, *Dorea*, *Eggerthella*, *Escherichia/Shigella*, *Eubacterium* [hallii group], *Eubacterium* [ventriosum group], *Fusicatenibacter*, *Holdemania*, *Intestinimonas*, *Lachnospiraceae UCG 004*, *Prevotella 7*, *Roseburia*, *Ruminiclostridium 5*, *Ruminiclostridium 9*, *Ruminococcus* [gnavus group], *Ruminococcaceae UCG 004*, *Ruminococcaceae UCG 013*, *Unknown genus* [i.d. 2041], *Unknown genus* [i.d. 1000000073], and *Unknown genus* [i.d. 826]) and four metabolites (tryptophan, betaine, phenylalanine, and propionic acid) were considered to exhibit causality with at least one disease, as per the MR method.

The MR analysis demonstrated positive associations between the gut microbiota of FamilyXIII (OR = 1.091, 95% CI: 1.005–1.185, $P = 0.038$), genera *Coprobacter* (OR = 1.112, 95% CI: 1.031–1.198, $P = 0.006$), *Dorea* (OR = 1.137, 95% CI: 1.005–1.286, $P = 0.041$), and *Ruminiclostridium 5* (OR = 1.196, 95% CI: 1.068–1.341, $P = 0.002$) and excessive, frequent, or irregular menstruation. Furthermore, inverse associations for phylum Lentisphaerae (OR = 0.941, 95% CI: 0.890–0.996, $P = 0.038$), genera *Fusicatenibacter* (OR = 0.887, 95% CI: 0.810–0.972, $P = 0.010$), and *Ruminococcaceae UCG 013* (OR = 0.887, 95% CI: 0.772–0.996, $P = 0.043$) concerning abnormal menstruation were found, as shown in Figure 8A and Supplementary Table 12.

In the case of oligomenorrhea, we found evidence for negative relationships with phyla Bacteroidetes (OR = 2.206, 95% CI: 1.363–3.573, $P = 0.001$) and Verrucomicrobia (OR = 1.898, 95% CI: 1.096–3.287, $P = 0.022$), classes Bacteroidia (OR = 1.838, 95% CI: 1.125–3.002, $P = 0.015$) and Betaproteobacteria (OR = 1.830, 95% CI: 1.031–3.248, $P = 0.039$), order Bacteroidales (OR = 1.838, 95% CI: 1.125–3.002, $P = 0.015$), Fusicatenibacter (OR = 1.798, 95% CI: 1.138–2.842, $P = 0.012$), and Intestinimonas (OR = 1.700, 95% CI: 1.059–2.728, $P = 0.028$), while positive relationships with genera *Ruminococcaceae UCG 004* (OR = 0.627, 95% CI: 0.408–0.963, $P = 0.033$) and *Unknown genus* (i.d. 826) (OR = 0.590, 95% CI: 0.384–0.908, $P = 0.017$) were observed, as shown in Figure 8B and Supplementary Table 13.

In terms of PCOS, class Betaproteobacteria (OR = 1.852, 95% CI: 1.076–3.189, $P = 0.026$) and genus *Clostridium sensustricto 1* (OR = 1.727, 95% CI: 1.028–2.901, $P = 0.039$) might be responsible for the disease pathogenesis. In contrast, class Clostridia (OR = 0.611, 95% CI: 0.378–0.987, $P = 0.044$), genera *Bilophila* (OR = 0.478, 95% CI: 0.270–0.847, $P = 0.011$) and *Holdemania* (OR = 0.645, 95% CI: 0.459–0.907, $P = 0.012$), and tryptophan (OR = 0.673, 95% CI: 0.492–0.920, $P = 0.013$) might reduce disease likelihood, as shown in Figure 8C and Supplementary Table 14.

Genera *Eggerthella* (OR = 2.878, 95% CI: 1.086–7.622, $P = 0.033$), *Escherichia/Shigella* (OR = 9.004, 95% CI: 1.965–41.258, $P = 0.005$), *Lachnospiraceae UCG 004* (OR = 5.285, 95% CI: 1.189–23.494, $P = 0.029$), and *Unknown genus* (i.d. 2041) (OR = 3.322, 95% CI: 1.172–9.416, $P = 0.024$) and betaine (OR = 1.429, 95% CI: 1.003–2.038, $P = 0.048$) and propionic acid (OR = 2.727, 95% CI: 1.415–5.254, $P = 0.003$) appeared to increase the likelihood of precocious puberty. In contrast, family Bacteroidales (S24.7 group) (OR = 0.341, 95% CI: 0.124–0.942, $P = 0.038$), genus *Ruminococcaceae UCG 013* (OR = 0.154, 95% CI: 0.034–0.706, $P = 0.016$), and phenylalanine (OR = 0.593, 95% CI: 0.395–0.890, $P = 0.012$) might decrease the likelihood of precocious puberty, as shown in Figure 8D and

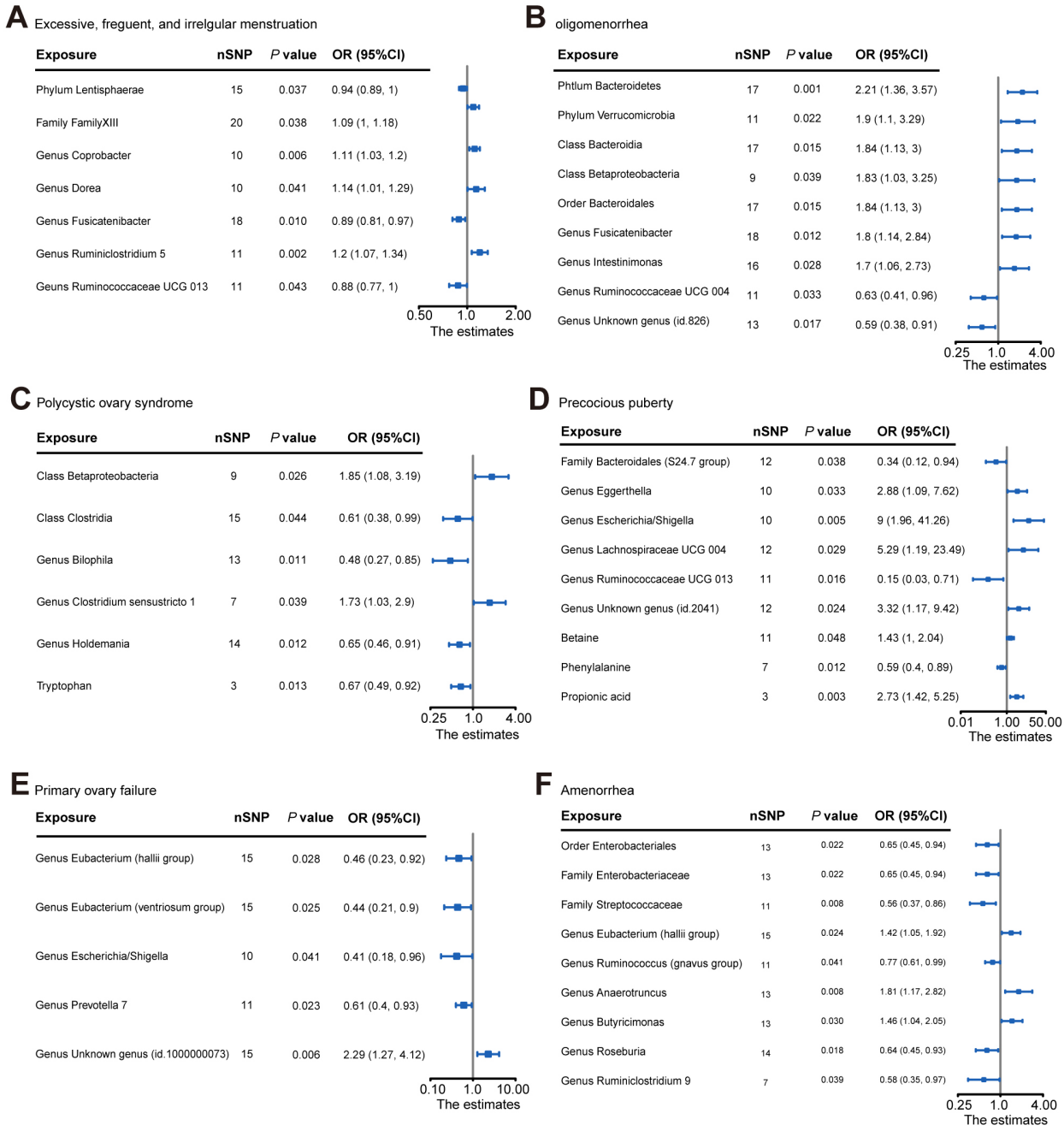

Figure 8. Forest plot of IVW estimates in endocrine disorders of the female reproductive system. (A) Excessive, frequent and irregular menstruation. (B) Oligomenorrhea. (C) Polycystic ovary syndrome. (D) Precocious puberty. (E) Primary ovary failure. (F) Amenorrhea. nSNP, number of single-nucleotide polymorphism; OR, odd ratio; 95% CI, 95% confidence interval; IVW, inverse-variance weighted.

Supplementary Table 15.

According to MR estimates, genus *Unknown Genus* (i.d. 1000000073) (OR = 2.285, 95% CI: 1.267–4.123, P = 0.006) increases the susceptibility of primary ovary failure. Genera *Eubacterium* (hallii group) (OR= 0.465, 95% CI: 0.235–0.921, P = 0.028), *Eubacterium* (ventriosum group) (OR = 0.436, 95% CI: 0.210–0.901, P = 0.025), *Escherichia/Shigella* (OR = 0.414, 95% CI: 0.178–0.964, P = 0.041), and *Prevotella 7* (OR = 0.609, 95% CI: 0.397–0.935, P = 0.023) decrease the likelihood of ovarian dysfunction, as shown in Figure 8E and Supplementary Table 16.

An increase in the abundance of order Enterobacteriales (OR = 0.651, 95% CI: 0.452–0.940, P = 0.022), genera *Eubacterium* (hallii group) (OR= 1.417, 95% CI: 1.046–1.919, P = 0.024), *Anaerotruncus* (OR = 1.813, 95% CI: 1.167–2.818, P = 0.008), and *Butyricimonas* (OR = 1.458, 95% CI: 1.037–2.051, P = 0.030) appeared

to increase the risk for developing amenorrhea, while inverse associations were observed for family Enterobacteriaceae (OR = 0.651, 95% CI: 0.452–0.940, *P* = 0.022) and Streptococcaceae (OR = 0.564, 95% CI: 0.369–0.861, *P* = 0.008), genera *Ruminococcus* (gnavus group) (OR = 0.775, 95% CI: 0.607–0.990, *P* = 0.041), *Roseburia* (OR = 0.644, 95% CI: 0.447–0.928, *P* = 0.018), and *Ruminiclostridium 9* (OR = 0.582, 95% CI: 0.348–0.973, *P* = 0.039), as shown in Figure 8F and Supplementary Table 17.

Cochran's IVW Q statistics and the MR-Egger intercept test were used to detect any underlying heterogeneity or pleiotropy. MR-PRESSO was used to exclude outliers. Furthermore, the leave-one-out test was used to exclude any potentially influential SNPs. The causal effects remain significant after all sensitivity analyses were considered.

Interestingly, specific effects of the gut microbiota on metabolic disorders in the female reproductive system appear to be related to specific endocrine mechanisms. Clinical manifestations of endocrine hyper- or hypofunction can be used to predict the fundamental effects of the same genus of the gut microbiota on different diseases.

DISCUSSION

The TSMR analysis results showed a causal relationship between different classification levels, including phylum, class, order, family, and genus of the gut microbiota, and benign diseases of the female reproductive system, including dysplasia, inflammatory diseases, proliferative polyps, hormone-dependent diseases, and uterine fibroids. These findings demonstrate novel biomarkers for possible screening tools and therapeutic targets for these diseases. Meaningful positive results were obtained at each classification level.

It should be highlighted that according to our results, more than half of the genera of gut microbiota of interest are causally involved in two or more benign diseases of the female reproductive system, suggesting that there is a greater possibility of early diagnosis or targeted treatment of diseases through these genera in the future. The results showed that the majority of disease-associated genera of gut microbiota belonged to phylum Firmicutes; however, a causal relationship to the diseases of the female reproductive system could not be established, based on the MR analysis on the level of phylum. The contradiction in these results could be because diverse genera belonging to phylum Firmicutes exerted different effects on the outcome of several diseases, which were subsequently counteracted. Similarly, previous MR studies had shown a strong causal relationship between more than 40 species of gut microbiota and adverse pregnancy outcomes, particularly pathological pregnancy and pregnancy complications such as eclampsia, gestational diabetes, spontaneous abortion, fetal appendage abnormalities, and postpartum hemorrhage.[18–20] Korpela *et al.*[21] conducted a well-characterized and longitudinally monitored cohort and reported that gut microbiota begins to shift toward adult-like composition during puberty, leading to an increased relative abundance of *Clostridia* and a lower abundance of *Bacteroidia*. Hence, the close relationship between the gut microbiota and the physiological or pathological changes in the female reproductive system at different stages should be given more attention.

According to the current knowledge, gut microbiota could be an important novel predictive biomarker for the prevention and treatment of both endometriosis and adenomyosis. Alterations in the abundance of the flora and gut microbiota-derived metabolites play a pivotal role in disease progression.[22,23] Yuan *et al.*[24] and Shan *et al.*[25] investigated a higher *Firmicutes*/*Bacteroidetes* ratio in women with endometriosis than that in controls through mouse models and clinical studies. Ata *et al.*[26] revealed a higher frequency of *Shigella*/*Escherichia* colonization in the colon of women with endometriosis than that in normal controls. Alterations in *Ruminococcus*, *Pseudomonas*, *Bacilli*, *Bacteroidia*, *Clostridia*, *Coriobacteriia*, and *Gammaproteobacteria* in women with endometriosis have been reported.[27,28]

In the case of PCOS, it has been reported that gut microbiota dysbiosis is strongly related to the pathogenic mechanisms of the progression of PCOS, which further indicates potential targets for its prevention, diagnosis, and treatment.[29,30] Various animal models have shown that the decrease in the number of gut microbiota belonging to *Bacteroides*, *Lactobacillus*, *Ruminococcus*, and *Clostridium*, while the increase in *Firmicus*, *Proteus*, and *Prevotella* is related to the pathogenesis of PCOS.[31,32] Existing multi-omics analyses identified 64 bacterial strains that were considered to change in women with PCOS in contrast to that in normal individuals.[33] The protective effect of *Lactobacillus*, *Bifidobacteria*, *Fusicatenibacter,* and *Clostridium* is also discussed in case-control studies.[34–36]

Very few studies have discussed the potential association of the diseases of the female reproductive system with gut microbiota. Using animal models, Bo *et al.*[37] investigated the relationship between a high-fat diet (HFD) and precocious puberty, and found it to be mediated by alterations in gut microbiota, including those belonging to *Desulfovibrio*, *Lachnoclostridium*, *Streptococcus*, *Anaerotruncus*, and *Bifidobacterium*. It has been reported that supplementing the HFD with short-chain fatty acids (SCFAs) derived from the gut microbiota, including acetate, propionate, butyrate, or their mixture, could significantly

reverse the precocious puberty in rats.[38] In addition, butyrate derived from the gut demonstrated a protective effect on endometritis induced by *Escherichia coli* and *Staphylococcus aureus*.[39,40] Elevated levels of *Ruminococcus*, *Roseburia*, *Coprococcus*, and *Clostridium* have been reported in women with idiopathic central precocious puberty.[41] A retrospective cross-sectional study on 651 Chinese women showed that the gut microbiota, particularly *Prevotella* and *Bacteroides*, were strongly related to abnormal levels of blood trace elements in women with infertility.[42] Similarly, a prospective study in 2020 showed a potent relationship between *Prevotella* and endometrial polyps in women with infertility.[43] Mao *et al.*[7] reported significant alterations in multiple bacterial phyla in the gut of women with uterine fibroids, including *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*. An increase in *Pseudomonas* and *Prevotella* and a decrease in *Bifidobacteria*, *Lactococcus*, and *Ligilactobacillus* were observed.

The exact findings vary based on the existing clinical observational studies. Some studies reported that results based on TSMR analysis lack sufficient support from existing evidence derived from observational studies, which could be attributed to the small number of participants, the limited size of the stool sample, and bias derived from population stratification. Further research is needed to correct the discrepancy between our findings and those from observational studies. Based on the available evidence, how alterations in gut microbiota established a causal relationship with the benign diseases of the female reproductive system can be summarized in three ways, where bacterial metabolites are possible crucial regulators that cannot be ignored:(i) disturbance in the balance of sex hormones;(ii) metabolic disorders characterized by hyperinsulinemia or insulin resistance (IR); and (iii) immune regulation and systemic chronic inflammation.

First, an imbalance between estrogen and androgen, or the dysregulation at any level of the hypothalamic-pituitary-gonadal (HPG) axis, plays an essential role in the pathogenesis of hormone-dependent diseases of the female reproductive system. A "vicious circle"-like relationship between the abnormal levels of estrogen or androgen and the alterations in gut microbiota has been reported in the pathogenesis of several diseases.[1,7] Proper levels of estrogen and androgen in the gut are responsible for maintaining the composition of the gut microbiota.[44,45] Some genera of the gut microbiota participate in the metabolism of sex hormones. For instance, β-glucuronidase and β-glucosidase derived from the gut increase the reabsorption of free estrogen, thereby improving estrogen bioavailability.[46,47]

Betaproteobacterium is related to androgen catabolic processes *via* the inhibition of enterohepatic circulation.[48] As the gut microbiota could be regarded as a potent target in alternative therapies for imbalanced states of estrogen or androgen, the underlying mechanisms and therapeutic effects should be further explored.

IR, or hyperinsulinemia, is a predisposing factor for diminished ovarian reserve and is considered a common endocrine characteristic in individuals with PCOS, especially in the obese group.[49,50] Current data emphasize that gut microbiota disorder and subsequent changes in SCFAs are key regulators in the pathogenesis of IR.[51] Animal models showed that antibiotic cocktails improved insulin sensitivity and hyperinsulinemia in women with PCOS, while gut microbiota transplantation aggravated lipid metabolic disorders and IR.[52] Similarly, probiotic supplementation showed a positive relationship with improving homeostatic model assessment of IR (HOMA-IR) levels in women with PCOS.[53–55] Therefore, it seems feasible to improve metabolic disorders of women with PCOS by targeting their gut microbiota. Microbiome transplantation or a reduction in the pathogen load seems to be an alternative approach.

Third, elevated translocation of lipopolysaccharides (LPS) to the circulation due to the disorder in gut microbiota is highly responsible for the imbalance between anti-inflammatory and pro-inflammatory responses.[56] The accumulation of LPS, activation of inflammatory cytokines, and infiltration of pro-inflammatory immune cells such as macrophages promote the development of endometriosis.[57,58] Qi *et al.*[59] revealed the potential role of the gut microbiota-bile acid-interleukin-22 (IL-22) axis in the disruption of ovarian functions and related metabolic disorders, including insulin resistance. Altering the gut microbiota, improving bile acid metabolic health, and increasing circulating IL-22 are of great significance for the amelioration of PCOS. However, it has not been established whether chronic low-grade inflammation present in individuals with PCOS is caused by an obese state, this intrinsic facet of PCOS is still unclear.[60] Therefore, future research should focus on the underlying mechanisms involving systemic immunity and local inflammation to understand how gut microbes and their metabolites affect disease progression. Polyps and other proliferative lesions of the female genital tract are more likely to be associated with the local inflammatory status. Thus, the protective effect of the vaginal microbiome should be highlighted.[61–63]

As the development of the microbial populations involves both genetic predisposition and the acquired lifestyle, the gut microbiota is considered a modifiable risk factor, particularly during the first few years of life. Thus, the stability of the gut microbiota should be established along with the maturation of each system of the human body.[64,65] The composition and colonization of

gut microbiota in childhood, adolescence, and adulthood are strongly related to lifestyles, antibiotic application, and environmental conditions.[66–69] In other words, more attention should be paid to the potential causal effects of the specific genera of gut microbiota on the development or pathogenesis of the female reproductive system, shedding light on better detection, prevention, and treatment of these diseases, further providing a promising management paradigm.

Compared to previous studies, key strengths of our study include sufficient data and study sample from GWAS, rigorous screening of IVs for the TSMR design, and meticulous analysis of causal outcomes to exclude possible violations. MR studies that satisfy the above-mentioned basic assumptions can effectively avoid confounding biases and mimic RCT in an observational setting. Moreover, we considered a diverse classification hierarchy including phylum, class, order, family, and genus of the gut microbiota, which further confirms the significance of several genera of the flora for the prevention, management, and treatment of associated diseases of the female reproductive system.

The current TSMR analysis has several limitations too. Firstly, all GWAS data were derived from European populations. Hence, the present findings cannot be directly extended to all populations. In addition, because of population stratification, it is not clear whether the causal association described herein is consistent with other populations. Secondly, we should focus on the complexity and the variety of the pathological phases of the diseases of the female reproductive system. Hence, broader studies for subgroups of the diseases need to be performed, such as various types of endometrioses, PCOS, and different stages of tumors of the female reproductive system. Third, there were contradictions or limitations among previous observational studies, leading to a lack of support for the TSMR findings. Thus, large-scale independent studies are necessary to rule out any coincidental findings.

CONCLUSION

This study assessed the causal crosstalk between gut microbiota and benign diseases of the female reproductive system *via* TSMR analysis. Based on the selection for genetic instruments, alterations in gut microbiota, including both the composition and metabolites, on the levels of phylum, class, order, family, and genus, positive or negative effects on the pathogenesis of the diseases of the female reproductive system were observed, which further affected female fertility. An in-depth understanding of the roles of alterations in the gut microbiota on these diseases will provide new insights into the pathological mechanisms, promising preventive management, and related targeted therapies for those diseases. Additionally, according to existing data, the role of several genera of the gut microbiota on the development of the female reproductive system has been reported, while the underlying mechanisms are still unclear, which are possibly related to the integrity of the intestinal barrier and the crosstalk among various systems. Hence, future studies should focus on mechanisms and signaling pathways that lead to various diseases of the female reproductive system.

DECLARATION

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Author contributions
Dai F and Sagnelli M conceived and designed the study. Dai F and Sagnelli M contributed to the data management and analysis. Dai F wrote the paper. Sagnelli M have accessed and verified the data. The authors both had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Ethical approval
Our investigation utilized GWAS summary statistics that are available to the public. No novel data were gathered, and thus, there was no need for additional ethical approval.

Informed consent
Not applicable.

Conflict of interest
The authors confirm that there are no conflicts of interest.

Use of large language models, AI and machine learning tools
No artificial intelligence (AI) tools or large language models (LLMs) were used in the design, conduct, analysis, or writing of this study.

Data availability statement
The data that support the findings of this study are openly available in the MiBioGen consortium at https://mibiogen.gcc.rug.nl/andtheFinnGendatabase at https://r7.finngen.fi/.

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