

Gut Microbiota, Circulating Metabolites and Risk of Endometriosis:
A Two-Step Mendelian Randomization Study

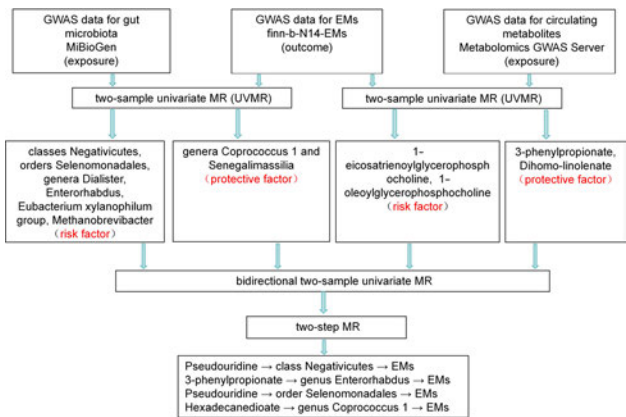
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Abstract

Epidemiological studies and animal models have suggested a possible link between gut microbiota (GM), circulating metabolites, and endometriosis (EMs) pathogenesis. However, whether these associations are causal or merely due to confounding factors remains unclear. We conducted a two-sample and two-step Mendelian randomization (MR) study to elucidate the potential causal relationship between GM and EMs, and the mediating role of circulating metabolites. Our MR analysis revealed that higher abundances of class Negativicutes, and order Selenomonadales, as well as genera *Dialister*, *Enterorhabdus*, *Eubacterium xylanophilum* group, *Methanobrevibacter* were associated with an increased risk of EMs (Odds Ratio (OR) range: 1.0019–1.0037). Conversely, higher abundances of genera *Coprococcus* 1 and *Senegalimassilia* were linked to reduced risk of EMs (OR range: 0.9964–0.9967). Additionally, elevated levels of circulating metabolites such as 1-eicosatrienoylglycerophosphocholine and 1-oleoylglycerophosphocholine were found to be associated with heightened risk of EMs (OR range: 2.21–3.16), while higher concentrations of 3-phenylpropionate and dihomolimonate were protective (OR range: 0.285–0.535). Two-step MR analysis indicated that specific microbial taxa, notably genus *Enterorhabdus* and order Selenomonadales, might function



as mediators linking circulating metabolites to the risk of EMs. Our findings suggest a probable causal relationship between GM, circulating metabolites, and EMs, indicating that GM may mediate the influence of circulating metabolites on the pathophysiology of EMs. These results offer new leads for future mechanistic studies and could inform clinical translational research.

Key words: gut microbiota, circulating metabolites, mendelian randomization, endometriosis, causal relationship, genome-wide association studies

Introduction

Endometriosis (EMs) is characterized by the abnormal presence of endometrial tissue outside the uterus, which can lead to chronic pain, subfertility, and the formation of nodules or masses due to recurrent bleeding and inflammatory responses. This condition affects approximately 10% of women of reproductive age, equating to an estimated 196 million women worldwide (Allaire et al. 2023; Frayne et al.2023; Garcia Garcia et al. 2023). Given its estrogen-dependent nature and the lack of curative treatment, current medical inter-

ventions are primarily symptomatic, with both hormonal therapies and surgical excision failing to prevent recurrences. Consequently, affected women may endure physical and mental health challenges until menopause, contributing to substantial social and economic burdens (Della et al.2020).

The definitive diagnosis of EMs requires surgical intervention with histological confirmation, as there is no non-invasive diagnostic method available despite ongoing research into various biomarkers in preclinical studies (Ahn et al. 2017; Bjorkman and Taylor 2019; Anastasiu et al.2020). The identification of EMs is often

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challenging, leading to delayed diagnoses. Despite extensive research efforts over the past several decades, the pathogenesis and etiology of EMs remain unclear (Czyzyk et al. 2017; Asghari et al. 2018; Shafir et al. 2018). While the theory of retrograde menstruation has gained widespread acceptance (Guo et al. 2023), it still needs to explain the biological processes and characteristics of EMs fully. Alternative hypotheses have been proposed to supplement and refine the retrograde menstruation theory, including the concepts of abnormal remnants of embryonic Müllerian ducts (Lambrinoudaki et al. 2009), coelomic metaplasia (Vercellini et al. 2014), metastatic spread via vascular or lymphatic vessels (Jerman and Hey-Cunningham 2015), and determinism of the eutopic endometrium (Maia et al. 2012). However, none of these theories have conclusively elucidated the relationship with EMs. It is generally accepted that EMs are likely the result of multiple determinant factors involving various genetic and epigenetic modifications, hormonal influences, environmental factors, and immunological aspects (Laganà et al., 2019).

The gut microbiota (GM) naturally coexists with the host. It plays a pivotal role in physiological processes, such as nutrient absorption, metabolism, detoxification, maintaining the integrity of the intestinal mucous barrier, and regulating the immune and endocrine systems (Thaiss et al. 2016; Shi et al. 2017; Régnier et al. 2021; Wiertsema et al. 2021). These microbial communities are essential for maintaining host health. Dysbiosis of the GM, manifesting as alterations in composition and abundance, can compromise the mucosal barrier, facilitate bacterial and endotoxin translocation (Ha et al. 2020), trigger inflammation (Clemente et al. 2018), disrupt the immune environment (Hou et al. 2022), and alter the metabolome (Barko et al. 2018). Intestinal dysbiosis can affect not only the local gastrointestinal tract but also elicit systemic responses and has been associated with various diseases, including multiple sclerosis (Preiningerova et al. 2022), type-II diabetes (Del et al. 2022), Grave's disease (Jiang et al. 2022), systemic lupus erythematosus (Zhang et al. 2021), reproductive disorders (Azpiroz et al. 2021), cancers (Tong et al. 2021; Zhao et al. 2021; Chen et al. 2022; Park et al. 2022), among others. Certain bacteria within the GM possess genes encoding estrogen-metabolizing enzymes, which may regulate circulating estrogen levels (Lephart and Naftolin 2022). Given the direct link between estrogen and the onset and progression of EMs, it is plausible that GM could contribute to the development of EMs.

Evidence from animal models and human samples increasingly suggests a correlation between the abundance of GM and the risk of EMs (Bailey and Coe 2002; Ata et al. 2019; Chadchan et al. 2019; Ni et al. 2020; Shan et al. 2021; Svensson et al. 2021). Concurrently, growing evidence points to potential links between metabolic

disorders and the occurrence of EMs. With the etiological and risk factors for EMs still largely unknown, the direct impact and pathogenic mechanisms of GM and circulating metabolites on EMs remain elusive. The relationships between them have not been adequately addressed due to the limitations inherent in conventional observational studies, which are susceptible to confounding biases or reverse causation.

Mendelian randomization (MR) is a statistical approach designed to overcome the limitations of observational studies by minimizing bias risks due to residual confounding or reverse causation (Sekula et al. 2016; Bowden and Holmes 2019; Birney 2022). It employs germline single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to estimate causal effects between exposures and outcomes. To elucidate the association and pathogenic mechanisms between GM, circulating metabolites, and EMs, I employed a two-sample and two-step MR analysis in this study.

Experimental

Materials and Methods

Genome-wide association study (GWAS) statistics of EMs. This study utilized the most comprehensive summary of GWAS data available: finn-b-N14-EMs (Liu et al. 2024), encompassing 16,377,306 SNPs. The cohort included 8,288 individuals with EMs and 68,969 non-gender-specific healthy controls, originating from the European population. Patient consent was obtained in the respective studies, and the present report adhered to the guidelines of the Strengthening the Reporting of Observational Studies in Epidemiology.

GWAS statistics of gut microbiota. Data on GM abundance was retrieved from the IEU Open GWAS (MR Base) public database (<https://gwas.mrcieu.ac.uk>) (Hemani et al. 2018). The international GWAS dataset MiBioGen (Wang et al. 2018), consisted of a sample size of 14,306 individuals from the European population. Gut microbiota abundance was profiled using 16S ribosomal RNA gene sequencing, comprising 197 taxa (120 genera, 33 families, 19 orders, 16 classes, and 9 phyla), with 14 unknown taxa (11 genera, 3 families) excluded.

GWAS data for human serum metabolites. The GWAS data for 486 metabolites were sourced from the Metabolomics GWAS Server (<http://metabolomics.helmholtz-muenchen.de/gwas>), published by Shin et al. (2014), covering 7,824 adult individuals from European cohorts. A total of 309 known metabolites were assigned to eight subgroups (lipids, cofactors and vitamins, amino acids, peptides, carbohydrates, energy, nucleotides, and xenobiotic metabolism), while an additional 177 unknown metabolites were omitted.

Instrumental variables (IVs) selection. The selection of IVs should adhere to the key assumptions of MR: 1) IVs must be associated with exposures, 2) IVs must not be associated with confounders, and 3) IVs must affect outcomes exclusively through exposures, not via alternative pathways. To ensure the robustness and veracity of association inferences linking, circulating metabolites, and the risk of EMs, stringent quality control measures were employed for optimal IVs extraction. Initially, single nucleotide polymorphisms (SNPs) meeting the statistical significance threshold ($p < 5 \times 10^{-8}$) closely associated with GM and circulating metabolites were extracted. However, this resulted in the inclusion of only a limited number of gut bacterial taxa and circulating metabolites. To explore more extensive relationships, a less stringent p -value threshold ($p < 5 \times 10^{-6}$) was applied. The minor allele frequency (MAF) threshold for variants of interest was set at 0.01. Key MR analysis rules eliminated linkage disequilibrium (LD) effects among IVs, as strong LD could introduce bias. During the current study, clumping was performed ($R^2 < 0.001$ and clumping distance = 10,000 kb) to assess LD among chosen SNPs. It was also ensured that SNP effects on exposure corresponded to the same allele effect on the outcome, avoided palindromic SNPs to prevent ambiguity in strand directionality or allele coding, and calculated F statistics to avoid weak IVs bias (Burgess et al. 2011). The formula used was $F = [R^2 \times (N - 1 - K)] / [(1 - R^2) \times K]$, where SNPs with F -values below 10 were discarded in subsequent MR analyses. Alleles were harmonized with the human genome reference sequence, and any ambiguous or duplicate SNPs were removed. Phenotype-genotype associations during the website PhenoScanner (<https://www.repository.cam.ac.uk/handle/1810/293487>) (Staley et al. 2016) for each SNP were checked, and those SNPs related to potential confounding factors of EMs were removed.

Horizontal pleiotropy was tested using MR-PRESSO (Verbanck et al. 2018) and MR-Egger regression (Burgess and Thompson 2017; Sang et al. 2022; Zeng et al. 2023). The MR-PRESSO outlier test calculated a p -value indicating pleiotropy significance for individual SNPs, whereas the global test assessed overall horizontal pleiotropy. SNPs were ranked by ascending order of their outlier test p -values and then iteratively removed. After each removal, the global test was rerun on the remaining SNPs. This process was repeated until the global test p -value exceeded 0.05. SNPs that remained after removing those with pleiotropic effects were used in subsequent MR analyses.

Mendelian randomization analysis. Two-sample univariate Mendelian randomization (UVMR) was employed to infer causal relationships between GM, circulating metabolites, and EMs. Additionally, two-step mediation Mendelian randomization (TSMR) was used

to decompose the indirect effects of GM or circulating metabolites on EMs, assuming no interaction between exposure and mediator. Firstly, the effect of exposure on outcome (β_0) was estimated via UVMR. Secondly, the effect of exposure on mediator (β_1) was determined using UVMR. Thirdly, the effect of mediators significantly associated with exposure on the outcome was assessed using UVMR. Finally, mediators significantly related to the outcome were screened and multivariable Mendelian randomization (MVMR) was conducted to evaluate the mediator's effect on the outcome after genetic effect adjustment (β_2). The proportion of mediation in the association between exposure and outcome was computed as $(\beta_1 \times \beta_2) / \beta_0$ (Sanderson 2021).

Three mainstream Mendelian randomization methods were used for analyses involving multiple instrumental variables (IVs): the inverse-variance weighted (IVW) test, the weighted median test, and the MR-Egger test (Cao et al. 2022; Zhao et al. 2022). The IVW method is often more potent under certain conditions; therefore, results with multiple IVs primarily rely on IVW, with the other methods serving as complementary. The Wald ratio (Gnona and Stewart 2022) test was used for analyses with only one IV.

Several sensitivity analyses were conducted to assess the robustness of the association. A leave-one-out analysis (Cheng et al. 2017) evaluated whether the association was driven by a single SNP. The causal direction test compared the variance caused by IVs in both exposure and outcome, considering the identified causality directionally robust if IVs caused greater variance in exposure than in outcome.

Horizontal pleiotropy was tested using Egger regression, with IVs affecting the outcome through pathways other than exposure considered pleiotropic and violating MR assumptions. Cochran's Q statistics and the two-sample MR package were used for heterogeneity testing. A Q value higher than the number of IVs minus one or a p -value < 0.05 indicated heterogeneity and invalid IVs.

All statistical analyses were performed using R software version 3.5.3 (R Core Team 2019), with MR analyses conducted using the "MendelianRandomization", "TwosimpleMR", "MVMR", and "MR-PRESSO" packages. The Benjamini-Hochberg (B-H) corrected p -value was used to control the false discovery rate (FDR) in multiple testing (Jung et al. 2023), with $p < 0.05$ deemed statistically significant. A flowchart of the current MR analysis is displayed in Fig. 1.

Results

SNP Selection. The analysis was initiated by extracting 1 to 11 SNPs per GM taxon and 1 to 126 SNPs per circulating metabolite, adhering to a stringent

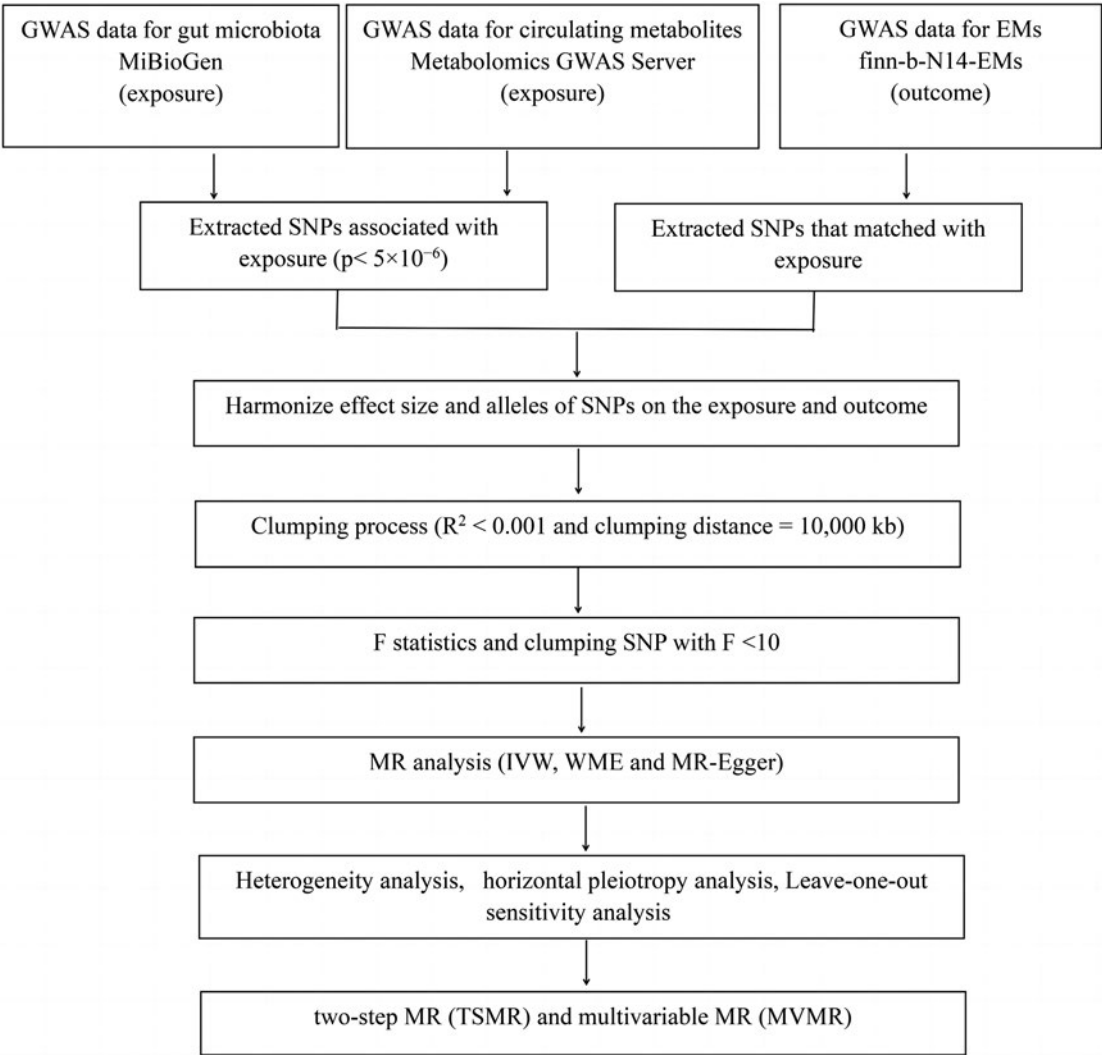


Fig. 1. The detailed flowchart of present mendelian randomization analysis.

significance threshold of $p < 5 \times 10^{-6}$ after a comprehensive series of quality control procedures. The distribution of SNPs across each taxon is meticulously documented in Tables SI and SII. Subsequently, upon the removal of pleiotropic SNPs as identified through MR-PRESSO outlier tests and MR-Egger regression, an absence of horizontal pleiotropy among the instrumental variables (IVs) was observed, confirmed by both the MR-PRESSO global test ($p > 0.05$) and MR-Egger regression ($p > 0.05$).

Association of GM on the risk of EMs. Within the set of instrumental variables (IVs) ($p < 5 \times 10^{-6}$) and utilizing GWAS data with finn-b-N14-EMs as the outcome, it was discovered that an increased abundance of the class Negativicutes was significantly associated with a heightened risk of endometriosis (EMs) (OR = 1.00252418, $p = 0.01863$ by IVW test). This finding was corroborated by concordant results from MR Egger and Weighted median tests, which indicated no horizontal pleiotropy ($p = 0.359$) or heterogeneity ($p = 0.4014$) across the SNPs. The leave-one-out analy-

sis further revealed that the association was not driven by any single SNP, suggesting a potential relationship between the class Negativicutes and EMs. Similarly, it was found that increased abundances of the genus *Dialister*, genus *Enterorhabdus*, genus *Eubacterium xylanophilum* group, genus *Methanobrevibacter*, and order Selenomonadales were associated with an augmented risk of EMs (OR = 1.00372939, 1.002017031, 1.003390736, 1.001949899, and 1.00252418 respectively). Conversely, an increased abundance of the genus *Coproccoccus 1* was linked to a decreased risk of EMs (OR = 0.99671419, $p = 0.001354$ by IVW test), with homogeneous results from MR Egger and Weighted median tests, showing no horizontal polymorphism ($p = 0.657$) or heterogeneity ($p = 0.5713$) between SNPs. The leave-one-out analysis indicated that the association was not influenced by any singular SNP, suggesting a potential relationship between the genus *Coproccoccus 1* and EMs. Additionally, it was found that a higher abundance of the genus *Senegalimassilia* was associated with a reduced risk of EMs (OR = 0.996418429,

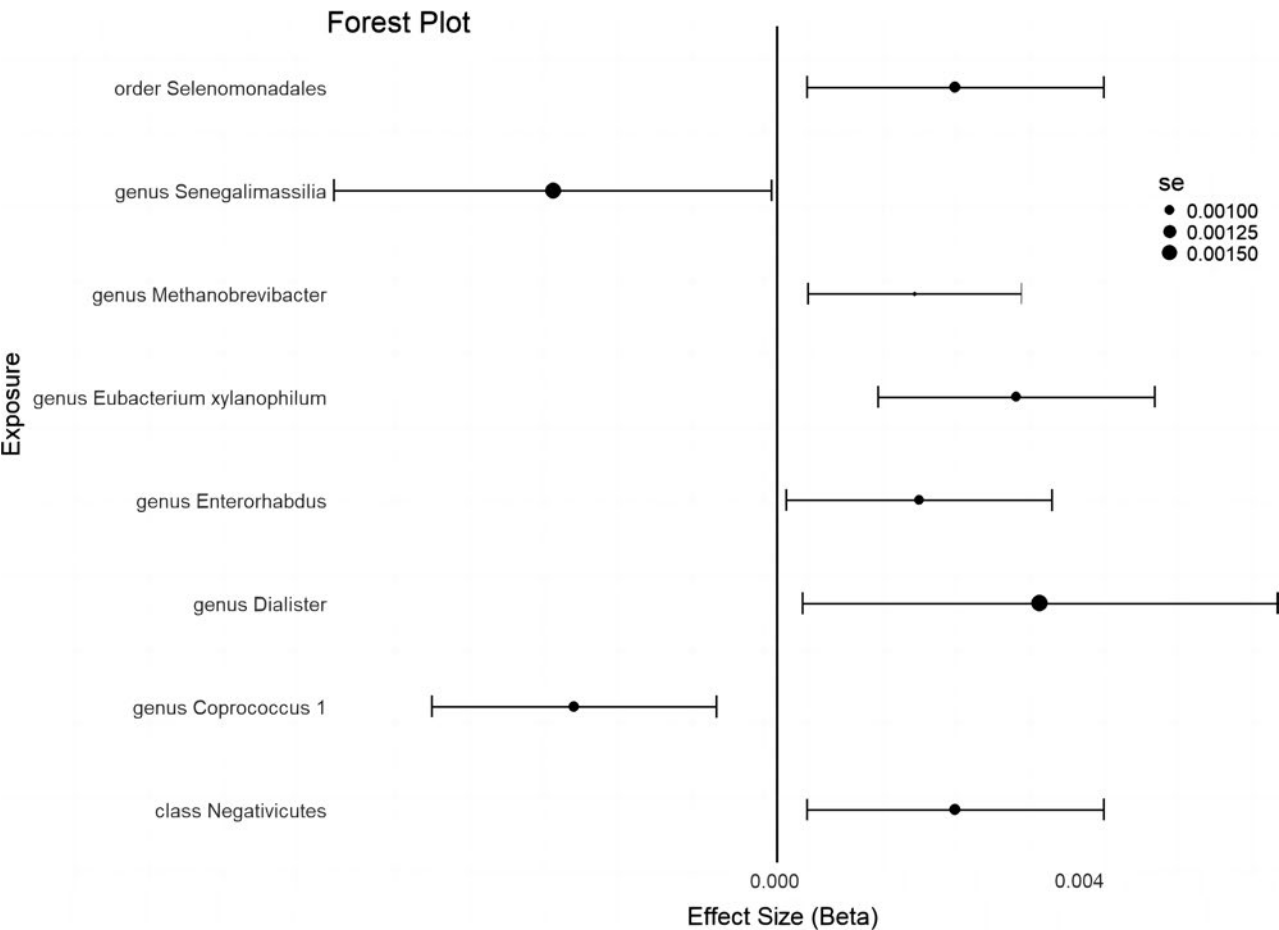


Fig. 2. The forest plot summarized the causality of gut microbiota on the risk of endometriosis.

$p=0.02319$ by IVW test), insufficient SNPs ($n=2$) precluded MR Egger and Weighted median tests. These findings are summarized in Fig. 2 and Table I. Following multiple testing corrections, the false discovery rate (FDR) for all causality assessments exceeded 0.05 (Table SI), indicating that these relationships should be interpreted cautiously.

Association of circulating metabolites on the risk of EMs. Within the defined set of instrumental variables (IVs) ($p < 5 \times 10^{-6}$) and employing GWAS data with finn-b-N14-EMs as the outcome, it was identified that elevated levels of 1-eicosatrienoylglycerophosphocholine were significantly associated with an increased risk of EMs ($OR=2.213998153$, $p=0.02416$ by IVW test). This association was consistently observed across MR Egger and Weighted median tests, revealing potential horizontal pleiotropy ($p=0.0253$) yet no heterogeneity ($p=0.593$) among the SNPs. The leave-one-out analysis further corroborated that the relationship was not driven by any singular SNP, suggesting a potentially robust link between 1-eicosatrienoylglycerophosphocholine and EMs. Additionally, it was discovered that higher concentrations of 1-oleoylglycerophosphocholine, 2-aminooctanoic acid, decanoylcarnitine, hexadecanedioate, hexanoylcarnitine, octanoyl-

carnitine, and threonine were also associated with an increased risk of EMs ($OR=3.15819291$, 1.421339874 , 1.591377511 , 1.422904208 , 1.611555723 , 1.584707741 , and 2.546704066 , respectively). Conversely, higher levels of 3-phenylpropionate were found to be protective against EMs ($OR=0.535154387$, $p=0.009951$ by IVW test), a finding that was uniformly supported by MR Egger and Weighted median tests, with no indication of horizontal polymorphism ($p=0.669$) or heterogeneity ($p=0.6729$) between SNPs. The leave-one-out analysis confirmed that this association was not influenced by any single SNP, suggesting a potentially meaningful relationship between 3-phenylpropionate and a reduced risk of EMs. Furthermore, it was noted that elevated levels of dihomolinolenate, dimethylarginine (SDMA + ADMA), ergothioneine, palmitate (16:0), pseudouridine, and stearate (18:0) were associated with a decreased risk of EMs ($OR=0.492825561$, 0.285075848 , 0.323033256 , 0.323033256 , 0.269280956 , and 0.477782343 , respectively). These results are summarized in Fig. 3 and Table II. After applying multiple testing corrections, the false discovery rate (FDR) for all causality assessments remained above 0.05 (Table SII), indicating that these findings should be interpreted with caution.

Table I
The causality of gut microbiota abundance on risk of endometriosis.

Exposure	n SNP	IVW			MR Egger			Weighted median			Horizontal pleiotropy			Heterogeneity	
		β	se	p-value	β	se	p-value	β	se	p-value	ERI	se	p-value	Q	p-value
class Negativicutes	7	0.002521	0.001072	0.01863	0.005405	0.003049	0.1364	0.002102	0.001445	0.1458	-0.00022	0.00022	0.359	6.198	0.4014
genus <i>Dialister</i>	2	0.003723	0.001718	0.03027	-	-	-	-	-	-	-	-	-	0.7413	0.3893
genus <i>Enterorhabdus</i>	3	0.002015	0.0009595	0.03575	0.0005135	0.002386	0.8651	0.001771	0.001182	0.134	0.00026	0.00038	0.617	0.7286	0.6947
genus <i>Eubacterium xylanophilum</i> group	7	0.003385	0.000999	0.0007027	-0.001805	-0.001805	0.5773	0.003223	0.001374	0.01897	0.00044	0.00025	0.131	6.42	0.3778
genus <i>Methanobrevibacter</i>	3	0.001948	0.0007719	0.0007719	0.002244	0.003202	0.6108	0.001635	0.001025	0.1107	-0.00005	0.00051	0.938	1.767	0.4134
order Selenomonadales	7	0.002521	0.001072	0.01863	0.005405	0.003049	0.1364	0.1364	0.001445	0.1458	-0.00022	0.00022	0.359	6.198	0.4014
genus <i>Coproccus</i> 1	7	-0.003294	0.001028	0.001354	-0.001902	0.003123	0.5692	-0.002869	0.001362	0.03517	-0.00011	0.00022	0.657	4.788	0.5713
genus <i>Senegalimassilia</i>	2	-0.003588	0.00158	0.02319	-	-	-	-	-	-	-	-	-	1.34	0.2471

SNP – single nucleotide polymorphisms, IVW – inverse variance weighted, MR – mendelian randomization, se – standard error, ERI – egger regression intercept

Table II
The causality of circulating metabolites on risk of endometriosis.

Exposure	Subset	IVW/Wald ratio			MR Egger			Weighted median			Horizontal pleiotropy			Heterogeneity	
		β	SE	p-value	β	SE	p-value	β	SE	p-value	ERI	SE	p-value	Q	p-value
1-Eicosatrienoylglycero-phosphocholine	lipid	0.7316	0.001575	0.02416	2.538	0.7409	0.006486	0.005782	0.002841	0.04843	-0.048	0.018	0.0253	8.367	0.593
1-Oleoylglycero-phosphocholine	lipid	1.15	0.4893	0.01871	-1.011	1.023	0.3685	1.157	0.652	0.07597	0.043	0.018	0.0658	1.725	0.8858
2-Aminooctanoic acid	amino acid	0.3516	0.1602	0.02819	0.2772	0.262	0.3716	0.3716	0.1729	0.06623	0.004	0.011	0.72	10.64	0.301
3-Phenylpropionate	amino acid	-0.6252	0.2425	0.009951	-0.4805	0.3796	0.3331	-0.5788	0.2893	0.04539	-0.0095	0.019	0.669	0.7925	0.6729
Decanoylcarnitine	lipid	0.4646	0.1842	0.01166	0.5798	0.3388	0.1307	0.5456	0.2251	0.01536	-0.0055	0.013	0.697	4.314	0.743
Dihomo-linolenate	fatty acid	-0.7076	0.3451	0.04032	0.05512	0.9978	0.9569	-0.4608	0.4847	0.3418	-0.015	0.018	0.43	12.96	0.3723
Dimethylarginine (SDMA + ADMA)	amino acid	-1.255	0.4615	0.006548	-2.798	1.317	0.04693	-2.063	0.6368	0.001197	0.017	0.014	0.226	14.04	0.7814
Ergothioneine	xenobiotics	-1.13	0.4685	0.4685	-	-	-	-	-	-	-	-	-	-	-
Hexadecanedioate	lipid	0.3527	0.128	0.005867	0.3823	0.1914	0.06711	0.4095	0.1608	0.01088	-0.002	0.0095	0.834	17.36	0.1832
Hexanoylcarnitine	lipid	0.4772	0.2379	0.04487	0.4677	0.4768	0.3523	0.4522	0.2656	0.08871	0.00032	0.014	0.982	12.08	0.2089
Octanoylcarnitine	lipid	0.4604	0.2092	0.02779	0.4957	0.3655	0.2466	0.4395	0.2324	0.05855	-0.0017	0.015	0.912	0.3964	0.9828
Palmitate (16:0)	fatty acid	-1.415	0.4277	0.0009344	-2.996	0.9716	0.006741	-0.9829	0.6468	0.1286	0.021	0.011	0.0878	11.85	0.809
Pseudouridine	nucleotide	-1.312	0.6122	0.03213	1.971	2.362	0.4164	-1.942	0.8565	0.02334	-0.035	0.024	0.17	16.41	0.4244
Stearate (18:0)	fatty acid	-0.7386	0.3701	0.04598	-0.1354	-0.1354	0.8967	-0.3944	0.5247	0.4522	-0.007	0.011	0.537	15.77	0.8962
Threonine	amino acid	0.9348	0.4587	0.04153	0.04153	1.501	1.501	0.6212	0.5832	0.2868	0.011	0.022	0.635	8.823	0.6382

SNP – single nucleotide polymorphisms, IVW – inverse variance weighted, MR – mendelian randomization, se – standard error, ERI – egger regression intercept

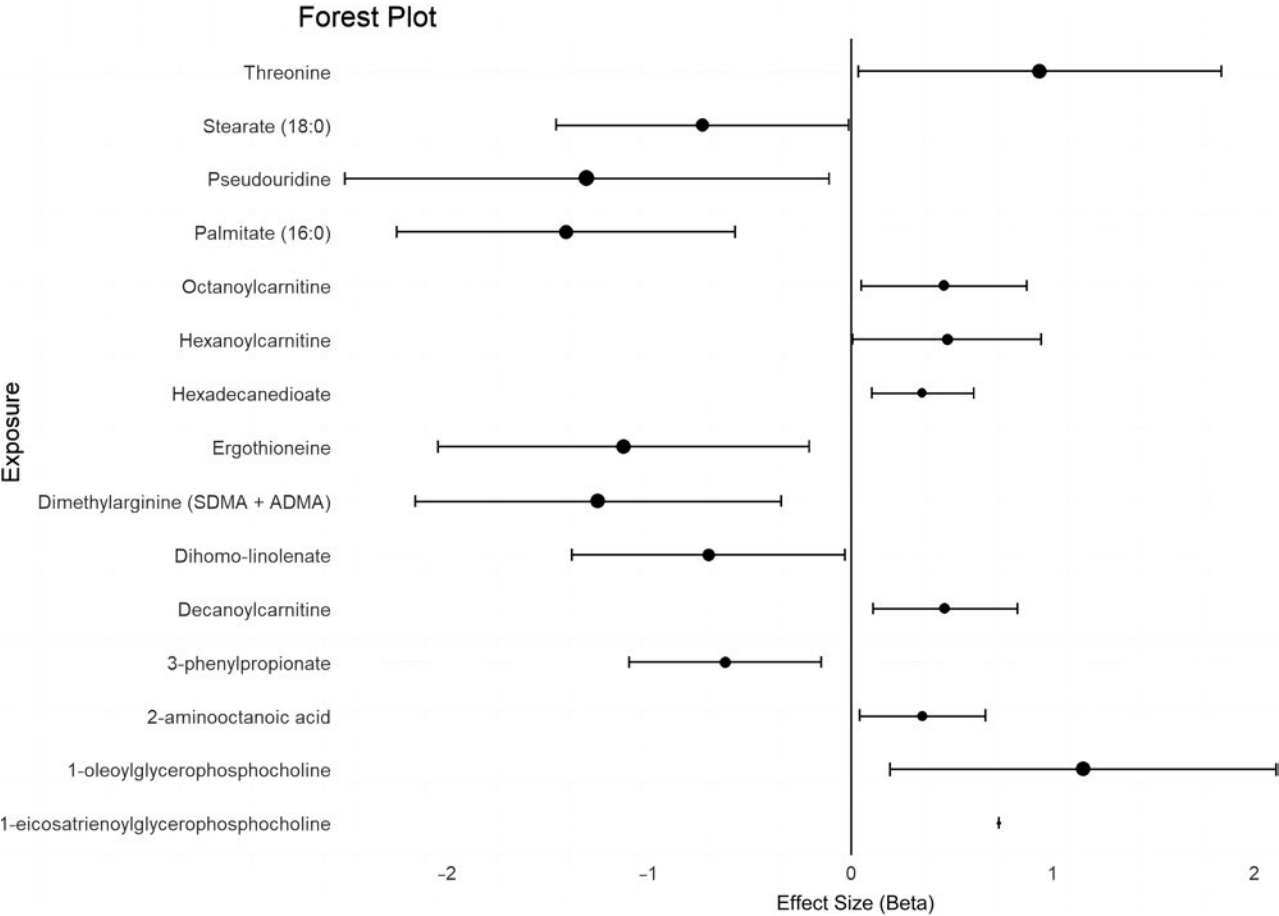


Fig. 3. The forest plot summarized the causality of circulating metabolites on the risk of endometriosis.

Mediation analysis with TSMR. Initially, the potential mediating role of circulating metabolites between GM and the risk of EMs was considered. It was observed that a higher abundance of the class Negativicutes corresponded to lower levels of hexadecanedioate ($\beta = -0.1311$, $se = 0.05139$, $p = 0.01074$ by IVW). Similarly, an increased abundance of the genus *Enterorhabdus* was associated with reduced levels of 1-oleoylglycerophosphocholine ($\beta = -0.06932$, $se = 0.03364$, $p = 0.03934$ by IVW). Furthermore, an elevated abundance of the order Selenomonadales linked to diminished levels of hexadecanedioate ($\beta = -0.1311$, $se = 0.05139$, $p = 0.01074$ by IVW) (Table SIII). After adjusting for the genetic effects of GM using MVMR, it was found that the mediation proportions of hexadecanedioate and 1-oleoylglycerophosphocholine exceeded 100%, which is logically untenable. Consequently, it was conjectured that circulating metabolites may not serve as the intermediary linking GM and EMs.

Subsequently, the examination of GM as a potential mediator between circulating metabolites and EMs was undertaken. A positive association was discovered between the level of pseudouridine and the abundance of class Negativicutes ($\beta = 1.206$, $se = 0.4339$, $p = 0.005453$ by IVW), with class Negativicutes exhib-

iting a mediation effect of 0.23% on the relationship between pseudouridine and EMs. The level of 3-phenylpropionate showed a negative correlation with the abundance of genus *Enterorhabdus* ($\beta = -0.9748$, $se = 0.4806$, $p = 0.04255$ by IVW), with genus *Enterorhabdus* having a mediation effect of 0.31% on the connection between 3-phenylpropionate and EMs. Additionally, a positive relationship was found between the level of pseudouridine and the abundance of order Selenomonadales ($\beta = 1.206$, $se = 0.4339$, $p = 0.005453$ by IVW), with order Selenomonadales contributing a mediation effect of 0.23% in linking pseudouridine to EMs. Lastly, the level of hexadecanedioate was positively associated with the abundance of genus *Coproccoccus 1* ($\beta = 0.09233$, $se = 0.0374$, $p = 0.01355$ by IVW), with genus *Coproccoccus 1* exerting a modest mediation effect of 0.06% on the relationship between hexadecanedioate and EMs (Fig. 4 and Table SIV).

Discussion

The present study constitutes the inaugural two-sample Mendelian randomization (TSMR) investigation into the interrelationships among GM, circulating

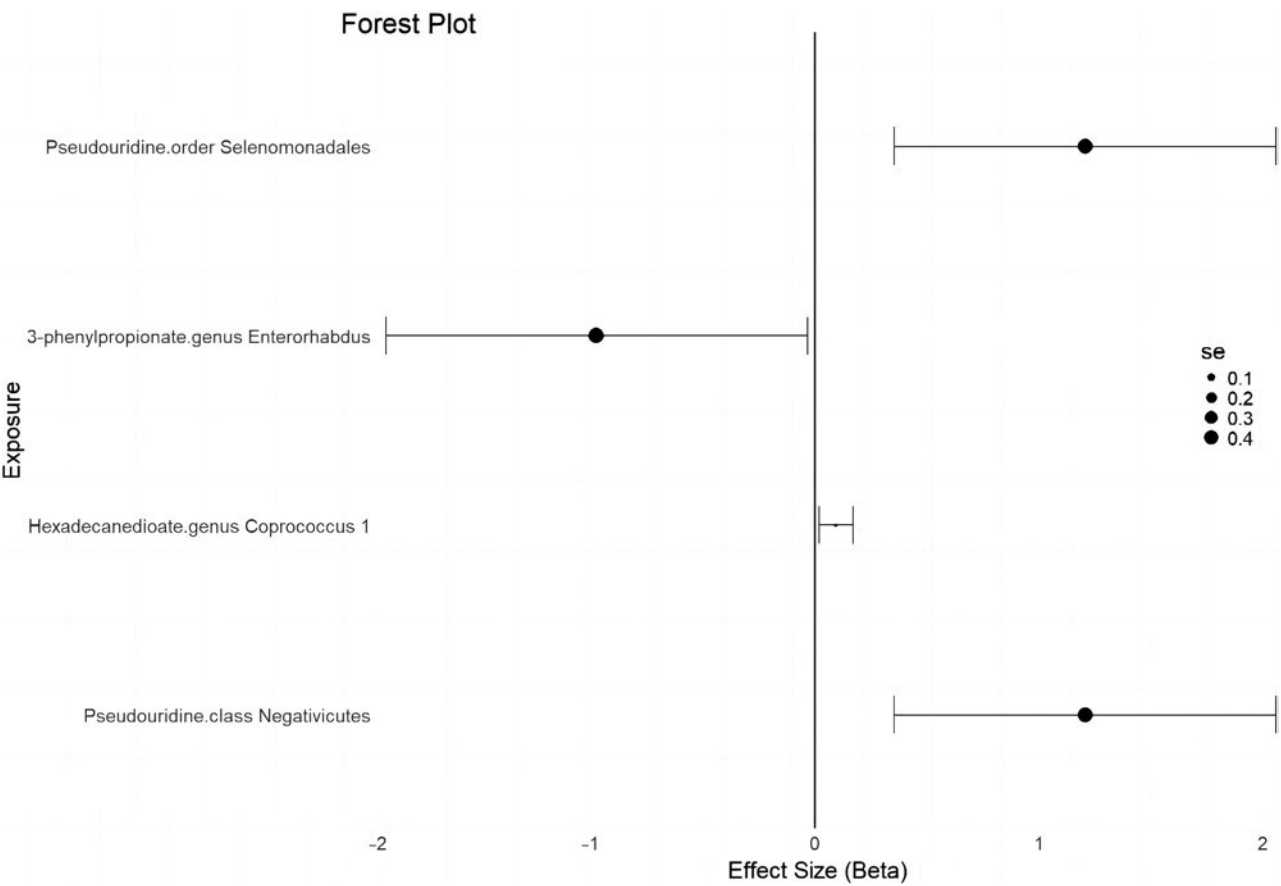


Fig. 4. The forest plot summarized the causality between circulating metabolites and gut microbiota.

metabolites, and EMs, revealing significant clinical relevance. Through the most extensive GWAS conducted to date on GM, circulating metabolites, and EMs, we identified robustly associated SNPs. Leveraging comprehensive genetic data from over 400,000 European individuals, we discovered a genetic predisposition to specific GM abundances that are causally linked with EMs. Similarly, we found a genetic liability to specific circulating metabolites associated with EMs. These findings suggest that circulating metabolites do not mediate the relationship between GM and EMs. Instead, GM may mediate between circulating metabolites and EMs, potentially guiding future mechanistic inquiries and clinical translational studies concerning EMs.

Endometriosis is a prevalent condition affecting women during their reproductive years, causing severe health and psychological distress. Symptoms such as chronic pelvic pain, infertility, and excessive bleeding are prevalent. Despite decades of research, the etiology and pathogenesis of EMs remain unclear, diagnosis is frequently delayed due to the requirement for invasive procedures, and curative treatments are elusive because the disease is estrogen dependent. Twin studies have estimated the heritability of EMs to be approximately 50% (Saha et al. 2015). Common genetic variations account for about 26% of the risk for EMs, making it

an ideal candidate for MR studies (Lee et al. 2013). The functional elucidation of variants implicated by GWAS in the pathogenesis of EMs necessitates integrated analyses of genomic data with epigenomic, metabolomic, proteomic, and transcriptomic data. The current study aims to reveal potential connections between gut microbiota, circulating metabolites, and EMs.

Over the past few decades, researches have consistently reported alterations in the abundance of GM among animal models and women afflicted with EMs. In a mouse model of EMs, established through the intraperitoneal injection of endometrial fragments, Ni et al. (2020) observed a significant correlation between EMs and altered gut microbiome profiles. Furthermore, Chadchan et al. (2019) discovered that metronidazole and broad-spectrum antibiotics could mitigate the development of EMs in a surgical mouse model. Comparable findings were noted in studies with Rhesus monkeys affected by EMs; Bailey and Coe (2002) identified a markedly different gut microbiota composition compared to healthy controls, with a higher prevalence of Gram-negative bacteria and reduced levels of lactobacilli. Corresponding patterns have been evident in human studies as well. Shan et al. (2021) reported notable disparities in the alpha diversity of gut microbiota and the Firmicutes/Bacteroidetes ratio between

individuals with stage III/IV EMs and those who are healthy. Ata et al. (2019) found an increased ratio of *Shigella/Escherichia* in the stool samples of women with advanced stage EMs which contrasted with healthy females. Additionally, Svensson et al. (2021) documented lower alpha and beta diversities, along with a decreased Firmicutes/Bacteroidetes ratio in EMs patients. However, these findings have not been uniform, leaving unclear the causal relationship between gut microbiota abundance and EMs.

The histopathological features of EMs were characterized by local inflammation. The inflammation and immune system dysfunction were crucial causes of EM pathogenesis. Recent studies presented strong evidence for an association between alteration of the gut microbiota and inflammatory bowel disease (Franzosa et al. 2019), neuropsychiatric diseases (Socała et al. 2021), psoriasis (Buhaş et al. 2022), arthritis (Xu et al. 2022), and some cancers. This was explained by the potential immunoregulation of gut microbiota on systemic inflammatory cellular responses. Since abnormal immune and inflammatory responses were thought to be involved in EM pathogenesis, the causality between microbiota and EMs was logically rational. The mouse model found that a fecal transplant from an EM mouse could alter EM progression and be accompanied by modulation of inflammatory and immune response. Lui et al. (2016) found that alteration of gut microbiota could influence mucosal T cell composition and function (TH1, TH17, TReg, etc.). Therefore, alteration of the gut microbiota might impact the mucosal immune balance, triggering inflammation and disease. Kogut et al. (2020) found that altering gut microbiota could cause elevated levels of systematic immune mediators. Macrophages played an essential role in EMs and were the predominant immune cell population in the ascites of EMs women. Elkabets et al. (2010), Lobo et al. (2018), and Rao et al. (2023) found that dysfunctional NK cells could down-regulate the phagocytic activity of macrophages and induce Treg lymphocytes, might promote ectopic endometrial cells to escape from immune surveillance. Recent studies have suggested that alteration of gut microbiota might cause the inappropriate activity of macrophages (Krishnan et al. 2018; Li et al. 2019; Sun et al. 2021), which might be involved in the EMs pathogenesis. Our MR study has revealed a significant association between specific GM taxa of the and the risk of developing EMs, thereby confirming the influence of the GM on the pathophysiology of EMs.

Numerous studies had yielded substantial evidence highlighting statistically significant differences in circulating metabolites between individuals with EMs and healthy controls. Dutta et al. (2012; 2018) observed that serum samples from EMs patients exhibited elevated levels of lactate, 3-hydroxybutyrate, alanine, leucine,

valine, threonine, lysine, glycerophosphatidylcholine, succinic acid, and 2-hydroxybutyrate, as well as decreased levels of lipids, glucose, isoleucine, and arginine. Additionally, they identified several dysregulated lipids, including phosphatidylcholines, sphingomyelins, phosphatidylethanolamines, and triglycerides in a mouse model induced with EMs. Conversely, Jana et al. (2013) reported decreased serum leucine levels in patients with EMs. Vicente-Muñoz et al (2015) discovered that the EMs patients' plasma metabolomic profile was characterized by increased concentrations of valine, fucose, choline-containing metabolites, lysine/arginine, and lipoproteins, along with decreased concentrations of creatinine. Vouk et al. (2012) found an association between the serum of EMs patients and elevated levels of sphingomyelins and phosphatidylcholines. Ghazi et al. (2016) noted significantly enhanced serum levels of 2-methoxyestron, 2-methoxy estradiol, dehydroepiandrosterone, androstenedione, aldosterone, and deoxycorticosterone in EMs, whereas cholesterol and primary bile acids were reduced. Letsiou et al. (2017) identified a panel of acylcarnitines that predicted the presence of endometriosis with 88.9% specificity and 81.5% sensitivity in human plasma, offering a positive predictive value of 75%. Present MR study revealed associations between specific circulating metabolites and the risk of EMs, encompassing lipids, amino acids, fatty acids, xenobiotics, and nucleotides. These findings align with previous research and underscore the influence of circulating metabolites on the pathophysiology of EMs.

The mechanisms through which gut microbiota may influence endometriosis largely remain unidentified. One potential hypothesis, as elaborated in a prior publication (Yang 2024), involves immunological dysfunction and alterations in estrogen homeostasis. Another hypothesis suggests that GM influences EMs through the action of circulating metabolites. However, illogical results were obtained when utilizing TSMR to evaluate the mediating effect of circulating metabolites linking GM and EMs. This led to the speculation that circulating metabolites are not the primary mediator. Instead, it is proposed that GM acts as the mediator between circulating metabolites and EMs. Notably, it was identified that pseudouridine and 3-phenylpropionate may mediate a small proportion (0.06–0.31%) of the GM's effect on EMs, representing a novel finding in the field that could guide future mechanistic and clinical translational studies on EMs.

The main strengths of the present study included the pioneering application of TSMR analysis to elucidate the causal relationships between GM, circulating metabolites, and EMs, enrolling the largest sample size to date, and employing multiple testing corrections to ensure the robustness of our results. The MR approach helps eliminate confounders typically observed in

epidemiological studies, attaining a level of evidence comparable to randomized controlled trials (RCTs). Furthermore, these selected SNPs showed strong associations with GM and circulating metabolites. Sensitivity analyses indicated no pleiotropy or heterogeneity, confirming the statistical robustness of our findings.

Present study provides valuable insights but is not without limitations. Firstly, the FDR for all causality assessments exceeded 0.05, indicating that they did not pass multiple testing corrections, which could undermine the robustness of results. Secondly, since the GWAS data originated from European populations, ethnic and geographical factors could influence GM abundance and the characteristics of circulating metabolites, possibly limiting the generalizability of findings to other populations. Thirdly, although MR is thought to mimic randomized controlled trials, the possibility of bias and confounding still exists. For example, if there is population stratification not properly controlled for, or if genetic markers associated with multiple biological pathways are used, it can lead to confounding. Fourthly, MR studies require large sample sizes to achieve sufficient statistical power, especially when estimating small effect sizes. Obtaining and analyzing large datasets while keeping type II errors (false negatives) low and controlling type I errors (false positives) is challenging. Fifth, the genotype and outcome measurements come from different populations, and selection bias may be introduced. Sixth, MR studies are limited to studying inheritable exposures and have limited capability to capture the effects of complex environmental exposures or behaviors.

Recent studies (Zheng et al. 2023) have revealed that histone deacetylase 8 (HDAC8) is progressively and aberrantly overexpressed in EMs, complementing the documented upregulation of HDAC1 in EMs. This dysregulation, alongside the promising therapeutic potential of histone deacetylase inhibitors (HDACIs), underscores the urgent need for a deeper investigation into the epigenetic alterations that drive EMs, mainly focusing on the roles of HDACs.

Melatonin, recognized for its ability to modulate crucial biochemical pathways involved in inflammation, oxidative stress, apoptosis, and energy metabolism, has been found in significantly higher concentrations in the gut than in the pineal gland. The GM, a critical player in multiple physiological functions, is increasingly implicated in various pathologies due to its imbalances. Emerging data (Iesanu et al. 2022; Sharifi et al. 2023; Ahmadi et al. 2024; Magadán-Corpas et al. 2024) suggest that melatonin may play a role in modulating the GM, offering potential therapeutic implications for these conditions. In the context of EMs, melatonin's anti-proliferative, antioxidant, anti-inflammatory, and anti-invasive properties make it a promising agent in

mitigating the disease's progression. These protective effects involve multiple pathways, including antiestrogenic, antioxidant, anti-inflammatory, and anti-apoptotic mechanisms, as well as inhibiting the growth of E2-induced endometriotic tissue.

Concurrently, EMs patients exhibit altered HPA-axis function (van Aken et al. 2018), potentially stemming from heightened chronic stress levels. The interplay between pro-inflammatory and anti-inflammatory cytokines is also evident in EMs pathogenesis, where these cytokines significantly influence cellular proliferation and differentiation (Oală et al. 2024). Dysbiosis of GM and reduced serum levels of acetic and propionic acids may contribute to glycolipid metabolism disorders associated with EMs.

Future research should aim to enroll larger sample sizes from diverse racial and geographical backgrounds to explore more robust causalities. Concurrently, deeper mechanistic investigations are warranted to uncover the complex interplay between the gut microbiome, circulating metabolites, and EMs.

Conclusions

In summary, the present study has comprehensively assessed the association between GM, circulating metabolites, and EMs. The results indicate that eight GM taxa and fifteen circulating metabolites have significant associations with EMs. Additionally, pseudouridine and 3-phenylpropionate have been identified as potential mediators of the GM's influence on EMs. This research contributes novel candidate factors that could serve as a foundation for future mechanistic inquiries and clinical translational efforts.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

HY wrote, discussed, reviewed the manuscript, and designed and created figures.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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