Original Article

Dietary, metabolic and gut microbiota influences on primary ovarian failure: a two-sample Mendelian randomization study

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Background and Objectives: Previous studies have reported there were associations between ovarian function and dietary factors, metabolic factors and gut microbiota. However, it is unclear whether causal associations exist. We aimed to explore the causal relationship of these factors with risk of primary ovarian failure (POF). **Methods and Study Design:** Two-sample Mendelian randomization (MR) analysis was performed to genetically predict the causal effects of dietary and metabolic factors and gut microbiota on POF. The inverse variance weighted (IVW) method was used as the primary statistical method. A series of sensitivity analyses, including weighted median, MR-Egger, simple mode, weighted mode methods, and leave-one-out analysis, were conducted to assess the robustness of the MR analysis results. **Results:** IVW analysis revealed that cigarettes smoked per day, coffee intake and cooked vegetable intake were not causally correlated with POF at the genetic level. However, POF were associated with fresh fruit intake, BMI, *Eubacterium* (*hallii group*), *Eubacterium* (*ventriosum group*), *Adlercreutzia*, *Intestinibacter*, *Lachnospiraceae* (*UCG008*), and *Terrisporobacter*. These findings were robust according to extensive sensitivity analyses. **Conclusions:** This study identified several dietary factors, metabolic factors and gut microbiota taxa that may be causally implicated in POF, potentially offering new therapeutic targets.

Key Words: primary ovarian failure, dietary factors, metabolic factors, gut microbiota, Mendelian randomization

INTRODUCTION

The ovary is essential for establishing and maintaining secondary sexual characteristics and fertility in females. However, primary ovarian failure (POF) negatively influences reproductive health and induces disorders of ovarian function. POF is defined as the presence of postmenopausal levels of follicle-stimulating hormone (FSH) (> 40 IU/L) in woman under 40 years of age, accompanied by four or more months of secondary amenorrhea, indicating the exhaustion of the ovarian reserve before age 40. In addition, women with POF experience menopausal symptoms and are adversely affected by long-term estrogen deprivation, which significantly affects their physical and mental health.[1] Given that the chance of spontaneous conception is 5%-10%,[2] adoption or *in vitro* fertilization and embryo transfer using donor oocytes are considered effective fertility treatments for women with POF. The etiology of POF is heterogeneous, including genetic defects, autoimmune diseases, iatrogenic factors (radiotherapy, chemotherapy, and ovarian surgery), and environmental factors.[3] However, most patients are idiopathic an-

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d the cause is unclear. Compared with immobile etiologies such as genetic and iatrogenic factors, learning and understanding the influence of modifiable factors such as diet, metabolic traits and gut microbiota in POF seem more valuable for the prevention and treatment of this disease. The most established and well-learned dietary factor associated with POF is smoking, while caffeine intake has been suggested as a potential factor.[4] Several observational studies have suggested that smoking duration,[5-8] caffeine consumption,[9-11] and fruit intake[12,13] were associated with the age of menopause. In addition, the gastrointestinal tract, which hosts ten trillion diverse symbionts (50 bacterial phyla and approximately 100–1000 bacterial species), has been extensively studied owing to its basic functions in the immunological, metabolic, structural and neurological landscapes in humans.[14] The interactions of the gut microbiota with estrogen, androgens, insulin, and other hormones appear to be crucial for the reproductive endocrine system.[15] Imbalance of the gut microbiota composition can lead to polycystic ovary syndrome (PCOS),[16-18] endometriosis,[19,20] ovarian dysfunction,[21] and ovarian cancer.[22] However, less is known about the exact role of diet and gut microbiota in ovarian physiology, and few studies have explored the causal relationship between the gut microbiota and POF.[23]

Although randomized controlled trials (RCTs) are the gold standard for establishing causal relationships, they can be costly, time-consuming and even impractical.[24] On the other hand, observational studies may not robustly reflect causal relationships owing to many potential biases, confounders and reverse causation.[25] Mendelian randomization (MR) is an approach that uses genetic variants associated with an exposure as instrumental variables (IVs) to examine the causality of exposure–outcome associations. MR can minimize potential confounders and reverse causality because genetic variants segregate randomly and independently, preceding the outcome of interest.[24] Furthermore, during the last decade, the publication of a large volume of genome-wide association studies (GWASs) has enabled MR studies to be conducted without recruiting new patients. Therefore, MR provides a suitable method for inferring the causal effect between risk factors and POF. Here, we conducted an MR study to investigate the associations between dietary and metabolic factors and gut microbiota with the risk of POF.

METHODS

We assessed the causal links between lifestyle-related exposure factors and POF using two-sample MR. An overview of the analytical approach is shown in Figure 1A.

Exposure data

Diet-related exposure factors used in this study included cigarettes smoked per day, coffee intake, fresh fruit intake, and cooked vegetable intake. Metabolism-related

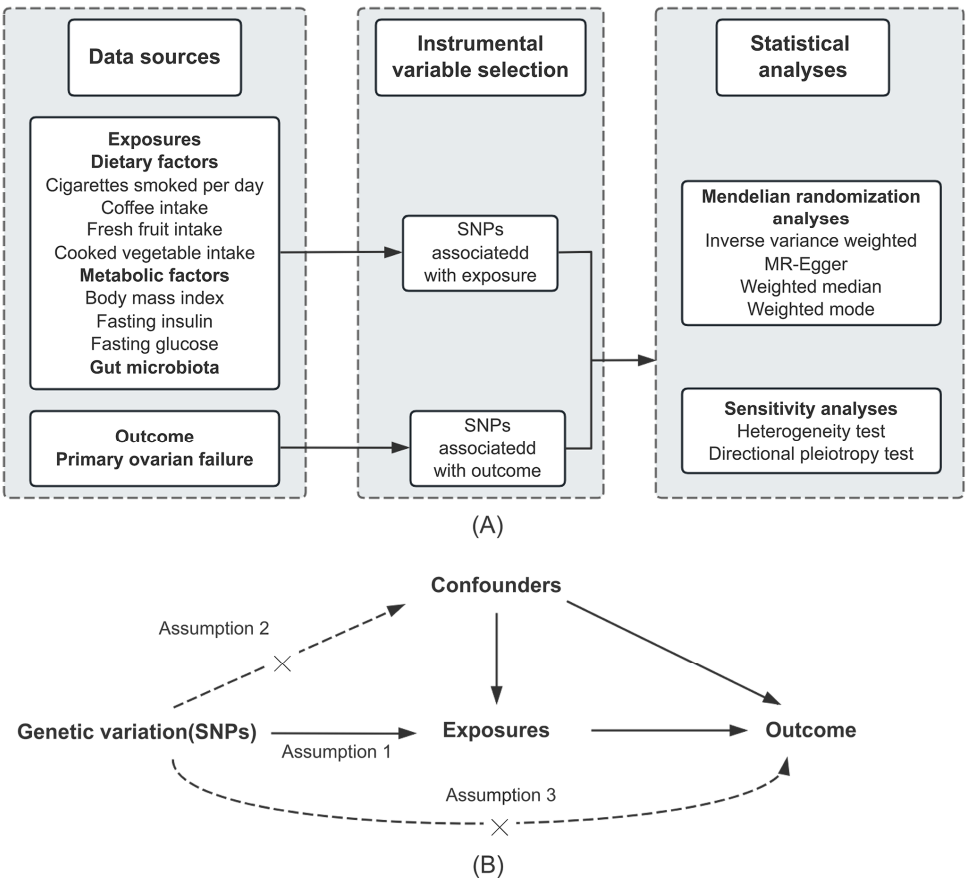


Figure 1. Study design (A) Flowchart showing the process for the MR analyses, including data collection, IVs selection, and statistical analysis. (B) Directed acyclic graph showing the assumptions of the MR methodology. MR relies on three assumptions: the genetic variants selected as instruments must (1) be associated with the exposures, (2) not be associated with confounders, (3) not directly affect the outcome, except through their effect on the exposures. SNP, single-nucleotide polymorphisms.

exposure factors used in this study included body mass index (BMI), fasting insulin, and fasting glucose. These GWAS summary-level data were extracted from IEU open GWAS project. We obtained genetic variant information related to the human gut microbiome composition from the latest large-scale genome-wide meta-analysis conducted by the MiBioGen consortium (https://mibiogen.gcc.rug.nl/.) based on European-dominated participants.[26] This study analyzed genome-wide genotypes and 16S fecal microbiome data from 18,340 individuals from 24 cohorts. Accordingly, the genus level was the lowest. A total of 131 genera with a mean abundance greater than 1% were identified, 12 of which were unknown genera.[26] As a result, we included 119 genus-level taxa in this study. More information about the exposure datasets is presented in Table 1.

Outcome data

GWAS summary statistics related to POF were obtained from the FinnGen Consortium release data (https: //www.r8.finngen.fi/.), which is one of the largest nationwide genetic studies with access to comprehensive electronic health register data of participants. Detailed information on used exposure datasets is presented in Table 1.

Instrumental variable selection

Single-nucleotide polymorphisms (SNPs) are used as IVs in MR analysis to provide evidence of causality between the exposure and outcome. To ensure the accuracy and robustness of the causal links, SNPs must satisfy three core assumptions to be used as IVs (Figure 1B).[27] Therefore, we selected independent SNPs (linkage disequilibrium $R^2 < 0.001$ and clumping distance=10,000 kb, based on the European-based 1000 Genome Projects reference panel) associated with each exposure factor at a genome-wide threshold of significance ($p < 5\times 10^{-8}$, diet-related and metabolism-related exposure factors) or at a locus-wide threshold of significance ($p < 1\times 10^{-5}$, gut microbiome-related exposure factors).

Statistical analysis

The inverse variance weighted (IVW) method was used as the primary statistical method and can provide the most accurate causal estimates provided that the pleiotropic effect is balanced and that all IVs meet the MR assumptions.[28] Given the difficulty in verifying that IVs influence the outcome solely through the exposure of interest, we performed a series of sensitivity analyses under various assumptions. These were designed to assess the robustness of the associations and to examine horizontal pleiotropy for exposures, using methods such as weighted median, MR-Egger, simple mode, and weighted mode. The weighted median of SNP-specific estimates provides valid results when more than 50% of the information is contributed by IVs.[29] MR-Egger regression provides a valid estimate of causal estimates under the instrument strength independent of direct effect (InSIDE) assumption.[30] However, this approach was used to detect and adjust for unbalanced horizontal pleiotropy rather than to produce causal estimates due to the low statistical power of MR-Egger. A MR-Egger intercept significantly different from 0 ($p < 0.05$) indicates the occurrence of directional pleiotropy and a potentially biased IVW estimate. To further test the robustness of our results, Cochran's Q test was used to evaluate heterogeneity among the SNPs included in each analysis. Q statistics significant at $p < 0.05$ provide evidence for heterogeneity between individual genetic variants and the existence of invalid instruments.[31] In addition, leave-one-out analysis was performed to assess whether an outcome was driven by a single outlying SNP,[32] indicating the presence of hetero-

Table 1. Information of the exposures and outcome datasets

Exposure or outcome	IEU GWAS id	Consortium	Cases	Controls	Sample size	Population
Cigarettes smoked per day	ieu-b-142	GSCAN	NA	NA	249,752	European
Coffee intake	ukb-b-5237	MRC-IEU	NA	NA	428,860	European
Fresh fruit intake	ukb-b-3881	MRC-IEU	NA	NA	446,462	European
Cooked vegetable intake	ukb-b-8089	MRC-IEU	NA	NA	448,651	European
BMI	ukb-b-19953	MRC-IEU	NA	NA	461,460	European
Fasting insulin	ebi-a-GCST90002238	NA	NA	NA	151,013	European
Fasting glucose	ebi-a-GCST90002232	NA	NA	NA	200,622	European
Gut microbiome	NA	MiBioGen consortium	NA	NA	18,340	European (N=13,266), Middle-Eastern (N=481), East Asian (N=811), American Hispanic/ Latin (N=1097), African American (N=114) multi-ancestry (N=2571)
POF	NA	FinnGen consortium	25,117	148,629	173,746	European

BMI, body mass index; POF, premature ovarian failure

geneous SNPs. Furthermore, if the genetic variants do not explain enough of the variance, there will be significant weak instrumental bias toward the confounded estimate.[33] To address this concern, SNP-specific F-statistics, approximated by the square of the beta divided by the variance for the SNP-exposure association, were calculated to evaluate the strength of the instruments used, and values exceeding the standard threshold of 10 are indicative of strong genetic instruments.[33]

All tests were two-sided and performed using R Version 4.2.1 with the R packages "TwoSampleMR" and "MendelianRandomization". A p value < 0.05 indicated statistical significance of the MR effect estimate. No ethical approval was required since we used publicly available summary data.

RESULTS

SNP selection

There were 22 SNPs associated with cigarettes smoked per day, 38 SNPs associated with coffee intake, 53 SNPs associated with fresh fruit intake, 17 SNPs associated with cooked vegetable intake, 414 SNPs associated with BMI, 37 SNPs associated with fasting insulin, 60 SNPs associated with fasting glucose, and 1508 SNPs associated with gut microbiota selected for the MR analyses according to the IV selection criteria. The detailed information and F-statistic for the selected instruments are shown in Supplementary Table 1. The overall instrument had a high F-statistic (>10), indicating the good strength of the genetic instruments used.

MR analysis

Figure 2 shows causal effect estimates of the dietary and metabolic factors and gut microbiota on POF from the IVW MR analyses. Associations for exposures using the different MR methods are presented in Supplementary Tables 2-4. Scatter and forest plots of the SNP-outcome associations against the SNP-exposure associations are shown in Supplementary Figures 1-6, allowing visualization of the causal effect estimate for each individual SNP on POF. Leave-one-out plots are shown in Supplementary Figures 7-9 to evaluate the influential outliers.

MR analysis via the IVW method showed that cigarettes smoked per day (OR = 1.00, 95% CI: 0.77–1.30, p = 0.982), coffee intake (OR = 2.05, 95% CI: 0.87–4.84, p = 0.103), cooked vegetable intake (OR = 3.13, 95% CI: 0.37–26.09, p = 0.292), fasting insulin (OR = 1.61, 95% CI: 0.66–3.96, p = 0.298), and fasting glucose (OR = 1.04, 95% CI: 0.67–1.60, p = 0.864) had no genetic causal relationship with POF (Figure 2). However, fresh fruit intake (OR = 7.33, 95% CI: 2.36–22.71, p = 0.001) and BMI (OR = 1.99, 95% CI: 1.60–2.48, p < 0.001) were related to an increased risk of POF. In addition, six gut microbiome taxa were significantly associated with POF risk (Figure 2). IVW method revealed that *Eubacterium* (*hallii group*) and *Eubacterium* (*ventriosum group*) were negatively associated with the risk of POF (OR = 0.49, 95% Cl: 0.26–0.90, p = 0.022; OR = 0.51, 95% Cl: 0.27–0.97, p = 0.040), while *Adlercreutzia*, *Intestinibacter*, *Lachnospiraceae* (*UCG008*), and *Terrisporobacter* were positively associated with the risk of POF (OR = 3.01, 95% Cl: 1.38–6.60, p = 0.006; OR = 1.82, 95% Cl: 1.04–3.20, p = 0.037; OR = 1.73, 95% Cl: 1.08–2.76, p = 0.023; OR = 2.47, 95% Cl: 1.14–5.36, p = 0.022) (Figure 2).

Sensitivity analyses

The observed causal associations were consistent in sensitivity analyses. MR-Egger regression showed no evidence of directional pleiotropic effect across the genetic variants (intercept, p > 0.05) (Table 2 and Supplementary Tables 5-7). There was no evidence of heterogeneity in the IVW analysis using Cochran's Q test (p > 0.05) (Table 2 and Supplementary Tables 5-7). Although there were outliers present on visual inspection in both scatter (Supplementary Figures 1-3) and forest plots (Supplementary Figures 4-6), the results of the leave-one-out sensitivity analysis indicated that the associations between dietary and metabolic factors and gut microbiota with POF were not substantially driven by any individual SNP (Supplementary Figures 7-9), suggesting the robustness of the results.

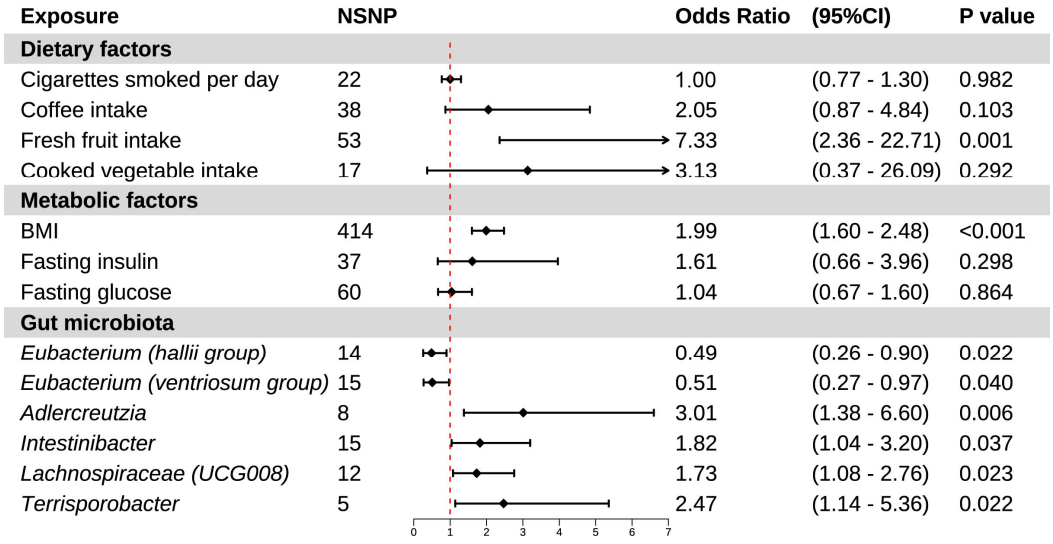


Figure 2. Associations of genetically predicted dietary and metabolic factors and gut microbiota with risk of POF. BMI, body mass index; POF, premature ovarian failure; SNP, single-nucleotide polymorphisms.

Table 2. Heterogeneity and directional pleiotropy tests from MR analysis of the dietary and metabolic factors and gut microbiota with risk of POF

Exposure	Heterogeneity		MR–Egger		
	Cochrane's Q	*p*	Egger Intercept	SE	$p_{intercept}$
Dietary factors					
Cigarettes smoked per day	12.09	0.937	0.000	0.02	0.990
Coffee intake	43.06	0.228	0.021	0.01	0.142
Fresh fruit intake	45.16	0.738	0.009	0.02	0.609
Cooked vegetable intake	24.40	0.081	0.000	0.13	0.999
Metabolic factors					
BMI	440.57	0.168	-0.006	0.01	0.233
Fasting insulin	43.27	0.189	0.002	0.02	0.944
Fasting glucose	50.14	0.788	0.003	0.01	0.758
Gut microbiota					
Eubacterium (hallii group)	9.01	0.773	-0.006	0.05	0.916
Eubacterium (ventriosum group)	10.02	0.761	-0.020	0.11	0.858
Adlercreutzia	9.55	0.215	0.279	0.14	0.085
Intestinibacter	12.90	0.535	-0.080	0.08	0.307
Lachnospiraceae (UCG008)	10.44	0.491	0.096	0.12	0.446
Terrisporobacter	2.93	0.570	0.050	0.12	0.703

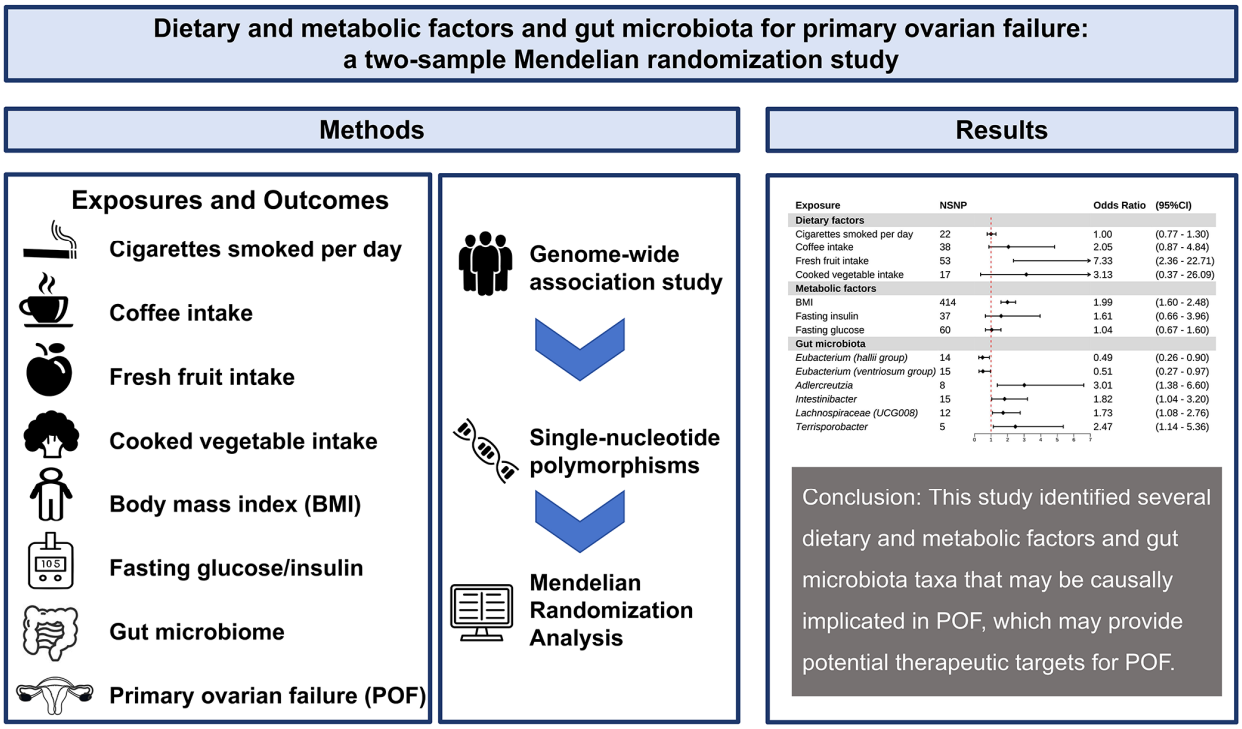
BMI, body mass index.

DISCUSSION

We conducted MR analyses by using the largest GWAS datasets to systematically investigate the causal relationship between the dietary and metabolic factors and gut microbiota with risk of POF. Our results showed that fresh fruit intake and BMI was associated with an increased risk of POF. Six gut microbiome taxa were associated with the risk of POF. *Eubacterium* (*hallii group*) and *Eubacterium* (*ventriosum group*) appeared to confer a protective effect against POF, while *Adlercreutzia*, *Intestinibacter*, *Lachnospiraceae* (*UCG008*), and *Terrisporobacter* increased the risk of POF. This study could provide important insight into the genetic relationship between dietary and metabolic factors and gut microbiota with POF and shed new light on the potential causes and therapeutic strategies for POF.

Altered gut microbial profiles have been observed in women with POF.[21] Additionally, Elgart et al.[34] reported that the gut bacteria of *Drosophila* can affect oogenesis and maternal-to-zygotic transition during embryo development. In this study, we found that *Eubacterium* (*hallii group*) and Eubacterium (*ventriosum group*) had protective effects on POF. *Eubacterium* produces short-chain fatty acids (SCFAs). SCFAs, including propionate, acetate and butyrate, are the main products of the fermentation of dietary fiber by the intestinal microbiota.[35] Butyrate can enhance the expression of tight-junction proteins and mucin to maintain the intestinal epithelial barrier,[36] which is the first line of defense in the intestine. The abundance of *Eubacterium* in the gut is strongly correlated with SCFA levels and the beneficial effects of SCFAs under a range of clinical conditions.[37] Several studies have shown that SCFAs play a major role in the modulation of inflammation through the inhibition of proinflammatory cytokines, such as interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor receptor-α (TNF-α), while upregulating the expression of anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β).[38,39] The human ovary is a ubiquitous target for autoimmune attack, leading to the consequent occurrence of POF.[40] Autoimmunity is responsible for approximately 4–30% of POF cases.[41,42] *E. hallii* and *E. ventriosum* may act as anti-inflammatory agents to protect the ovary from inflammation. On the other hand, we found that *Adlercreutzia*, *Intestinibacter*, *Lachnospiraceae* (*UCG008*), and *Terrisporobacter* increased the risk of POF. Other studies have shown that these 4 gut microbiome taxa are correlated with the risk of diabetic retinopathy, male infertility, periodontitis, and sepsis.[43-46] However, there is a lack of corresponding research evidence to clarify the underlying mechanism by which these gut microbiome taxa contribute to POF, thus providing new directions for future studies.

Smoking is a worldwide issue. Cigarette smoke contains several toxicants, including polycyclic aromatic hydrocarbons (PAHs), such as benzoapyrene (BaP), nitrosamines, heavy metals (cadmium), alkaloids and aromatic amines, which have different properties and targets. Therefore, these chemical compounds may exert hazardous effects on the entire reproductive system in women.[47] It has been documented that active smoking was associated with earlier menopause.[5-8] The tobacco-mediated ovarian injury characterizes by a significant decline in steroidogenesis,[47,48] and folliculogenesis.[49-54] Evidence from experimental models have shown that a single high dose of PAHs led to the loss of primordial and primary follicles.[55] In addition, *in-vitro* studies have demonstrated that BaP could induce follicular demise and alter the growth of rat and mouse follicles[50,52], and exposure to nicotine could cause decrease estradiol production in cultured granulosa bovine cells.[56] The pathophysiological mechanism behind tobacco-mediated ovarian injury involves a range of factors such as oxidative stress,[57-60] DNA damages,[61] and follicle loss through autophagy/apoptosis.[62-65] However, a meta-analysis comprising 15 studies found a relationship in earlier age of natural menopausal in current smokers but the association disappeared in former smokers.[66] The results of this study show that there was no causal relationship between cigarettes smoked per day and POF at the genetic level. The association between cigarettes smoked per day and premature ovarian failure (POF) might be attenuated due to the way



Graphical abstract.

the smoking phenotypes are defined, as they include both current and former smokers. Although several studies were devoted to investigating the relationship between drinking coffee and the age of menopause, data are lacking on POF. Current studies have suggested that there is no association between coffee intake and early menopause or ovarian age indicators such as anti-Müllerian hormone (AMH) and FSH.[9-11] Combined with the results of our study, we considered that there is no causal relationship between coffee intake and POF at the genetic level. A large prospective study involving 33,054 Shanghai women has found that a high level of fruit intake (>383.2 g/day) was associated with delayed menopause.[12] Another study also supported this finding.[13] The association of fruit intake with POF could, in part, be related to the antioxidant content in fruit. However, according to our study, fresh fruit intake was associated with an increased risk of POF. Potential mechanisms underlying this association needs to be explored in mechanistic studies. The association between BMI and POF remains much less understood and even controversial. Both overweight and underweight had been reported to be associated with earlier menopause. Our study can provide evidence of causal association. Various mechanisms could explain how overweight might influence the development of ovarian aging. It is known that being overweight can increase oxidative stress in the body through a number of potential mechanisms.[67,68] In addition, obesity is related to chronic low-grade inflammation in the body.[69] Adipose tissue is an important endocrine organ that produces adipokines contributing to a state of inflammation.

This study has several strengths. The major merit is MR design which can exclude the interference of confounding factors and reverse causality to a large extent. Furthermore, non-overlapping exposure and outcome summary-level data were used to avoid unnecessary bias.[70] In order to ensure the accuracy of MR analysis, horizontal pleiotropy was detected and excluded by the MR-Egger regression intercept test. Limitations should be considered when interpreting our results. First, our study analyzed only European populations, so the generalizability of the results should be approached with caution when extending them to other populations. Secondly, our study was only conducted at the genetic level, and did not explore the exact mechanisms behind the association. Finally, since genus was the lowest taxonomic level in the exposure datasets, we were unable to further explore the causal association between gut microbiota and POF at the species level.

Conclusion

To conclude, we provided evidence to show that fresh fruit intake and BMI was associated with an increased risk of POF. Six gut microbiome taxa were associated with the risk of POF. *Eubacterium* (*hallii group*) and *Eubacterium* (*ventriosum group*) appeared to confer a protective effect against POF, while *Adlercreutzia, Intestinibacter, Lachnospiraceae* (*UCG008*), and *Terrisporobacter* increased the risk of POF. Our results provided potential therapeutic targets for POF. At the same time, it is necessary to validate these findings and explore the underlying mechanisms in clinical trials and animal models.

SUPPLEMENTARY MATERIALS

All supplementary tables and figures are available upon request.

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CONFLICT OF INTEREST AND FUNDING DISCLOSURES

The authors report there are no competing interests to declare.

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