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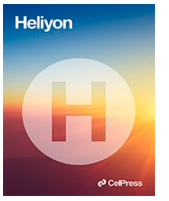

Research article

Calcium homeostasis and endometriosis: A Mendelian randomization study

Zhi-Min Deng [a,1], Fang-Fang Dai [a,1], Rui-Qi Wang [a], Gan-Tao Chen [b,**], Xiao Yang [c,***], Yan-Xiang Cheng [a,*]

[a] Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan University, Wuhan, Hubei, 430060, PR China
[b] Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan, Hubei, 430060, PR China
[c] Department of Obstetrics and Gynecology, Peking University People's Hospital, Beijing, 100000, PR China

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ABSTRACT

Background: Previous observational studies have investigated the correlation between calcium homeostasis modulator levels and endometriosis risk. Yet, the genetic association between body calcium homeostasis and endometriosis risk remains to be elucidated.

Methods: Four tiers of Mendelian randomization (MR) analysis were conducted, as follows: (1) single univariate MR and (2) multivariate MR to evaluate the correlation between calcium homeostasis regulators and endometriosis; (3) inverse MR to probe the influence of endometriosis on body calcium homeostasis; (4) two-sample MR to scrutinize the connection between calcium levels and endometriosis categories.

Results: The two-sample MR analysis unveiled a robust positive correlation between genetically inferred calcium levels and endometriosis risk (IVW: OR = 1.15, 95 % CI: 1.02–1.29, $p = 0.018$). The MVMR analysis corroborated that the positive correlation of calcium levels with endometriosis persisted after adjusting for 25(OH)D and PTH. The inverse MR analysis disclosed a significant association between endometriosis and 25(OH)D ($\beta = 0.01$, 95 % CI: 0.00–0.02, $p = 0.007$) and calcium ($\beta = 0.02$, 95 % CI: 0.00–0.04, $p = 0.035$). The two-sample MR analysis further demonstrated that calcium levels were positively linked solely to endometriosis of uterus (i.e. adenomyosis, IVW: OR = 1.23, 95 % CI: 1.01–1.49, $p = 0.038$), with no evidence of a influence on other endometriosis categories.

Conclusions: This study, employing various types of MR, offers some genetic evidence for the relationship between calcium homeostasis and endometriosis, augmenting the current comprehension of the complex association between the two and suggesting that calcium levels are a risk factor for endometriosis. These findings provide a unique genetic perspective that may spur further investigation and may inform future strategies for managing patients with endometriosis.

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: 3206539401@qq.com (G.-T. Chen), 1811110311@pku.edu.cn (X. Yang), yanxiangcheng@whu.edu.cn (Y.-X. Cheng).
[1] Contributed equally to this work.

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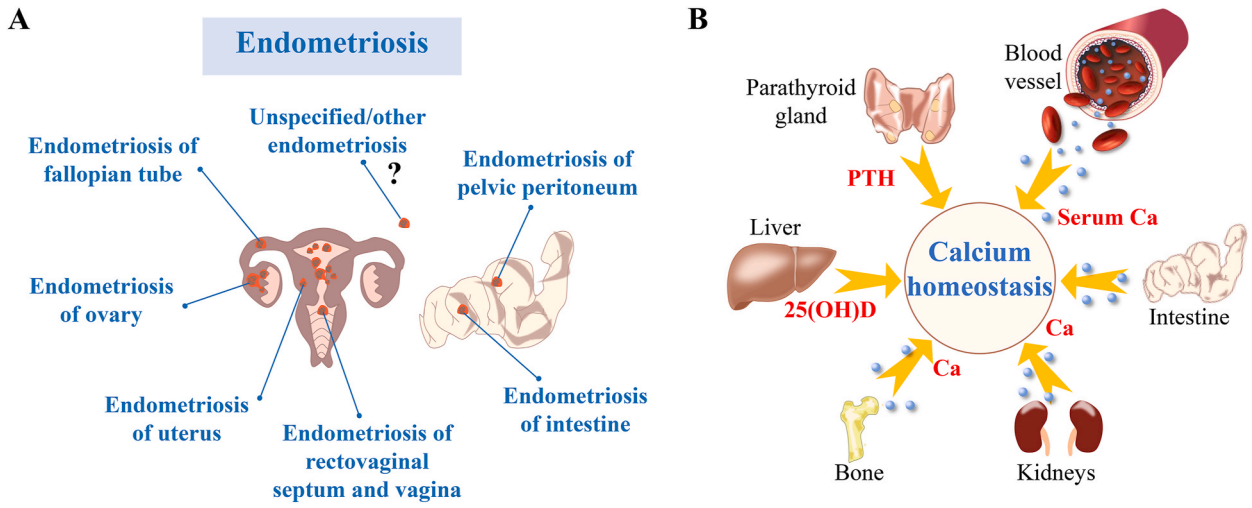


Fig. 1. (A) Classification of endometriosis categories in this article; (B) Schematic representation illustrating the regulatory pathways of calcium, 25-hydroxyvitamin D (25(OH)D), and parathyroid hormone (PTH) in maintaining calcium homeostasis, involving the blood, small intestine, parathyroid glands, liver, bone, and kidney.

1. Introduction

Endometriosis, a chronic inflammatory condition characterized by discomfort and infertility, significantly impairs the health and life quality of women in their reproductive years [1]. It is characterized by the deposition of endometrial tissue outside of its normal location, such as the ovaries, fallopian tubes, uterine surface, pouch of Douglas, vagina, pelvic peritoneum, intestine, etc. [2]. This displaced tissue exhibits proliferative and invasive characteristics (Fig. 1A). Although various factors, including genetics, hormones, inflammation, and environmental influences, are believed to contribute to the development of endometriosis [3], its etiology and pathogenesis remain incompletely understood. There is a pressing need to deepen our understanding of endometriosis pathophysiology to devise more effective therapeutic strategies.

Calcium homeostasis is crucial to human health, participating in physiological processes such as cell signaling, muscle contraction, bone and tooth formation, and blood coagulation [4]. Calcium homeostasis regulatory factors, including calcium, 25-hydroxyvitamin D (25(OH)D), and parathyroid hormone (PTH), interact intimately to regulate calcium absorption, transportation, and excretion (Fig. 1B). Specifically, PTH increases the blood calcium level by promoting calcium absorption in the small intestine, mobilization of bone calcium, and calcium reabsorption in the renal tubules [5,6]. 25(OH)D, the storage form of vitamin D in the liver, similarly augments calcium absorption in the small intestine [7]. Prior research has evidenced the existence of disrupted calcium metabolism in patients with endometriosis [8], with serum calcium levels significantly lower in these patients compared to controls [9]. Moreover, several in vitro studies have endeavored to inhibit endometriosis at the cellular level by modulating calcium homeostasis [10,11]. These observational and experimental studies imply that calcium homeostasis may play a pivotal role in the pathogenesis of endometriosis. However, current studies have yielded conflicting conclusions. For instance, Joanna et al. discovered that dairy products fortified with calcium and 25(OH)D might mitigate the risk of developing endometriosis [12]. In contrast, a recent systematic review encompassing four human studies, four animal studies, and four in vitro studies found no benefit of vitamin D supplementation in patients with endometriosis or dysmenorrhea [13]. Therefore, further clarification of the relationship between calcium homeostasis and endometriosis risk could have significant implications for the prevention and treatment of endometriosis. In recent years, Mendelian randomization (MR) studies have increasingly been employed to investigate causality in observational studies, which may be a viable approach to address this issue.

MR analysis is an innovative statistical method that utilizes genetic variants (i.e., single nucleotide polymorphisms (SNPs)) as instrumental variables (IVs) to assess the causal relationship between an exposure of interest and an outcome [14]. Given that these SNPs are randomly assigned at conception and exert lifelong effects, MR can effectively circumvent the confounding bias inherent in traditional epidemiological studies [15,16]. Drawing on previous articles employing Mendelian randomization to study calcium homeostasis [17,18] and the availability of data in existing genome-wide association studies (GWASs) databases, our study selected calcium, 25(OH)D, and PTH as calcium homeostasis regulatory factors to address the following two key questions via MR analysis: (1) what is the association between calcium homeostasis regulatory factors and endometriosis; (2) what is the impact of calcium levels on different types of endometriosis.

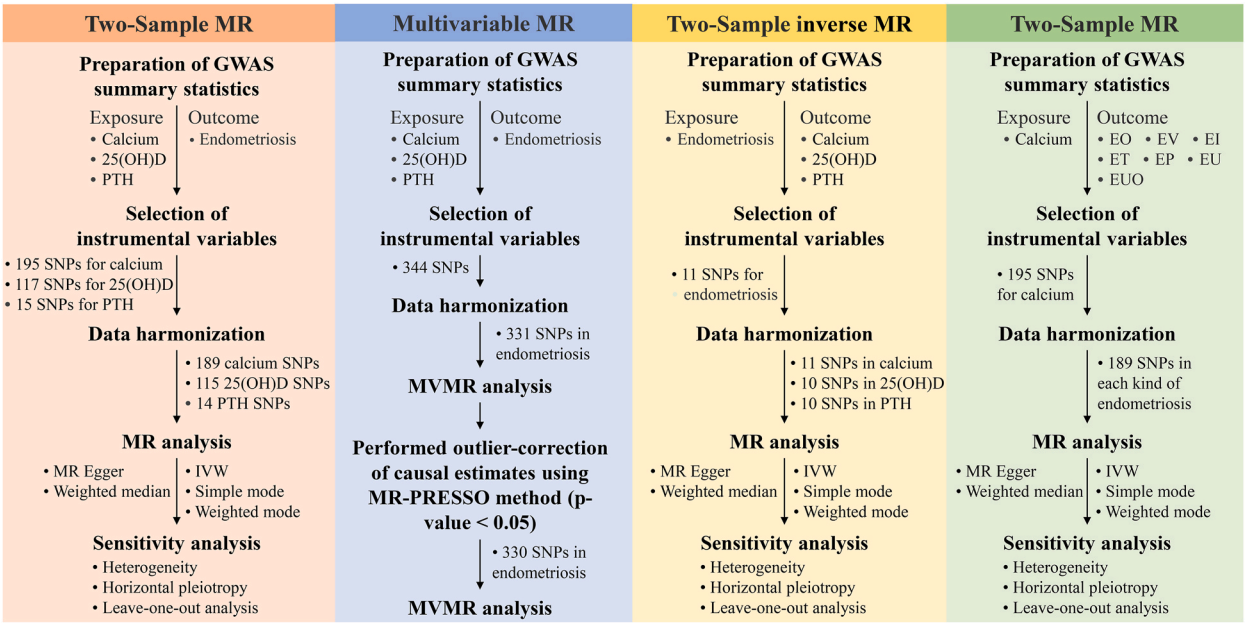


Fig. 2. Overview and rationale of the research design. Notes: EO, Endometriosis of ovary; EV, Endometriosis of rectovaginal septum and vagina; EI, Endometriosis of intestine; ET, Endometriosis of fallopian tube; EP, Endometriosis of pelvic peritoneum; EU, Endometriosis of uterus (Adenomyosis); EUO, Unspecified/other endometriosis.

2. Materials and methods

2.1. Study design

This study utilizes Mendelian randomization, a technique for causal inference, to investigate the relationship between calcium homeostasis regulators (calcium, 25(OH)D, and PTH) and endometriosis, leveraging publicly accessible GWAS datasets. We initially conducted a two-sample MR analysis, followed by the application of both single univariate MR (SVMR) and multivariate MR (MVMR) methods to the SNPs of each calcium homeostasis regulator to enhance the robustness of the findings. Concurrently, a reverse MR analysis was implemented to examine the effects of endometriosis on calcium homeostasis in patients. This reverse analysis aims to shed light on the underlying mechanism linking the two. Furthermore, we delved into the association between calcium levels and various categories of endometriosis, specifically endometriosis of the ovary (EO), endometriosis of rectovaginal septum and vagina (EV), endometriosis of intestine (EI), endometriosis of fallopian tube (ET), endometriosis of pelvic peritoneum (EP), unspecified/other endometriosis (EUO), endometriosis of uterus (EU, i.e. adenomyosis). The research design and rationale are depicted in Fig. 2.

2.2. Data source

The data for our study were sourced from the IEU OpenGWAS database (https://gwas.mrcieu.ac.uk/, accessed on 01/2024), which currently encompasses 346, 549, 749,467 genetic associations from 50,044 pooled GWAS datasets available for researcher query or download. Among these, the GWAS data for endometriosis (8288 cases and 68,969 controls) were procured from the FinnGen Research Program's Finnish Biobank. Endometriosis was defined using physician diagnostic information from International Classification of Diseases (ICD) codes 8 through 10 (i.e., ICD-10 N80, ICD-9 617, and ICD-8 6253). Data on specific categories of endometriosis were also extracted from the FinnGen public database, which included EO (3231 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.1 and ICD-9 6171), EV (1360 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.4, ICD-9 6174, and ICD-8 6253), EI (177 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.5, and ICD-9 6175), ET (116 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.2, ICD-9 6172, and ICD-8 6253), EP (2953 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.3, ICD-9 6173, and ICD-8 62531), EUO (1435 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.8/N80.80/N80.81/N80.89/N80.9, ICD-9617 [8,9], and ICD-8 6253 [8, 9]), and EU (2372 cases, 68,969 controls, diagnostic criteria: ICD-10 N80.0, ICD-9 6170, and ICD-8 62533).

We sourced GWAS data for PTH from a previously published GWAS dataset of European families (prot-a-2431) [19]. Summary statistics of serum 25(OH)D levels were derived from the European Bioinformatics Institute (EBI) GWAS data with a sample size of 496, 946 Europeans (EBI-a-GCST90000618), and blood samples were quantified using a chemiluminescent immunoassay (Diasorin Liaison) for 25(OH)D levels [20]. GWAS data for calcium levels were obtained from the UK Biobank (UKBB) Neale Laboratory's summary statistics (ukb-d-30680_irnt) for 315,153 European participants.

There was no overlap in samples between the exposure and outcome datasets, and all data samples were of European ancestry. All

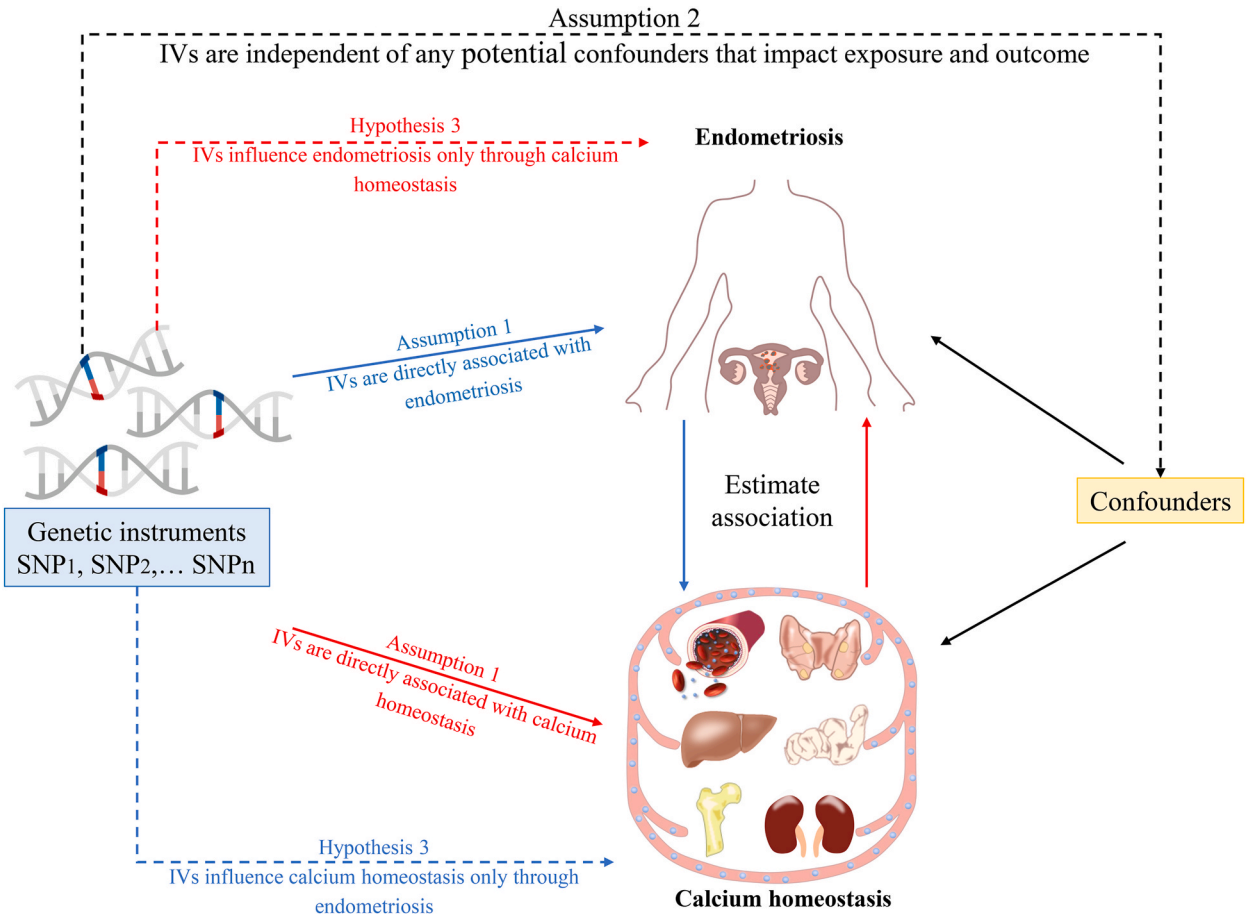


Fig. 3. Assumptions of the bidirectional Mendelian randomization (MR) study in this article. Red lines represent forward MR, while blue lines indicate reverse MR.

original studies secured ethical approval and informed consent records. The details of the utilized data sources are presented in Table 1.

2.3. Instrumental variables (IVs) selection

Selection of Instrumental Variables (IVs) IVs were derived from the exposure dataset for MR analysis through the following quality control steps to ensure the precision of the causal inference between the exposure and outcome factors.

1) Genome-wide significant SNPs for exposure traits were selected (Two-sample MR and inverse MR: $p < 5 \times 10^{-8}$ for calcium/25(OH)D/endometriosis, $p < 5 \times 10^{-6}$ for PTH; MVMR: $p < 5 \times 10^{-6}$ for calcium/25(OH)D/PTH/endometriosis).
2) The chosen SNPs were tested for linkage disequilibrium (LD) to validate the data. We utilized a clumping procedure to filter independent SNPs (clumping distance = 10,000 kb and $r^2 < 0.001$ threshold).
3) SNPs with F-statistics<10 was excluded. The formula for F- statistic was as follows:

$$F = \frac{R^2 \times (N - K - 1)}{K \times (1 - R^2)}$$

where N represents the sample size of the included GWAS data, K denotes the number of SNPs subjected to MR analysis, and R2 corresponds to the variance associated with each exposure SNP.

4) SNPs associated with confounders/outcomes at the genome-wide significance level ($p < 1 \times 10^{-5}$) were excluded using the Phenoscanner tool [21]. Upon obtaining the IVs, we extracted crucial information of SNP instruments from the GWAS outcome data.

Exposure Traits	Methods	OR	95%LCI	95%UCI	*P* Value
25(OH)D	MR Egger	1.218399	0.9358203	1.586304	0.145074
	IVW	1.004382	0.8452704	1.193444	0.960373
PTH	MR Egger	1.115792	0.8828838	1.410143	0.37709
	IVW	0.975762	0.9006119	1.057183	0.548466
Calcium	MR Egger	1.088325	0.8780806	1.348910	0.440596
	IVW	1.149646	1.0242314	1.290418	**0.017969**

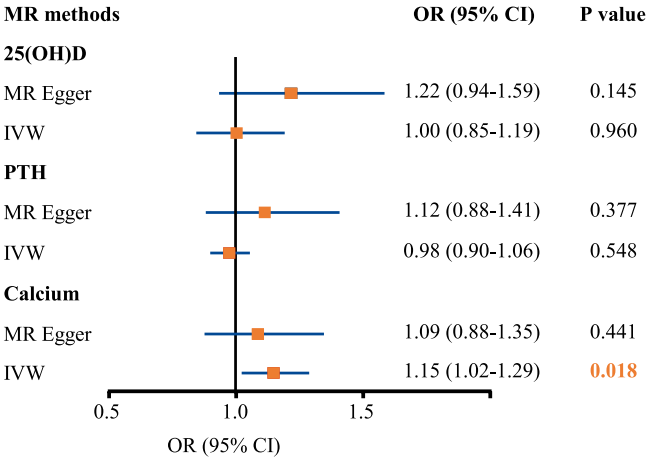


Fig. 4. Table and forest plot illustrating the effects of three calcium homeostasis regulators (25(OH)D, PTH, and calcium) on endometriosis. The charts display results from the Inverse Variance Weighting (IVW) and MR Egger methods. Horizontal bars denote 95 % confidence intervals (CIs). Significant P values are highlighted in orange.

5) Lastly, we proceeded with data harmonization and excluded those palindromic and ambiguous SNPs with intermediate allele frequencies (i.e., allele frequencies ranging from 0.01 to 0.30) [22]. By rigorously screening for IVs, we minimized the weak associations between potential confounders and genetic variants.

2.4. Mendelian randomization analysis

The MR analysis hinges on three fundamental assumptions [23]: 1) Correlation Assumption: The SNPs selected as IVs should exhibit a strong correlation with the exposure, thereby circumventing weak instrumental bias in the model. This correlation is determined by the p and LD values of the SNPs, with a strong correlation necessitating an F-statistic >10 [24]; 2) Exclusion Assumption: Each IV should influence the outcome solely through the exposure and not via any other pathways; 3) Independence Assumption: The chosen IVs should be independent of any potential confounders of the exposure and outcomes. The bidirectional MR study in this paper adheres to these assumptions, as depicted in Fig. 3.

In this study, we employed Inverse Variance Weighted (IVW) as the primary method to probe the potential relationship between calcium homeostasis regulators and endometriosis. We also implemented four validated methods, namely MR-Egger, Weighted Median, Weighted Mode, and Simple Mode, to estimate the robust effects. *Zhang* et al. provided an explanatory note on these five approaches [25]. We further applied MVMR to dissect the effect of the levels of potential calcium homeostasis regulators on the causal estimation of endometriosis. SNPs associated with specific calcium homeostasis regulators were selected as candidate IVs for subsequent MVMR analysis, with $p < 5 \times 10^{-6}$ serving as the cutoff value. The remaining screening criteria were as previously described. For the horizontal pleiotropy test under MVMR, we utilized the MR-PRESSO method. If the p value of the global test in MR-PRESSO results exceeded 0.05, it indicated the absence of horizontal pleiotropy. If it existed, the outliers should be removed and MR-PRESSO analysis should be performed again until the *p* value was less than 0.05, which was then followed by MVMR analysis.

2.5. Sensitivity analyses

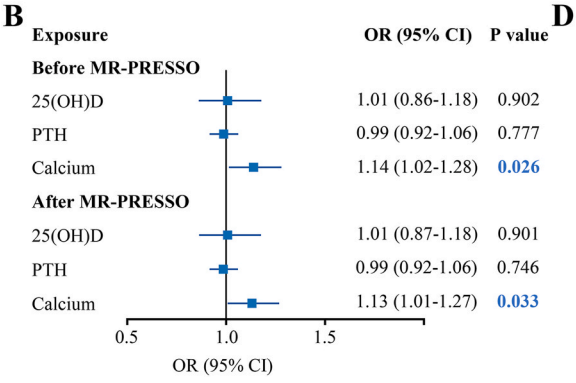
Sensitivity analyses were conducted to verify the reliability of the MR analysis results. Firstly, Cochran's Q test was utilized to quantify the heterogeneity among SNPs, with a *p*-value exceeding 0.05 indicating the absence of heterogeneity. Secondly, the MR-Egger regression test was employed to ascertain the presence of horizontal pleiotropy via the intercept index. If horizontal pleiotropy was detected in selected SNPs, the analysis was reiterated after the removal of those pleiotropic SNPs. Finally, a leave-one-out analysis was performed to evaluate the impact of individual SNPs on the population, verifying the presence of outliers that might significantly influence the results.

All MR analyses were executed in R (version 4.2.2) software using the R packages "TwoSampleMR" and "MRPRESSO". Scatter plots, forest plots, and funnel plots were employed to visualize the MR analysis results. Estimates were expressed as an odds ratio (OR) if the outcome indicator was a binary variable, or as a beta value (representing the effect size of the SNP on the phenotype) if it was a continuous variable.

A

	Exposure	OR	95%LCI	95%UCI	P Value
Before MR-PRESSO	25(OH)D	1.009901	0.863717	1.180828	0.901709
	PTH	0.98943	0.919377	1.064821	0.776691
	Calcium	1.141481	1.016119	1.28231	**0.025787**
After MR-PRESSO	25(OH)D	1.009809	0.865353	1.178379	0.901372
	PTH	0.988091	0.918984	1.062394	0.746034
	Calcium	1.132831	1.009806	1.270844	**0.033472**

B



C

MR-PRESSO-1	Exposure	MR analysis	Casual estimate	Sd	T-Stat	P Value
Main MR	25(OH)D	Raw	0.010	0.080	0.124	0.902
	PTH	Raw	-0.011	0.037	-0.284	0.777
	Calcium	Raw	0.132	0.059	2.229	**0.026**
	25(OH)D	Outlier-corrected	NA	NA	NA	NA
	PTH	Outlier-corrected	NA	NA	NA	NA
	Calcium	Outlier-corrected	NA	NA	NA	NA
RSSobs of Global Test in MR-PRESSO results	381.591					
P value of Global Test in MR-PRESSO results	0.042					

D

MR-PRESSO-2	Exposure	MR analysis	Casual estimate	Sd	T-Stat	P Value
Main MR	25(OH)D	Raw	0.010	0.079	0.124	0.901
	PTH	Raw	-0.012	0.037	-0.324	0.746
	Calcium	Raw	0.125	0.059	2.126	**0.034**
	25(OH)D	Outlier-corrected	NA	NA	NA	NA
	PTH	Outlier-corrected	NA	NA	NA	NA
	Calcium	Outlier-corrected	NA	NA	NA	NA
RSSobs of Global Test in MR-PRESSO results	370.957					
P value of Global Test in MR-PRESSO results	0.078					

Fig. 5. (A–B) Results of multivariate Mendelian randomization (MR) analysis with and without MR-PRESSO, along with the corresponding forest plot; (C) First MR-PRESSO analysis indicating horizontal pleiotropy; (D) Second MR-PRESSO analysis after removing outlier instrumental variables (IVs), demonstrating the absence of horizontal pleiotropy.

3. Results

3.1. Selection of instrumental variables

Employing our stringent screening criteria, we obtained 189 significant IVs for the two-sample MR between calcium levels and endometriosis and its categories. Concurrently, 115 and 14 independent and genome-wide significant SNPs were utilized to construct IVs for the two-sample MR of 25(OH)D and PTH on endometriosis, respectively. In the inverse MR analysis, 11 SNPs of endometriosis were identified. Please refer to **Supplementary Data Sheet 1** for further details.

3.2. SVMR and MVMR

We initially conducted a two-sample MR analysis to estimate the effects of three calcium homeostasis regulators on endometriosis risk. The MR estimates for the different methods are displayed in **Supplementary Data Sheet 2.** The two-sample MR analysis unveiled a robust relationship between genetically predicted calcium levels and endometriosis risk (IVW: OR = 1.15, 95 % CI: 1.02–1.29, p = 0.018) (Fig. 4). However, the remaining four models exhibited no significant association, and the other calcium regulators (25(OH)D and PTH) had no significant impact on endometriosis ($p > 0.05$). The findings suggest that only calcium levels were positively associated with endometriosis.

We tested for horizontal pleiotropy in the MR Egger intercept test and found no significant evidence (P-intercept = 0.553) (Fig. S1A, upper left panel). Heterogeneity was assessed by Cochrane's Q test, which demonstrated heterogeneity in endometriosis for calcium (MR Egger: Cochran's Q = 220.64, P-heterogeneity = 0.047; IVW: Cochran's Q = 221.06, P-heterogeneity = 0.050), despite the macroscopic funnel diagram showing a relatively symmetrical distribution on both sides (Fig. S1A, lower left panel). Further leave-one-out analyses were performed to identify potential outliers in the instrumental variables, and the leave-one-out chart suggested that the positive association between calcium levels and endometriosis was highly stable and unlikely to be influenced by individual SNPs (Fig. S1A, right panel). **Supplementary Data Sheet 2** contains detailed information on each of these sensitivity analyses.

The MVMR analysis were performed to estimate the effects of calcium levels on endometriosis after adjusting for 25(OH)D and PTH, which revealed a positive correlation that remained significant (Fig. 5A and B, OR = 1.14, 95 % CI: 1.02–1.28, p = 0.026). To ensure the reliability of the MVMR, we implemented MR-PRESSO for further analysis, setting the value of NbDistribution to 10,000 iterations, and detected horizontal pleiotropy (Fig. 5C, RSSobs of Global Test in MR-PRESSO results = 381.591, P value of Global Test in MR-PRESSO results = 0.042), and an outlier (rs28520334). Consequently, we performed MR-PRESSO again after removing the

Outcome Traits	Methods	β	95%LCI	95%UCI	P Value
25(OH)D	MR Egger	0.033929	0.000676	0.067181	0.080533
	IVW	0.013547	0.003783	0.023311	**0.00654**
PTH	MR Egger	-0.30781	-0.8076	0.191982	0.261864
	IVW	-0.02331	-0.17432	0.127703	0.762251
Calcium	MR Egger	0.03735	-0.02894	0.103642	0.29811
	IVW	0.020866	0.001431	0.0403	**0.035349**

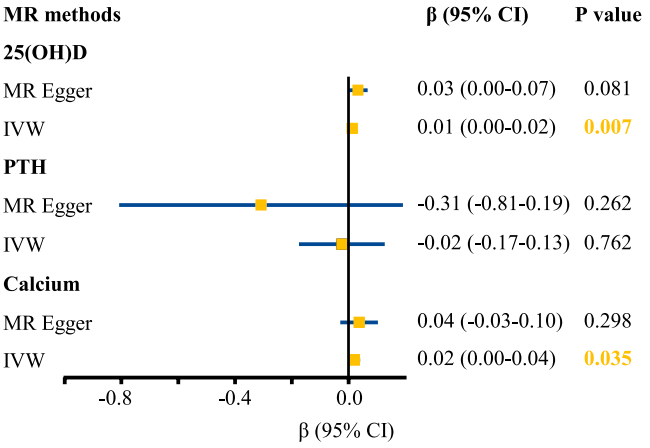


Fig. 6. Inverse Mendelian randomization (MR) analysis. The table and forest plot illustrate the effects of endometriosis on three calcium homeostasis regulators (25(OH)D, PTH, and calcium). Results from the Inverse Variance Weighting (IVW) and MR Egger methods are presented. Horizontal bars represent 95 % confidence intervals (CIs). Significant P values are highlighted in yellow.

Outcome Traits	Methods	OR	95%LCI	95%UCI	P Value
EO	MR Egger	1.006363	0.7157919	1.414889	0.9709335
	IVW	1.129968	0.9405162	1.357581	0.1918874
EV	MR Egger	1.1588043	0.7242811	1.854014	0.53950751
	IVW	1.2398018	0.9630812	1.596032	0.09530023
EI	MR Egger	0.4002257	0.12001593	1.334661	0.1378518
	IVW	0.8252414	0.43136783	1.578753	0.5616849
ET	MR Egger	1.6740735	0.3539012	7.918939	0.5165683
	IVW	1.2809649	0.5546558	2.958359	0.5620352
EP	MR Egger	1.0705172	0.7849536	1.459968	0.667361
	IVW	1.1023966	0.9325261	1.303211	0.2535387
EU	MR Egger	1.082108	0.7558406	1.549211	0.66695112
	IVW	1.225207	1.0099780	1.486303	**0.03932846**
EUO	MR Egger	0.9770209	0.6336725	1.506409	0.9163016
	IVW	1.0232461	0.8105507	1.291754	0.8467315

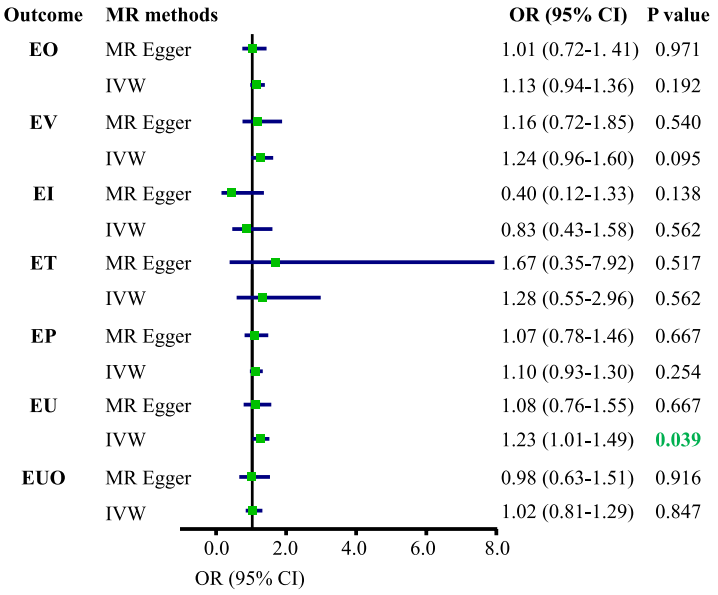


Fig. 7. Category-specific Mendelian randomization (MR) analysis. The table and forest plot depict the effects of calcium levels on various endometriosis categories, including endometriosis of ovary (EO), endometriosis of rectovaginal septum and vagina (EV), endometriosis of intestine (EI), endometriosis of fallopian tube (ET), endometriosis of pelvic peritoneum (EP), endometriosis of uterus (EU, Adenomyosis) and Unspecified/other endometriosis (EUO). Results from the IVW and MR Egger methods are displayed. Horizontal bars indicate 95 % confidence intervals (CIs). Significant P values are marked in green.

outlier, and eliminated horizontal pleiotropy (Fig. 5D, RSSobs of Global Test in MR-PRESSO results = 370.957, P value of Global Test in MR-PRESSO results = 0.078). Based on this, we repeated the MVMR analysis, and the association between calcium levels and endometriosis remained significant after correcting for other calcium homeostatic modifiers (Fig. 5A and B, **Supplementary Data Sheet 2**, OR = 1.13, 95 % CI: 1.01–1.27, $p = 0.033$).

By integrating the results of the SVMR and MVMR analyses, we concluded that genetically predicted calcium levels are associated with an increased risk of endometriosis, and that calcium levels constitute a risk factor for the development of endometriosis.

3.3. Inverse MR

We conducted an inverse MR analysis to explore the effects of endometriosis on body calcium homeostasis. Endometriosis was considered as the exposure, with calcium levels, 25(OH)D, and PTH as outcomes. We identified 11 SNPs ($p < 5 \times 10^{-8}$) closely

associated with endometriosis risk, and discovered an association between endometriosis and 25(OH)D (β = 0.01, 95 % CI: 0.00–0.02, $p = 0.007$) and calcium (β = 0.02, 95 % CI: 0.00–0.04, $p = 0.035$). The inverse analysis of endometriosis and PTH was not significant (Fig. 6, **Supplementary Data Sheet 2**).

A sensitivity analysis was performed, and no significant evidence of horizontal pleiotropy was detected in either calcium (P-intercept = 0.621) (scatter plot in Fig. S1B) or 25(OH)D (P-intercept = 0.244) (scatter plot in Fig. S1C). Heterogeneity in endometriosis for calcium was revealed (MR Egger: Cochran's Q = 27.27, P-heterogeneity = 0.001; IVW: Cochran's Q = 28.07, P-heterogeneity = 0.002), but not in 25(OH)D (MR Egger: Cochran's Q = 4.84, P-heterogeneity = 0.775; IVW: Cochran's Q = 6.42, P-heterogeneity = 0.698). The funnel plot results were not intuitive due to the small number of IVs included in the inverse MR analysis (funnel plots in Figs. S1B and S1C). The leave-one-out analysis showed a high overall stability (the red line representing the overall effect did not cross the null line) (leave-one plots in Figs. S1B and S1C). **Supplementary Data Sheet 2** contains the details of the above results.

3.4. Calcium levels and endometriosis categories

Given that endometriotic lesions can manifest in various body locations, we gathered GWAS aggregated statistics from the FinnGen database for six categories of endometriosis, encompassing EO, EV, EI, ET, EP, EU, and EUO. Building upon prior research, we employed a two-sample MR analysis to further probe the effects of genetically predicted calcium levels on different categories of endometriosis. The results indicated that calcium levels were positively associated solely with EU, i.e., adenomyosis (IVW: OR = 1.23, 95 % CI: 1.01–1.49, $p = 0.038$), with no evidence of a causal relationship with the risk of other endometriosis categories (Fig. 7, **Supplementary Data Sheet 2**). Fig. S1D visualizes the sensitivity analyses between calcium levels and EU, including scatter plots (horizontal pleiotropy), funnel plots (heterogeneity), and leave-one-out analyses (stability).

4. Discussion

We conducted a Mendelian Randomization (MR) study to investigate the estimated association of serum 25-hydroxyvitamin D (25(OH)D), calcium, parathyroid hormone (PTH), and endometriosis and its categories, utilizing genetic data from the EBI, UKBB, FinnGen databases, and published GWAS literature. To the best of our knowledge, this is the inaugural MR study to examine the relationship between calcium homeostasis regulators and endometriosis risk, based on large-scale GWAS summary data. Our findings indicated that higher genetically predicted calcium levels were associated with an increased risk of endometriosis, particularly in situ endometriosis, and inverse MR analysis suggested that endometriosis influences the levels of 25(OH)D and calcium.

Few prior studies have explored the role of 25(OH)D, calcium, and PTH as etiological factors of endometriosis. *Parazzini* et al. reported no association between the intake of high-calcium dairy products and endometriosis risk [26]. In contrast, a prospective cohort study by Harris et al., in 2013 [27], which has been widely cited in subsequent reviews [12,28], demonstrated that higher 25(OH)D levels and high calcium intake were associated with a lower risk of endometriosis, seemingly contradicting our findings. However, we note that this inverse association was only observed for women with symptomatic endometriosis causing abdominal pain, not for those diagnosed during infertility evaluation or those with asymptomatic endometriosis. *Sylvie* et al. proposed that this inverse association might be due to reverse causality, suggesting that women with unexplained abdominal pain who were suspected of lactose intolerance might avoid milk, leading to a lower intake of dairy products [29]. Reverse causality, coupled with the complexity and heterogeneity of endometriosis, increased the risk of bias in these observational studies.

Indeed, the conclusions of various observational studies differ due to disparate definitions of exposure and outcome, study designs, patient selection, and analytical methods. For instance, some studies suggest that vitamin D deficiency may contribute to endometriosis [9,30] while others report a positive association between high 25(OH)D levels and endometriosis risk [8,31]. However, the study by *Emanuela* et al. [6] and a recent systematic review of 21 studies [32] found no significant correlation between them, aligning with our MR study. We conclude that despite the beneficial effects of 25(OH)D on endometrial tissue in animal and in vitro studies, the genetic prediction of the role of 25(OH)D in endometriosis diagnosis and treatment based on GWAS data remains inconclusive.

In fact, previous observational studies could not definitively determine whether calcium or 25(OH)D influences the development of endometriosis, or vice versa. In contrast, MR analysis leverages natural genetic variation to assign individuals to different genotypes, simulating a randomized trial to evaluate the effect of a factor on a disease [14]. This approach bolsters the confidence of causal inferences by circumventing confounding and reverse causality inherent in observational studies [33], a strength further amplified by our study of multiple MR types. We also applied multiple rigorous methods to screen IVs and conduct various sensitivity analyses, such as heterogeneity, pleiotropy, and leave-one-out analyses, to affirm the robustness of the MR results. MR-Egger intercept analysis and F-statistics were also employed to mitigate horizontal pleiotropy and weak instrumental variable bias. In conclusion, our study revealed calcium level as a risk factor for endometriosis, and reducing calcium levels may provide a direction for future basic and clinical research on endometriosis. Larger multicenter randomized controlled prospective studies are needed to determine the effect and specific concentration of calcium on endometriosis and to validate our results through more multifaceted approaches (i.e., larger sample size GWAS data, computer prediction models [34,35], or basic biological experiments).

This study has some limitations. Firstly, we were unable to obtain instrumental variables (IVs) for PTH that met the genome-wide significance threshold (5×10^{-8}) for further studies. Therefore, when selecting IVs for PTH, we relaxed the cut-off value to 5×10^{-6}, which increased the risk of horizontal pleiotropy in MR analysis. However, even with this more lenient threshold, the F-statistics did not detect weak IVs. Secondly, while endometriosis is a disease specific to women, the GWAS data of calcium homeostasis regulators included in our study encompasses both men and women. There is currently no GWAS data exclusively for women, and although existing research has found no significant association between gender and 25(OH)D [20], indicating that it may not be affected by

Table 1
Basic characteristics of summary-level genome-wide association studies (GWASs) utilized in the Mendelian randomization (MR) analysis.

GWAS ID	Traits	Year	Ethnicity	Sample size	Cases	SNP size	Author/ consortium	Link
ebi-a-GCST90000618	Serum 25-Hydroxyvitamin D levels	2020	European	496,946	NA	6,896,093	Revez JA	https://gwas.mrcieu.ac.uk/datasets/ebi-a-GCST90000618/
ukb-d-30680_irnt	Calcium	2018	European	315,153	NA	13,585,309	Neale lab	https://gwas.mrcieu.ac.uk/datasets/ukb-d-30680_irnt/
prot-a-2431	Parathyroid hormone	2018	European	3301	NA	10,534,735	Sun BB	https://gwas.mrcieu.ac.uk/datasets/prot-a-2431/
finn-b-N14_ENDOMETRIOSIS	Endometriosis	2021	European	77,257	8288	16,377,306	FinnGen team	Trait: Endometriosis - IEU OpenGWAS project (mrcieu.ac.uk)
finn-b-N14_ENDOMETRIOSIS_OVARY	Endometriosis of ovary	2021	European	72,200	3231	16,376,686	FinnGen team	Trait: Endometriosis of ovary - IEU OpenGWAS project (mrcieu.ac.uk)
finn-b-N14_ENDOMETRIOSIS_RECTPVAGSEPT_VAGINA	Endometriosis of rectovaginal septum and vagina	2021	European	70,329	1360	16,376,472	FinnGen team	https://gwas.mrcieu.ac.uk/datasets/finn-b-N14_ENDOMETRIOSIS_RECTPVAGSEPT_VAGINA/
finn-b-N14_ENDOMETRIOSIS_INTESTINE	Endometriosis of intestine	2021	European	69,146	177	16,376,157	FinnGen team	https://gwas.mrcieu.ac.uk/datasets/finn-b-N14_ENDOMETRIOSIS_INTESTINE/
finn-b-N14_ENDOMETRIOSIS_FALLOPIAN_TUBE	Endometriosis of fallopian tube	2021	European	69,085	116	16,376,156	FinnGen team	https://gwas.mrcieu.ac.uk/datasets/finn-b-N14_ENDOMETRIOSIS_FALLOPIAN_TUBE/
finn-b-N14_ENDOMETRIOSIS_PELVICPERITONEUM	Endometriosis of pelvic peritoneum	2021	European	71,922	2953	16,376,599	FinnGen team	Trait: Endometriosis of pelvic peritoneum - IEU OpenGWAS project (mrcieu.ac.uk)
finn-b-N14_ENDOMETRIOSIS_NOS	Unspecified/other endometriosis	2021	European	70,404	1435	16,376,331	FinnGen team	https://gwas.mrcieu.ac.uk/datasets/finn-b-N14_ENDOMETRIOSIS_NOS/
finn-b-N14_ENDOMETRIOSIS_UTERUS	Endometriosis of uterus	2021	European	71,341	2372	16,376,529	FinnGen team	Trait: Endometriosis of uterus - IEU OpenGWAS project (mrcieu.ac.uk)

gender, there may still be gender bias. Thirdly, all the data in this study originate from European ancestry, and it remains to be verified whether our research results are applicable to other populations. Fourthly, in this study, we referred to other Mendelian randomization articles on calcium homeostasis [17,18], and included PTH, 25(OH)D, and serum calcium as calcium homeostasis regulators in this study. Another common calcium homeostasis regulator was excluded in the end due to the lack of sufficiently strongly correlated instrumental variables. Fifthly, due to our reliance on whole-genome association study (GWAS) data from public databases, we are currently unable to provide additional individual-level information, such as surgical staging of endometriosis for subjects/samples, as well as whether subjects/samples belong to the category of deep infiltrating endometriosis (DIE) or non-DIE. Consequently, we cannot delve deeper into exploring the relationship between calcium homeostasis and the severity of endometriosis. Lastly and most importantly, although Mendelian randomization studies based on SNPs have certain advantages in elucidating the causal relationship between exposure and outcome, helping to reduce genetic propensity and some confounding factors, Mendelian randomization still has limitations in causal inference, i.e., the genetic variation explained by SNPs only represents a part of the total variance of exposure, and all possible interference factors and the impact of horizontal pleiotropy cannot be completely ruled out. This means that even if our Mendelian randomization study confirms that calcium levels may increase the genetic risk of endometriosis, we still need to interpret the results cautiously. And in the future, if possible, further verification through large-scale randomized controlled studies.

5. Conclusion

This is the first multi-type Mendelian randomization study to explore the impact of calcium homeostasis on the risk of endometriosis. Our findings corroborate the association between calcium levels and endometriosis, indicating that elevated calcium levels are a risk factor for endometriosis. However, the findings should be interpreted cautiously due to certain limitations. Future multicenter randomized controlled trials and larger GWAS data are necessitated to confirm our results, and further investigation into the underlying biological mechanisms is warranted.

Conflicting interests

The authors declare that they have no competing interests.

Consent for publication and participation

Not applicable.

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Ethics approval

Not applicable.

Data availability statement

The data used in this study can be accessed through the links provided in Table 1. Additional data are included in the article/supplementary material. Further queries can be directed to the corresponding author.

CRediT authorship contribution statement

Zhi-Min Deng: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fang-Fang Dai:** Writing – original draft, Formal analysis, Data curation. **Rui-Qi Wang:** Methodology, Investigation, Formal analysis. **Gan-Tao Chen:** Writing – review & editing, Writing – original draft, Validation. **Xiao Yang:** Writing – review & editing, Validation, Formal analysis. **Yan-Xiang Cheng:** Writing – review & editing, Validation, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Yan-Xiang Cheng reports administrative support was provided by Cross-innovation talent project in Renmin Hospital of Wuhan University. Yan-Xiang Cheng reports statistical analysis was provided by Undergraduate education quality construction comprehensive reform project. Yan-Xiang Cheng reports administrative support was provided by National Natural Science Foundation of China.

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Appendix A. Supplementary data

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