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RESEARCH ARTICLE

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# An inverse causal relationship between serum 25-hydroxyvitamin D levels and pulmonary hypertension: A two-sample Mendelian randomization study

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**Abstract**

Observational studies have confirmed that 25-hydroxyvitamin D (25(OH)D) is associated with pulmonary hypertension (PH), but the causal association between each other is unclear. Therefore, Mendelian randomization (MR) method was performed to validate the causal association between PH and serum 25(OH)D levels. The summary data for 25(OH)D and PH were from the National Human Genome Research Institute-European Bioinformatics Institute. Catalog of human genome-wide association studies and FinnGen biobank consortium. MR analysis was utilized to explore the potential causal association between PH and 25(OH)D. To evaluate this association, inverse variance weighting was considered as the primary method. Cochran's Q test, MR-Egger intercept test, and "leave-one-out" sensitivity analyses were utilized to control the pleiotropy and heterogeneity in the study. Two-sample MR analysis revealed an inverse causal relationship between 25(OH)D and PH (odds ratio: 0.376, 95% confidence interval: 0.162–0.876, $p = 2.334 \times 10^{-2}$). There was no significant heterogeneity and pleiotropy. The present study confirmed the inverse causal relationship between 25(OH)D and PH. This pathway may provide another treatment pathway in PH. Further studies to elucidate this pathway is indicated.

**KEYWORDS**

genome-wide association study, Mendelian randomization, pulmonary hypertension, the causal link, vitamin D

Ce Chao and Min Wang contributed equally to this study.

## INTRODUCTION

Pulmonary hypertension (PH) is a serious and incurable pulmonary vascular disease and ultimately leading to right heart failure and death. Its pathological features are progressive increase in pulmonary artery resistance and obstructive vascular remodeling.[1,2] At present, numerous medications have received international approval for managing PH, including prostacyclin pathway drugs, nitric oxide pathway drugs, and endothelin pathway drugs.[3] Through PH-specific treatment, the 5-year survival rate of PH patients increased from 34% to over 60%.[4,5] However, PH remains progressive and fatal due to the lack of effective cure methods. Therefore, accurately controlling the PH risk factors is particularly important.

Vitamin D is a steroid hormone that is deficient in humans and is associated with conditions such as hypertension, cardiovascular events, myocardial infarction, stroke, and cancer.[6] It is biologically inactive and needs to function via two-step hydroxylation to 1,25-hydroxyvitamin D (25(OH)D). Furthermore, 25(OH)D is an intermediate product, and the level of vitamin D is typically assessed by measuring its concentration in the human body.[7] At present, observational studies have indicated that individuals diagnosed with PH exhibited lower levels of vitamin D in comparison to individuals who are in good health and that vitamin D levels showed a direct correlation with 6-min walk distance.[8] Supplementing vitamin D can ameliorate pathological right ventricular hypertrophy and improve the survival in rats with PH.[9] It is unclear whether 25(OH)D levels are associated with the incidence of PH. Previous studies have mostly been observational and susceptible to confounding factors. Therefore, further investigation is required to determine if there exists a causal connection between levels of 25(OH)D and PH.

Mendelian randomization (MR) is a method for assessing the causal relationship between risk factors and disease.[10,11] Allele frequencies are known to have been assigned from parents to offspring, and genotypes that are fixed during the formation of spermatovum are also unaffected by disease.[12] MR utilizes genetic variation as an instrumental variable (IV) and avoids interference from confounding factors that are difficult to control for in observational studies.[13,14] Thus, MR analysis is similar to a natural randomized controlled trial. This method of analysis excludes the possibility of reverse causation by assessing the causal relationship between exposure and outcome at the genetic level.[15,16] Genome-wide association studies (GWAS) have identified thousands of variants associated with complex exposures, which opens up the possibility of widespread use of MR.[17,18] In the present study, two-sample MR was used to evaluate the causal relationship between 25(OH)D levels and PH via GWAS summary statistics.

## MATERIALS AND METHODS

### Study design and data source

In this study, two-sample MR method was utilized to explore the causal connection between levels of 25(OH)D and PH (Table 1). MR analyses are based on the premise that IVs must fulfill three prerequisites: (1) be reliably associated with the risk factor studied (relevance assumption); (2) not be associated with any known or unknown confounders (independence assumption); and (3) affect outcomes only through the risk factor and not through any other direct causal pathway (exclusionary restriction assumption). Based on these criteria, MR was conducted to explore the causal relationship between 25(OH)D and PH. The whole process of the study consisted of five main steps: (1) obtaining exposure factor GWAS data, (2) screening for appropriate IVs, (3) inputting the outcome GWAS data and mapping the single-nucleotide polymorphisms (SNPs) of the above IVs, (4) preprocessing the exposure factor and the outcome GWAS data to ensure the consistency of the format, and (5) performing MR and sensitivity analyses.

The GWAS summary data for genetic variants linked to levels of 25(OH)D were obtained from 496,946 European participants.[19] The Diasorin assay was used to determine 25(OH)D levels. In the UKB, 25(OH)D concentrations below or above the assay validation range (10–375 nmol $L^{-1}$) were excluded. The average within-laboratory coefficient of variation (and standard deviation) ranged from 5.04 (4.73) to 6.14 (2.21). The distribution of 25(OH)D concentrations was right skewed and showed the seasonal fluctuations.

The summary-level genetics information for PH was obtained from the FinnGen biobank (Finn) consortium that included 208 PH patients and 243,756 controls from European[20] ancestry database (https://r8.finngen.fi/pheno/I9_HYPTENSPUL). The FinnGen database mainly includes people within the Nordic healthcare

**TABLE 1** Characteristics of data sources.

| Traits | Data sources | Sample size | Ancestry |
|---|---|---|---|
| 25-hydroxy vitamin D | PMID: 32242144 | 496,946 | European |
| Pulmonary hypertension | FinnGen | 208/243,756 | European |

system. All PH is primary PH according to ICD 8th–10th edition disease coding.

## IV

The eligible SNPs were regarded as IVs based on the following standards (Figure 1): (1) SNPs were strongly associated with 25(OH)D, (2) SNPs were unaffected by interfering variables, and (3) SNPs did not affect the outcome.[21] To meet these standards, the following operations were implemented. First, SNPs significantly associated with 25(OH)D ($p < 5 \times 10^{-8}$) and instrument strengths (F) of >10 were screened.[22,23] Second, SNPs in potential linkage disequilibrium (LD) with $r^2 \geq 0.001$ and LD distance of <10,000 kb were removed. Third, the PhenoScanner website (http://www.phenoscanner.medschl.cam.ac.uk/) was used to look up and exclude the SNPs directly associated with PH.[24] Finally, MR pleiotropy residual and outlier (MR PRESSO) test was used to screen and delete outlier SNPs.[25] According to the above criteria, multiple eligible SNPs closely related to exposure factors were screened as IVs.

## Statistical analysis

In the present study, all MR methods were carried out through "TwoSampleMR"[26] and "MR-PRESSO"[25] package in R software (version 4.2.3, USA).

Inverse-variance weighting (IVW) method was mainly utilized to evaluate the causal relationship between 25(OH)D and PH, and weighted median, MR-Egger regression, simple mode, and weighted mode methods were also utilized to assist in the evaluation.[25,27] Among them, IVW is the most commonly used and the most important method. It utilized meta-analysis to obtain the overall estimate by combining the Wald estimates for each SNP, followed by forced intercept of zero in weighted linear regression. The final MR results were represented as odds ratio (OR) and 95% confidence interval (CI).

MR-Egger and IVW were employed in Cochran's Q statistics to evaluate the heterogeneity of IVs in the GWAS database outcomes, where $p > 0.05$ showed no significant heterogeneity.[28] The pleiotropy test was carried out by MR-Egger intercept, where $p > 0.05$ indicated that the IV had no significant pleiotropy in the GWAS data set outcomes.[29] The MR-PRESSO methods were used to detect the outlier SNPs and horizontal pleiotropy. To ensure the stability of MR results, the leave-one-out method was utilized to avoid SNP bias.

# RESULTS

According to the screening criteria for IVs, 106 SNPs significantly associated with 25(OH)D were considered IVs with no linkage imbalances and outliers, and all F-statistic values were >30 (Supporting Information S1: Table S1). The rs2511279, rs57601828, and rs7955128 were removed for incompatible alleles or being palindromic with intermediate allele frequencies.

In IVW, SNPs associated to serum 25(OH)D levels were inversely correlated with incidence of PH and the relative risk of PH was reduced by 62.4% for every one standard deviation increase in 25(OH)D level (OR: 0.376, 95% Cl: 0.162–0.876, $p = 2.334 \times 10^{-2}$). Therefore, a decrease in abnormal serum 25(OH)D levels increases the risk of developing PH. Other MR estimates showed similar results but with larger CIs due to lower statistical power (Table 2). A scatter plot also demonstrated that elevated serum 25(OH)D levels reduced the risk of PH

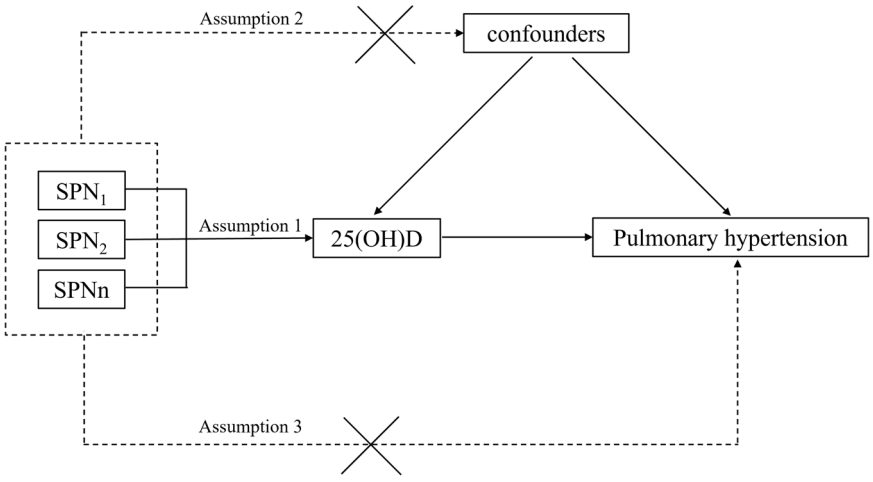


**FIGURE 1** Study design flowchart of the Mendelian randomization study. The Mendelian randomization method is based on three hypotheses: (1) the instrumental variables are closely related to exposure; (2) instrumental variables are independent of any confounding factor; (3) instrumental variables affect the results only through exposure but not through other ways.

**TABLE 2** MR of serum 25(OH)D levels and the risk of PH.

| Exposure | Outcome | MR method | SNP (*n*) | OR (95% CI) | *p* Value |
|---|---|---|---|---|---|
| 25(OH)D | PH | MR Egger | 106 | 0.655 (0.177, 2.424) | 0.527 |
| | | Weighted median | 106 | 0.911 (0.234, 3.419) | 0.890 |
| | | Inverse variance weighted | 106 | 0.376 (0.162, 0.876) | 0.023 |
| | | Simple mode | 106 | 0.020 (0.001, 0.328) | 0.007 |
| | | Weighted mode | 106 | 0.769 (0.228, 2.586) | 0.672 |

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; MR, Mendelian randomization; OR, odds ratio; PH, pulmonary hypertension; SNP, single-nucleotide polymorphism.

(Figure 2). The effect size and 95% CI of each SNP related to 25(OH)D levels were represented in the form of forest plots, and the overall effect evaluation of the MR-Egger and IVW models was also included (Figure 3).

Subsequently, sensitivity analysis was used to assess the pleiotropy and heterogeneity of the MR results. The Cochran's Q test results showed that there was no significant heterogeneity ($P_{\text{MR-Egger}} = 0.576$, $P_{\text{IVW}} = 0.571$, Table 3). Using MR-Egger intercept test, no significant gene directional pleiotropy was observed ($p = 0.280$). The outlier SNPs and horizontal pleiotropy were not noted by the MR-PRESSO test. The approximate symmetric funnel plot also confirmed this result (Figure 4). The results remained unchanged after removing one SNP in the sequence and evaluating the total effect of the remaining SNPs on PH (Figure 5).

## DISCUSSION

The present study is the first genetic research to evaluate the causal relationship between levels of serum 25(OH)D land PH using two-sample MR analysis. The final results demonstrated that the relative risk of PH decreased with genetically predicted increasing serum 25(OH)D levels. The role of vitamin D may be a key factor in the occurrence and development of PH.

Recently, more and more researches have explored the relationship between 25(OH)D and PH. The present study further validated the conclusions of prior observational studies, where the levels of serum vitamin D were lower in patients with PH compared to healthy individuals and approximately 68% of PH patients were diagnosed with vitamin D deficiency.[8,30] In addition, lower serum 25(OH)D levels can predict poor prognosis for patients with PH.[31] The present investigation supplemented and confirmed the findings regarding the harm of vitamin D deficiency in PH. In a family-based study, Bai et al. have reported that there was causal association between vitamin D deficiency and hypertension.[32] An MR study has also shown that increasing plasma 25(OH)D levels reduced the relative risk of hypertension.[33] These results suggest that vitamin D may be associated with altered vascular tone. A recent meta-analysis has confirmed that supplementation with vitamin D did not effect on cardiovascular disease or type 2 diabetes risk.[34] There also has been no clear conclusion on the clinical significance of vitamin D supplementation in PH treatment.[35] A clinical observation has suggested that vitamin D deficiency affected the effectiveness of sildenafil in treating PH, and restoring vitamin D levels improved this symptom.[28] Callejo et al. have explained that the lack of vitamin D may reduce nitric oxide-dependent cGMP production,[36] which also suggested that there is a benefit of vitamin D supplementation in patients with PH. MR analysis estimates the lifetime effects of exposure, which is not possible in randomized clinical trials. Therefore, MR analysis would be a good choice to answer the question of whether vitamin D supplementation is beneficial in preventing PH.[37] Based on existing literature, there may be multiple potential mechanisms between vitamin D and PH. In a rat model, vitamin D deficiency did not increase pulmonary artery pressure in normal oxygen conditions, and the same conclusion was reached in vitamin D receptor knockout mice.[38,39] However, vitamin D deficiency can lead to characteristic changes similar to those of PH in experimental animals, such as smooth muscle cell and endothelial dysfunction, increased muscularization, increased KCNE4 and survivin expression, increased sensitivity to Kv7 channels, downregulated TASK−1 channels, and reduced potassium two-pore domain channel subfamily K member 3 (KCNK3) expression.[38,40,41] Research has found that dietary supplementation with vitamin D significantly improved prognosis in rats with PH, which may be regulated by the eNOS signaling pathway. Increasing vitamin D levels can promote the expression of KCNK3 and the activity of

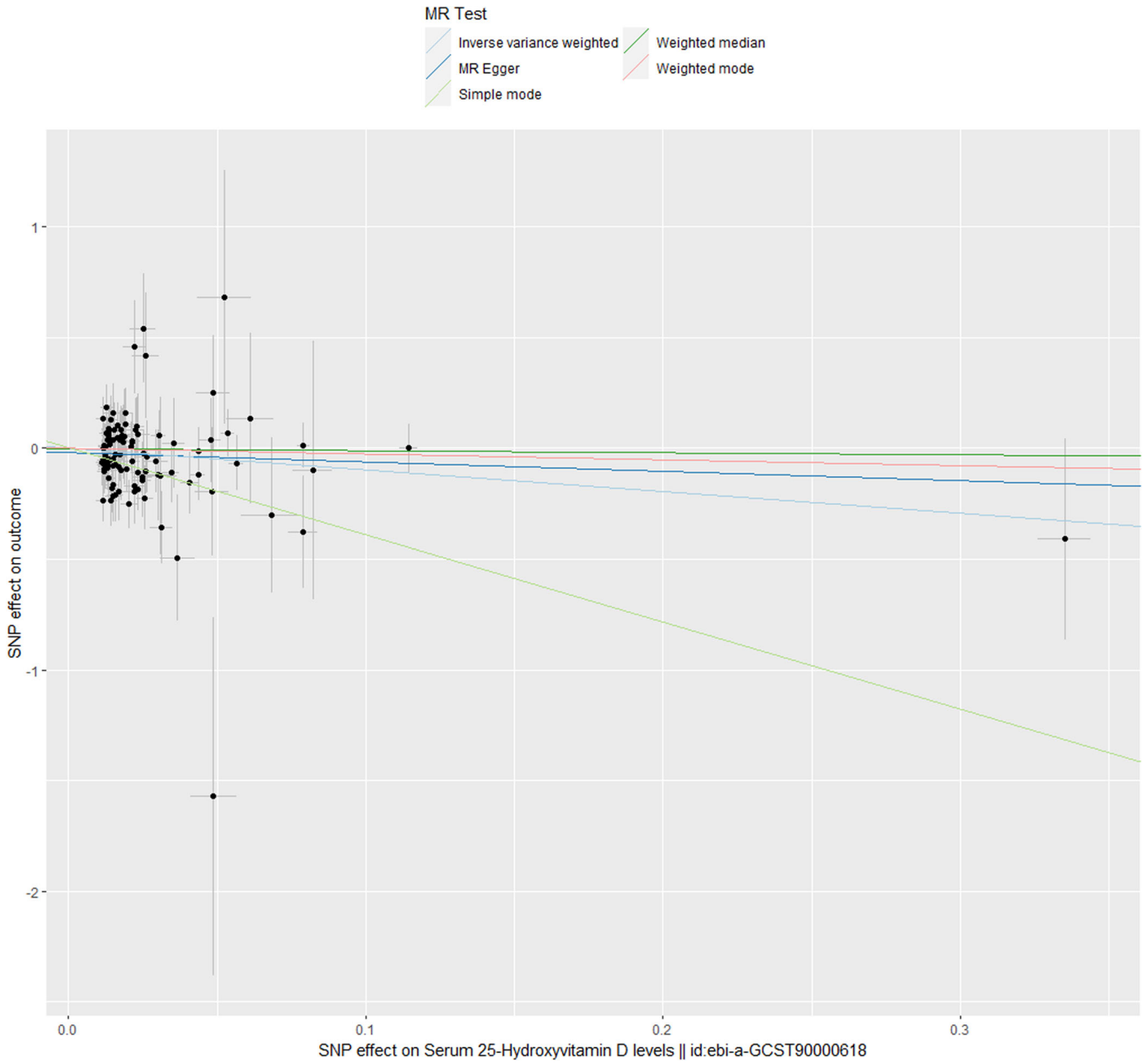


**FIGURE 2** Scatterplot of 25-hydroxyvitamin D (25(OH)D)-pulmonary hypertension (PH) risk in Mendelian randomization study. *X*-axis, the single-nucleotide polymorphism (SNP) effect and standard errors (SEs) on each of the selected SNPs from vitamin D 25(OH)D genome-wide summary association study (GWAS) data set. *Y*-axis, the SNP effect and SEs on PH from PH GWAS data sets. The dark blue vertical line represents the Mendelian randomization-Egger method-derived causal effect estimate, while the light blue line signifies the equivalent estimate derived via the inverse-variance weighting method.

TASK-1 channels, which may be targets for the treatment of PH.[39,41,42] Although restoring vitamin D levels cannot reduce the pulmonary artery pressure, it can improve the prognosis and some pathological changes in PH.[41,43] Therefore, supplementing with vitamin D has clinical benefits for PH patients. However, more clinical studies are needed to verify this statement.

The main advantage of the present study was the use of two-sample MR to avoid selection bias while using SNPs as IVs to exclude other confounding factors. However, the study has several limitations. First, all cohorts were from the European population, and it is unclear whether this conclusion applied to other populations and individuals. Second, there was only one measurement of serum 25(OH)D levels and the effect of the use of vitamin D supplements is not yet clear. Third, this study ignored the moderating effects of factors such as age, gender, and environment due to the fact that

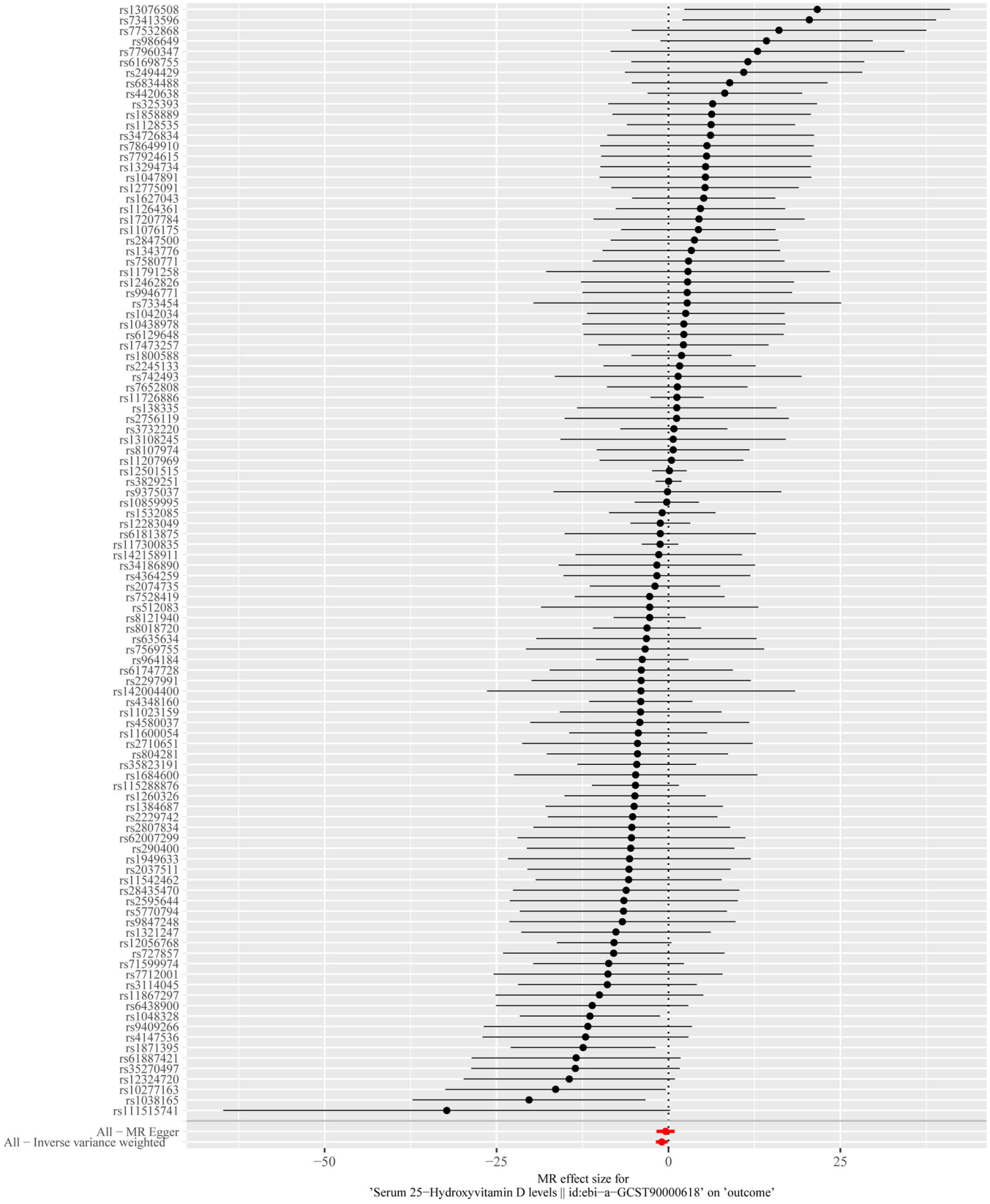


**FIGURE 3** Forest plot of 25-hydroxyvitamin D-pulmonary hypertension risk in Mendelian randomization study.

**TABLE 3** Sensitivity analysis of the causal association between 25(OH)D and the risk of pulmonary hypertension.

| Pleiotropy test MR-Egger | | | Heterogeneity test | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | MR-PRESSO | MR-Egger | | | Inverse-variance weighted | | |
| Intercept | SE | *p* Value | *p* Value | Q | Q_df | Q_*pval* | Q | Q_df | Q_*pval* |
| −0.020 | 0.018 | 0.280 | 0.563 | 100.59 | 104 | 0.576 | 101.77 | 105 | 0.571 |

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; *df*, degrees of freedom; MR, Mendelian randomization; PRESSO, pleiotropy residual and outlier.

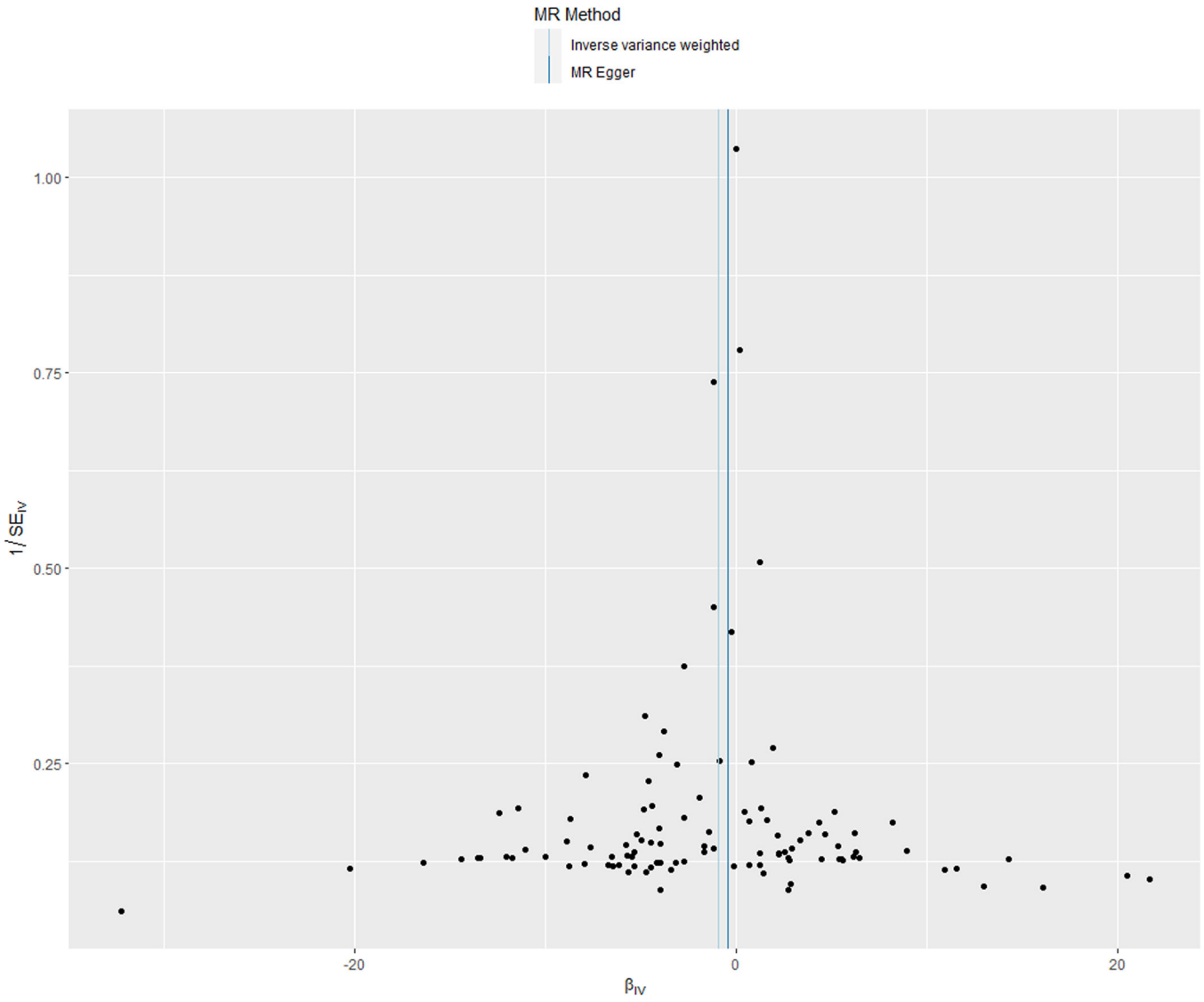


**FIGURE 4** Funnel plot of 25-hydroxyvitamin D-pulmonary hypertension risk in Mendelian randomization study.

25(OH)D expression levels in population data are influenced by age and seasonal factors. Fourth, PhenoScanner identifies disease-associated SNPs based on available data. Most of these data are from European populations. Therefore, some bias is unavoidable. Finally, MR analysis has some inherent shortcomings and is unable to eliminate the effects of confounding factors and horizontal pleiotropy.

## CONCLUSION

In conclusion, the present study confirmed the causal association between serum 25(OH)D levels and PH from a genetic perspective. Higher levels of serum 25(OH)D levels reduced the relative risk of PH. Supplementing with Vitamin D may show clinical benefits to PH patients.

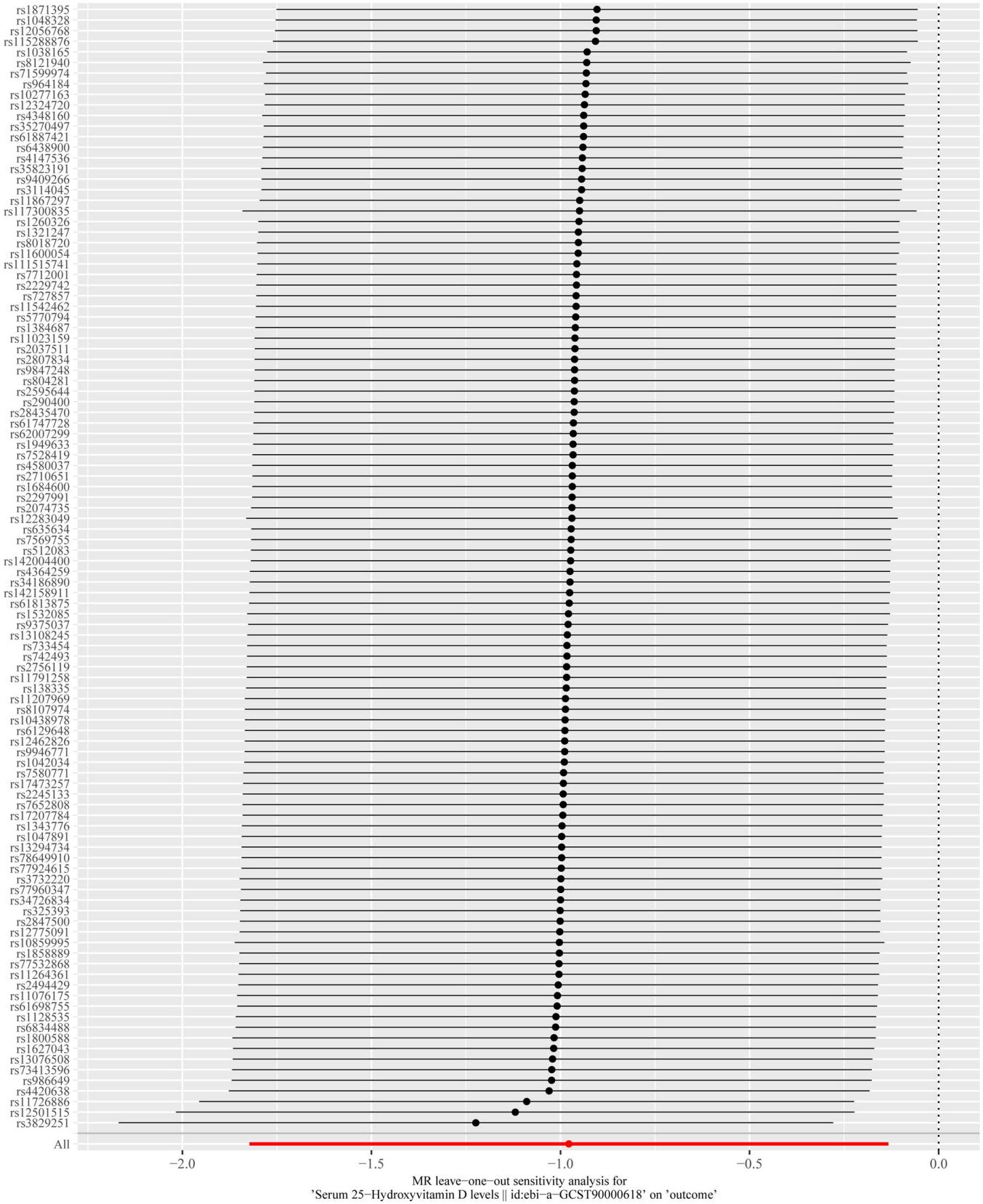


**FIGURE 5** Mendelian randomization leave-one-out sensitivity analysis for 25-hydroxyvitamin D on pulmonary hypertension.

## AUTHOR CONTRIBUTIONS

**Ce Chao**: Conceptualization; data curation; formal analysis; funding acquisition; methodology; software; writing—original draft. **Xiaoying Zhang**: Conceptualization; writing—review and editing. **Min Wang**: Data curation; formal analysis; methodology. **Kun Mei**: Data curation; formal analysis. **Chao Ma**: Methodology. **Yongxiang Qian**: Visualization. All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

All data in this study are from public databases, and the method of obtaining them has been described in the article. The analysis code can be obtained by contacting the first author.

## ETHCIS STATEMENT

The authors have nothing to report.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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