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ORIGINAL RESEARCH

Causal Associations of the Alterations in Peripheral Blood Immune Cell Characteristics on the Incidence of Osteoporosis: A Bidirectional Mendelian Randomization Study

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Purpose: Osteoporosis is closely related to specific immune cell, yet the causal mechanism has not been clarified. Previous studies mostly adopted traditional unidirectional Mendelian randomization (MR) for analysis, failing to fully clarify their relationship. This study is the first to analyze the bidirectional causal relationship between the characteristics of peripheral immune cells and the risk of osteoporosis.

Methods: A bilateral two-sample MR was performed, with immune cells serving as instrumental variables and the incidence of osteoporosis as the outcome. We used five algorithms to evaluate the causal relationship between immune cells and the incidence of osteoporosis (inverse-variance weighted [IVW], MR-Egger, simple mode, weight median, and weight mode). The Cochran Q and leave-one-out tests were used to evaluate heterogeneity and stability, and the MR-Egger intercept test was used to evaluate horizontal pleiotropy.

Results: The eosinophil percentage of granulocytes (odds ratio [OR] = 1.25, 95% confidence interval [CI] = 1.08–1.44, P = 0.002), eosinophil percentage of white cells (OR = 1.17, 95% CI = 1.02–1.35, P = 0.027), and sum eosinophil basophil counts (OR = 1.16, 95% CI = 1.02–1.32, P = 0.027) had positive causal associations with the incidence of osteoporosis. The lymphocyte counts (OR = 0.83, 95% CI = 0.71–0.97, P = 0.016), neutrophil percentage of granulocytes (OR = 0.78, 95% CI = 0.67–0.90, P < 0.001) played negative effect on osteoporosis. The reverse direction showed that osteoporosis had no causal effect on the characteristics of the immune cells. Non-significant heterogeneity and horizontal pleiotropy indicated the results were robust.

Conclusion: This study identified a unidirectional causal link between five immune cell traits and osteoporosis, providing new insights into osteoporosis pathogenesis and potential targeted immunotherapy.

Keywords: immune cells, osteoporosis, Mendelian randomization, genome-wide association study

Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and degeneration of the bone tissue, and it leads to a decline in patients' quality of life and an increase in medical expenses owing to the increased risk of fractures with aging,[1] which causes substantial harm and economic burden to society. The prevalence of osteoporosis worldwide is 21.7%, with the highest incidence in the elderly population of Asia, reaching 24.3% in a global meta-analysis of the incidence of osteoporosis in an elderly cohort.[2] Osteoporosis has become a prominent public health issue owing to the increasing aging population. Pharmacological treatments primarily involve antiresorptive drugs, such as bisphosphonates and denosumab, and anabolic agents, such as teriparatide and abaloparatide, which aim to restore the balance between bone resorption and formation. Notably, romosozumab, a monoclonal antibody targeting sclerostin, uniquely combines

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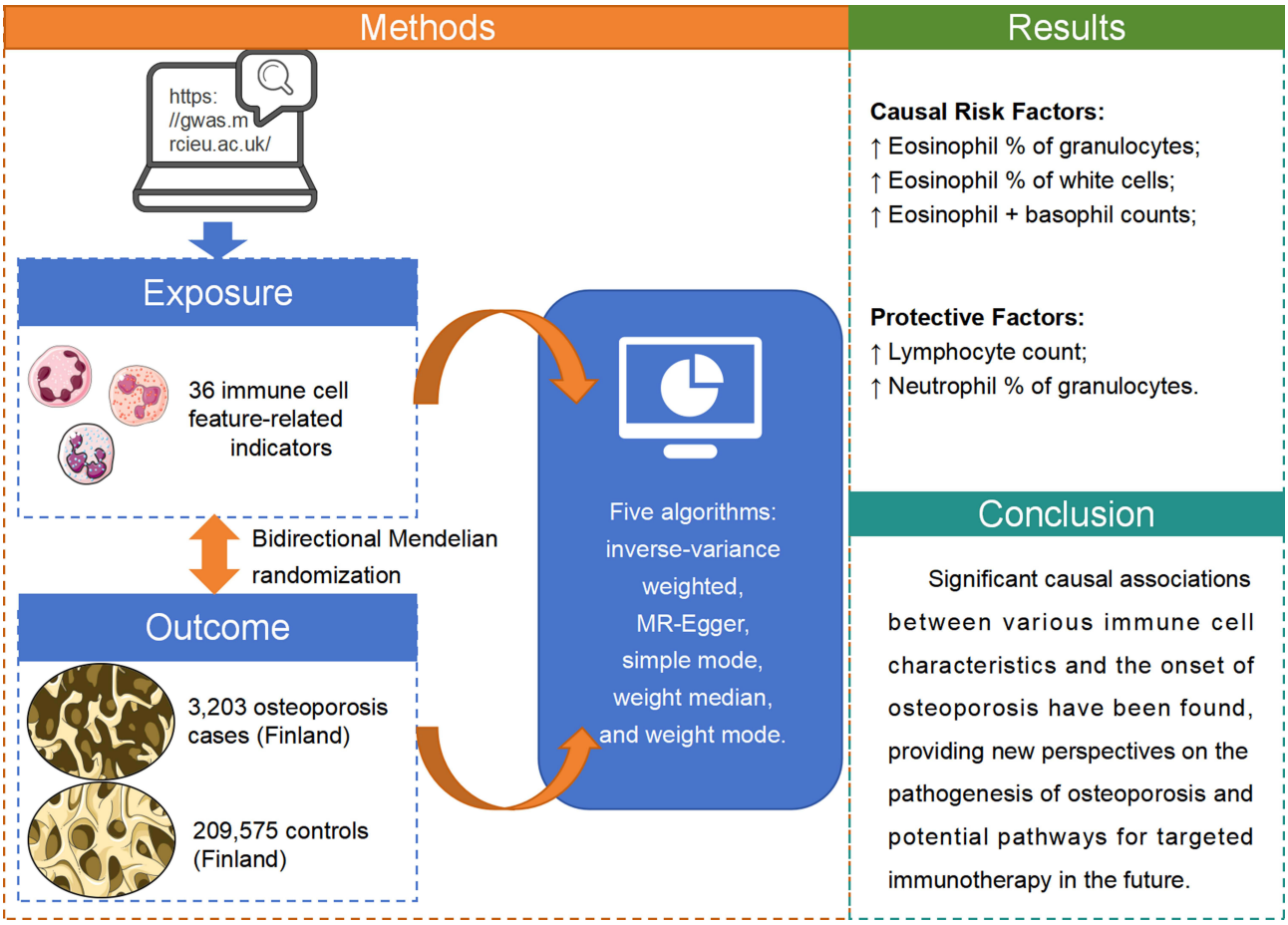
Graphical Abstract



dual-action effects by simultaneously stimulating bone formation and suppressing bone resorption, offering a novel therapeutic strategy to enhance bone density and reduce fracture risk in severe osteoporosis. However, it may lead to serious adverse events, including cardiovascular disease.[3,4] Although significant progress has been made in the diagnosis and treatment of osteoporosis, it is still difficult for patients to achieve long-term pathological reversal due to the incomplete elucidation of its pathological mechanism. Therefore, it is urgent to conduct in-depth research on the mechanism of osteoporosis.[5]

Recently, immunotherapy has received increasing interest as a novel therapeutic approach for osteoporosis,[6] as the immune system is intrinsically involved in bone metabolism.[7] In the concept of bone immunology, early scholars pointed out that inflammation could lead to increased bone resorption and subsequent bone loss.[8] Subsequently, Srivastava et al[9] introduced the term "immunoporosis", highlighting the key role of the immune microenvironment and innate and adaptive immune cells in osteoporosis. Innate immune cells, including macrophages, monocytes, dendritic cells, neutrophils, eosinophils, and mast cells, play a key role by producing inflammatory mediators that affect the activity of osteoclasts and bone resorption, and some of them are capable of transforming into osteoclasts.[10] Adaptive immune cells, such as B and T lymphocytes, can directly or indirectly regulate bone metabolism and affect bone remodeling through cytokine production.[11,12] Various immunotherapeutic targets are expected to play a role in inducing bone regeneration or inhibiting osteoclastogenesis.[13] T cell therapy could be considered an innovative treatment strategy for osteoporosis,[14] indicating that immune-based therapies have the potential to reverse this potentially devastating aging disease. However, the role of immune cells in the development of different types of osteoporosis varies owing to the

complexity of the immune system,[15] which poses difficulties for targeted immunotherapy in patients with osteoporosis. Eosinophils contribute to bone metabolism regulation by secreting interleukin (IL)-31, a cytokine implicated in transcriptional modulation of osteogenic pathways, with elevated IL-31 levels correlating with reduced bone mineral density(BMD) in postmenopausal populations.[16,17] Bone mineral density decreased with the decrease of CD8 T lymphocytes in senile osteoporosis.[18] Neutrophil-mediated inflammatory cascades, exemplified in rheumatoid arthritis (RA), drive bone destruction via sustained inflammation,[19] with clinical studies confirming that elevated IgM rheumatoid factor independently predicts amplified fracture risk in RA patients.[20] Accumulating evidence has established a significant association between HIV infection and an elevated risk of osteoporosis.[21] Research findings indicate that T cells infected with HIV exhibit altered cytokine production profiles, characterized by reduced osteoprotegerin (OPG) secretion and increased receptor activator of nuclear factor kappa - B ligand (RANKL) expression. This dysregulation of OPG/RANKL balance plays a pivotal role in the pathogenesis of bone loss among HIV-positive patients.[22] These observations underscore the critical role of immune cells in the etiopathogenesis of osteoporosis. Therefore, exploring the relationship between immune cells and osteoporosis is of great significance for further exploring the pathogenesis and developing new treatment plans.

Current observational studies encounter challenges in understanding the causal dynamics between immune dysregulation and the development of osteoporosis, as traditional approaches are inadequate for addressing reverse causation or residual confounding. Mendelian randomization (MR) is a research method that uses genetic variants as instrumental variables (IVs) to assess causal relationships between exposure factors and diseases. Because of the characteristics of Mendel's second law and the independent assortment law, it could reduce the bias caused by confounding factors and reverse causality and ensure a long-term impact from the identified causal relationship.[23] Observational studies have shown a close link between RA and osteoporosis (OP). However, no evidence of a causal relationship between RA and OP/BMD (bone mineral density) has been found in Mendelian randomization studies,[24] which contradicts the findings of traditional observational studies. The association between RA and OP/BMD may be related to secondary effects such as anti-rheumatic treatment and reduced physical activity. The previous observations are limited for causal inference due to the potential bias introduced by mixed factors, and MR can overcome these limitations to provide more robust causal inferences. While previous MR investigations have explored inflammatory biomarkers' roles in bone metabolism,[25] the critical knowledge gaps remain. To date, the study remains conspicuously vacant which has systematically implemented bidirectional MR analysis to determine whether immune cell characterises drive osteoporotic pathogenesis or conversely represent secondary effects of progressive bone loss. Our study innovatively applies two-sample bidirectional MR evaluate the causal effects of immune cell characteristics on the development of osteoporosis, with the goal of providing new directions for potential future targeted immunotherapy pathways.

Materials and Methods

Study Design

This study utilized two-sample Mendelian randomization (MR) and publicly available datasets to investigate the impact of peripheral immune cell traits on the pathogenesis of osteoporosis. This research was performed in accordance with the MR guidelines for "Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization".[26] The technology roadmap used in this study is shown in Figure 1.

Data Source

Genetic instruments for 36 immune cell characteristics were derived from a study.[27] Summary statistics for these immunophenotypes were sourced from the MRC IEU OpenGWAS database[28] (https://www.ebi.ac.uk/gwas/home, GWAS IDs listed in a Table S1 in the Supplementary Materials File). Subsequently, 36 indicators associated with immune cell characteristics were identified by the first author through a literature PMID search (Shown in a Table S1 in the Supplementary Materials File). The data on immune cell characteristics are from cross - sectional studies with measurements taken at a single time point. Data from a total of 173,480 individuals of European ancestry, sourced from three large-scale UK research project were selected for analysis.[29–31] The outcome data for osteoporosis (212,778

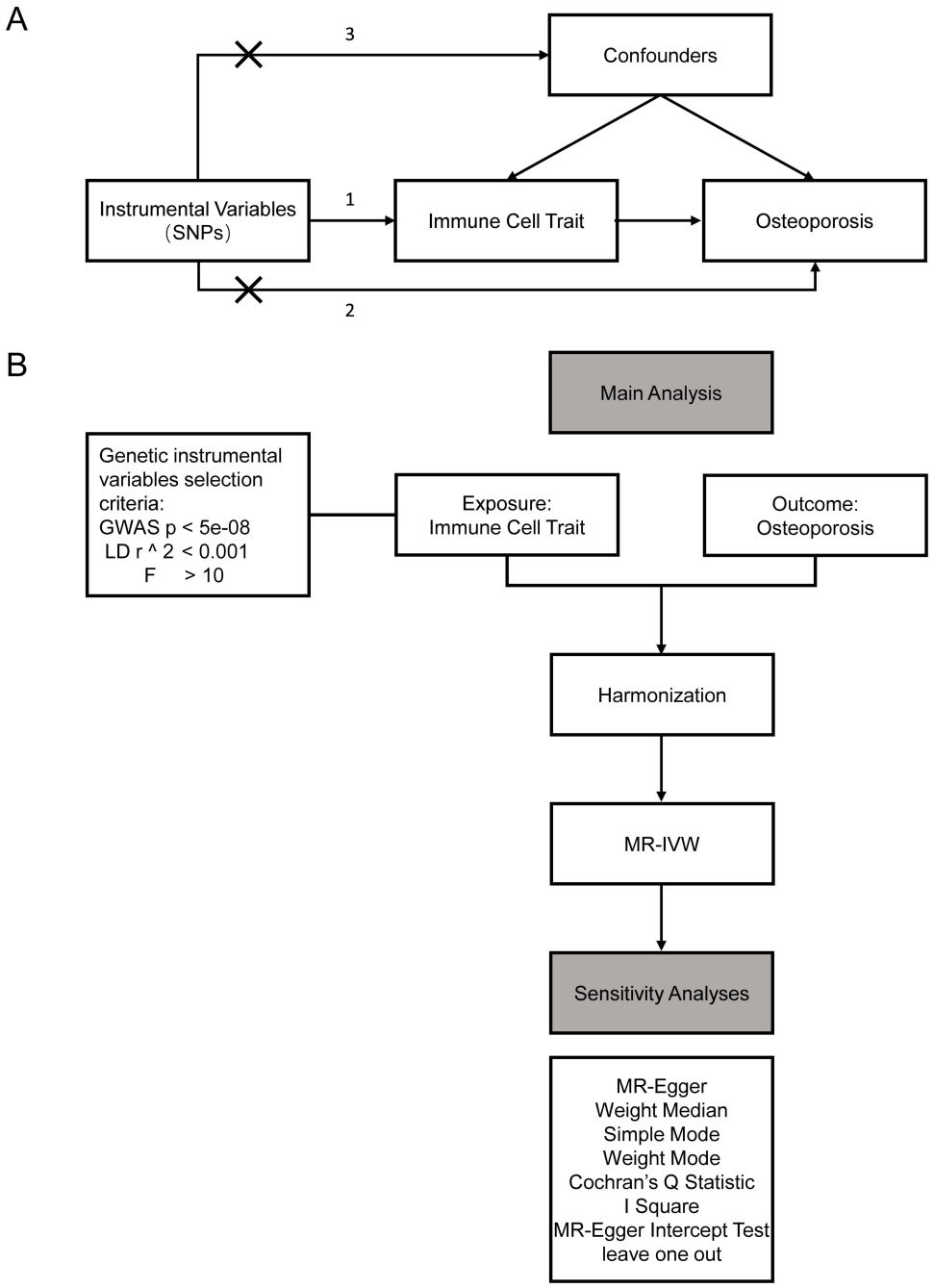


Figure 1 Technology roadmap. (**A**) Core assumptions in MR analysis. (**B**) Technology roadmap.
Abbreviations: SNPs, single-nucleotide polymorphisms; GWAS, genome-wide association study; LD, linkage disequilibrium; MR, Mendelian randomization; IVW, inverse variance weighted; MR-Egger, Mendelian randomization-Egger.

samples, 3,203 cases and 209,575 controls) were obtained from Finland. The osteoporosis data are based on prospective cohort designs. The GWAS datasets included in this study must meet the following criteria: (1) they must be derived from European populations; (2) they must have a sufficient sample size; (3) they must provide summary level data; (4) single-nucleotide polymorphisms (SNPs) must be genome-wide significant ($P < 5 \times 10^{-8}$) and independent of each other ($r^2 < 0.001$, window < 10,000 kb). SNPs with F-statistics < 10 or missing data are excluded. All GWAS summary data were obtained from publicly available databases that have already received ethical approval. Consequently, this study could be exempt from ethical review according to national regulatory guidelines, such as Item 1 of Article 39 of the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects, dated February 18, 2023,

China. The use of legally obtained public data or non-private personal information available through public channels is exempt from ethical review.

Selection of IVs (Instrumental Variables)

Efficient tools for genetic variation analysis must satisfy the following core assumptions: (1) the relevant assumptions, meaning that selected IVs must be associated with significant exposure; (2) independence assumption, meaning that IVs must not be significantly related to potential confounders that might affect exposure or outcome; and (3) exclusivity limitation, meaning that IVs could only affect the outcome through the path of "instrumental variable → exposure → outcome." The core assumptions of the MR analysis are presented in Figure 1.

The screening criteria for SNPs of the IVs were as follows. Initially, SNPs with $P < 5\times10^{-8}$ in the exposure GWAS were used as the screening criterion. This stringent threshold helps to identify SNPs with strong genetic associations with the immune cell traits of interest. Secondly, to avoid potential biases caused by redundant genetic information, SNPs in Linkage Disequilibrium (LD) were excluded. This step ensures that each selected SNP represents an independent genetic signal. Thirdly, after filtering SNPs based on exposure GWAS and LD, relevant data were extracted from the osteoporosis GWAS using the remaining SNPs. Finally, Fisher (F)-statistics were calculated to assess the weak IV bias. An F-statistic < 10 indicates that genetic variation is a weak instrumental variable, which might cause bias in the results and should be removed.[32] The formula for calculating the F-statistic is as follows: $F = (N - k - 1)/k \times R^2 / (1 - R^2)$, where N is the sample size, k is the number of instrumental variables used, and R^2 reflects the extent to which the instrumental variables explain exposure. $R^2 = 2 \times (1 - MAF) \times \beta^2 MAF$, where MAF stands for minor allele frequency and β represents the allele effect size. Leveraging summary data from 173,480 individuals for immune traits and 212,778 participants (3,203 osteoporosis cases and 209,575 controls) for the outcome, the study achieved 85% power ($\alpha = 0.0014$, Bonferroni-corrected for 36 tests) to detect an odds ratio (OR) of 1.15 for immune cell traits explaining ≥5% phenotypic variance.[33]

Causal Effect Estimation

Five algorithms were used to evaluate the causal relationship between immune cell characteristics and the incidence of osteoporosis, including the inverse-variance weighted (IVW) method, MR-Egger method, simple mode, weighted median, and weighted model. The IVW method is slightly more robust than other methods under certain conditions. The regression does not consider the existence of the intercept term and uses the inverse variance of the ending as a weight to fit.[34] Therefore, the IVW method was used as the main MR analysis method, with the other four methods serving as supplements, regardless of the presence of heterogeneity when there was no pleiotropy present. IVW was used as the primary method, providing maximum statistical power under the assumption of balanced pleiotropy. MR-Egger regression was used to detect and adjust for directional pleiotropy via intercept testing, valid when instrument strength is independent of pleiotropic effects. Weighted median was used to deliver consistent estimates even if ≤50% of instruments are invalid, enhancing robustness to heterogeneous pleiotropy. Simple and weighted mode methods were applied to identify causal signals resilient to outlier-driven pleiotropy through SNP effect clustering. This methodological triangulation allowed concordant results across methods to strengthen causal claims, while discrepancies highlight pleiotropic biases. Therefore, the IVW method was prioritized under the assumption of balanced pleiotropy (ie, pleiotropic effects are symmetrically distributed around zero), MR-Egger regression addressed directional pleiotropy (systematic bias in pleiotropic effects), whereas weighted median, simple mode, and weighted mode methods provided robustness to heterogeneous pleiotropy (ie, invalid instruments with non-null pleiotropic effects). The IVW random-effects model was used when heterogeneity was present. However, when pleiotropy was present, the results were calculated using the MR-Egger method to obtain more accurate causal effect estimates. The same set of methods were applied to evaluate the possible causal effects in the reverse direction, ie, from osteoporosis to the immune cell traits. Significance in the IVW model ($P < 0.05$) was set as the filter condition for significant causal relationships. Using multiple methods in this way allows us to comprehensively assess the causal relationship from different perspectives, mitigate potential biases, and obtain more robust causal estimates.

Sensitivity Analysis

Sensitivity analysis of the results was performed using heterogeneity, pleiotropy, and leave-one-out tests, as follows:

(1) The Cochran Q test was used to estimate the heterogeneity between each SNP. The statistical significance of the Cochran Q test indicated the presence of significant heterogeneity in the analysis results. Highly heterogeneous results were assessed using the IVW random-effects model to determine the causal effect size. Because the Cochran Q only determines the heterogeneity and cannot test heterogeneous distribution, the I^2 statistic was used to reflect the proportion of total variance attributed to the heterogeneity of IVs, with I^2 of 0 indicating no observed heterogeneity; 0–25%, mild heterogeneity; 25–50%, moderate heterogeneity; and >50%, high heterogeneity. The formula is as follows: $I^2 = (Q - df) / Q \times 100\%$.

(2) The MR-Egger method was used to test the pleiotropy of the IVs. If the P-value of the MR-Egger intercept is <0.05, it indicates significant horizontal pleiotropy of genetic variation, suggesting that the genetic variation might affect the outcome through pathways other than the exposure-outcome causal pathway.

(3) The leave-one-out test was used to assess whether a specific SNP affected the relationship between immune cell characteristics and the incidence of osteoporosis by successively eliminating each SNP and calculating the MR results for the remaining IVs. If the MR effect estimate changes significantly after removing an instrumental variable, this suggests that the MR effect estimates are sensitive to SNPs, suggesting that the results might be driven by a single SNP rather than a true causal relationship.

Statistical Analysis

The "TwoSampleMR" package (Version 0.5.7, The Comprehensive R Archive Network [CRAN], USA; https://cran.r-project.org/package=TwoSampleMR) was used to perform the MR analysis.[35] The Cochran Q and leave-one-out tests were employed to evaluate the robustness and reliability of the results, and the MR-Egger intercept test was used to assess horizontal pleiotropy. The data for the effect of immune cell characteristics on the incidence of osteoporosis are presented as odds ratio (OR) and 95% confidence interval (95% CI). All data calculations and statistical analyses were performed using R programming (version 4.3.0; R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/), and two-sided P-values < 0.05 indicated statistical significance.

Results

Genetic Instrument Selection and Validation

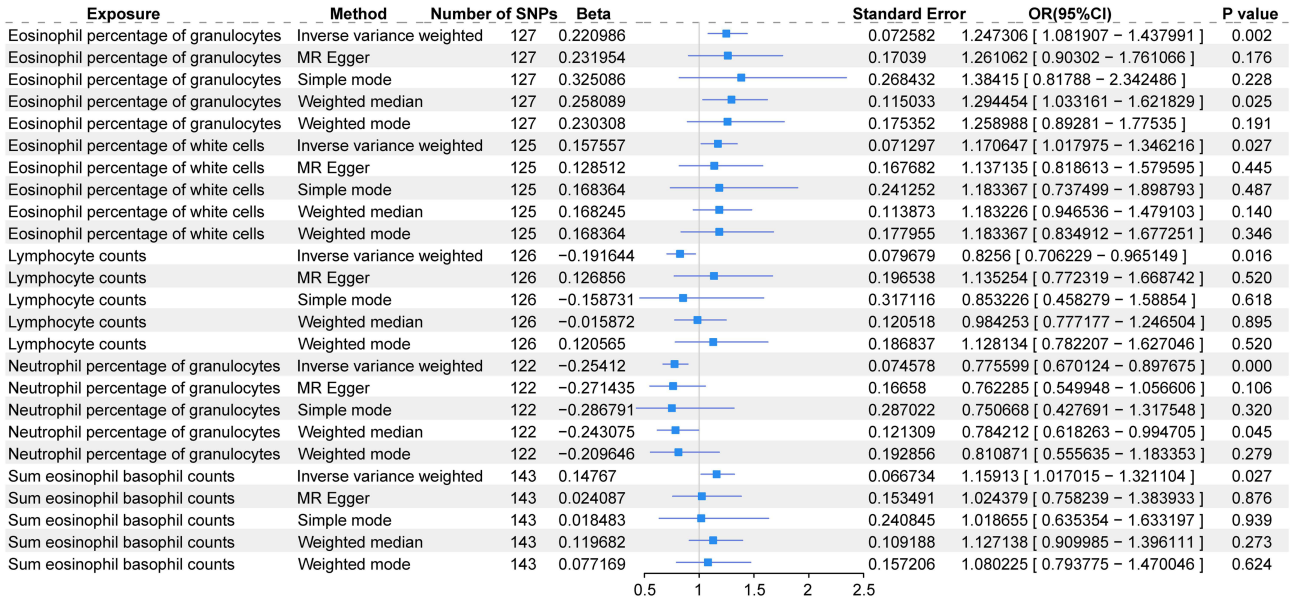
Selected IVs

According to the IV selection criteria, SNPs with linkage disequilibrium were excluded, and SNPs related to immune cell characteristics were included after matching with the GWAS data for osteoporosis. The indices of the IV selection results are presented in Table 1, which only displays significant indicators in MR analysis. More than 10 indicators of the IV F-test statistic were identified, indicating that this study's screening tool of SNP was strongly variable and that the weak IV of possible bias was limited.

Table 1 Immune Cell Characteristics and Strength of the Osteoporosis Tool Selection and Tool Variables According to the F-Statistic

Exposure	Outcome	Number of SNPs	Median F-Statistic	Minimum F-Statistic	Maximum F-Statistic
Eosinophil percentage of granulocytes	Osteoporosis	127	54.88	30.05	693.95
Eosinophil percentage of white cells	Osteoporosis	125	56.27	29.74	659.82
Lymphocyte counts	Osteoporosis	126	50.09	29.87	606.35
Neutrophil percentage of granulocytes	Osteoporosis	122	50.02	29.77	838.34
Sum eosinophil basophil counts	Osteoporosis	143	50.98	30.00	757.95

Abbreviation; SNPs, single-nucleotide polymorphisms; F, Fisher.



Exposure	Method	Number of SNPs	Beta	Standard Error	OR(95%CI)	P value
Eosinophil percentage of granulocytes	Inverse variance weighted	127	0.220986	0.072582	1.247306 [1.081907 − 1.437991]	0.002
Eosinophil percentage of granulocytes	MR Egger	127	0.231954	0.17039	1.261062 [0.90302 − 1.761066]	0.176
Eosinophil percentage of granulocytes	Simple mode	127	0.325086	0.268432	1.38415 [0.81788 − 2.342486]	0.228
Eosinophil percentage of granulocytes	Weighted median	127	0.258089	0.115033	1.294454 [1.033161 − 1.621829]	0.025
Eosinophil percentage of granulocytes	Weighted mode	127	0.230308	0.175352	1.258988 [0.89281 − 1.77535]	0.191
Eosinophil percentage of white cells	Inverse variance weighted	125	0.157557	0.071297	1.170647 [1.017975 − 1.346216]	0.027
Eosinophil percentage of white cells	MR Egger	125	0.128512	0.167682	1.137135 [0.818613 − 1.579595]	0.445
Eosinophil percentage of white cells	Simple mode	125	0.168364	0.241252	1.183367 [0.737499 − 1.898793]	0.487
Eosinophil percentage of white cells	Weighted median	125	0.168245	0.113873	1.183226 [0.946536 − 1.479103]	0.140
Eosinophil percentage of white cells	Weighted mode	125	0.168364	0.177955	1.183367 [0.834912 − 1.677251]	0.346
Lymphocyte counts	Inverse variance weighted	126	−0.191644	0.079679	0.8256 [0.706229 − 0.965149]	0.016
Lymphocyte counts	MR Egger	126	0.126856	0.196538	1.135254 [0.772319 − 1.668742]	0.520
Lymphocyte counts	Simple mode	126	−0.158731	0.317116	0.853226 [0.458279 − 1.58854]	0.618
Lymphocyte counts	Weighted median	126	−0.015872	0.120518	0.984253 [0.777177 − 1.246504]	0.895
Lymphocyte counts	Weighted mode	126	0.120565	0.186837	1.128134 [0.782207 − 1.627046]	0.520
Neutrophil percentage of granulocytes	Inverse variance weighted	122	−0.25412	0.074578	0.775599 [0.670124 − 0.897675]	0.000
Neutrophil percentage of granulocytes	MR Egger	122	−0.271435	0.16658	0.762285 [0.549948 − 1.056606]	0.106
Neutrophil percentage of granulocytes	Simple mode	122	−0.286791	0.287022	0.750668 [0.427691 − 1.317548]	0.320
Neutrophil percentage of granulocytes	Weighted median	122	−0.243075	0.121309	0.784212 [0.618263 − 0.994705]	0.045
Neutrophil percentage of granulocytes	Weighted mode	122	−0.209646	0.192856	0.810871 [0.555635 − 1.183353]	0.279
Sum eosinophil basophil counts	Inverse variance weighted	143	0.14767	0.066734	1.15913 [1.017015 − 1.321104]	0.027
Sum eosinophil basophil counts	MR Egger	143	0.024087	0.153491	1.024379 [0.758239 − 1.383933]	0.876
Sum eosinophil basophil counts	Simple mode	143	0.018483	0.240845	1.018655 [0.635354 − 1.633197]	0.939
Sum eosinophil basophil counts	Weighted median	143	0.119682	0.109188	1.127138 [0.909985 − 1.396111]	0.273
Sum eosinophil basophil counts	Weighted mode	143	0.077169	0.157206	1.080225 [0.793775 − 1.470046]	0.624

Figure 2 Forest plot of the causality of immune cell characteristics on osteoporosis. The effect sizes are presented as ORs with 95% CIs.
Abbreviations: SNPs, single-nucleotide polymorphisms; OR, odds ratio; CI, confidence interval; MR-Egger, Mendelian randomization-Egger; Beta, Mendelian randomization analysis effect coefficient.

Bi-Directional Two-Sample MR Causal Effect Estimates

The causal effect estimates from the five models are presented in the forest plot in Figure 2, and a scatter plot of the causal effect estimates from the selected SNPs after screening is shown in Figure 3. This figure presents only the results when the number of SNPs exceeded two. The fitting curves of the five scatterplot models were in the same direction, with most of the model slopes being consistent. The intercept of the IVW model was close to zero.

The MR results for the estimation of the causal effect of immune cell characteristics on the incidence of osteoporosis are shown in Table 2. The IVW model showed that an increased eosinophil percentage of granulocytes (OR=1.25, 95% CI=1.08–1.44, P=0.002), eosinophil percentage of white cells (OR=1.17, 95% CI=1.02–1.35, P=0.027), and sum eosinophil basophil counts (OR=1.16, 95% CI=1.02–1.32, P=0.027) were associated with an increased risk of osteoporosis, while higher lymphocyte counts (OR=0.83, 95% CI=0.71–0.97, P=0.016) and a higher neutrophil percentage of granulocytes (OR=0.78, 95% CI=0.67–0.90, P<0.001) had protective effects against the disease. To evaluate potential reverse causation (the causal effect of osteoporosis on immune cell traits), we performed bidirectional MR analysis using genetic instruments for osteoporosis (212,778 samples) and outcome data for 36 immune cell characteristics. No significant causal effects of osteoporosis on any immune trait were observed across all MR methods (P>0.05; Figure 4). In this study, the forward MR was found to be significant, while the reverse MR was non-significant. This indicates that the five inflammatory factors identified in the above findings are the main causes rather than the results of osteoporosis, suggesting that the pathway from the exposure factors to the outcome variable is unique.

Sensitivity Analysis

The heterogeneity of the significant results was tested using the Cochran Q test and I^2 statistic, as shown in Table 3. The indicators we selected had non-significant heterogeneity (Cochran Q, $P > 0.05$) in the MR results for osteoporosis, and the proportion of heterogeneity was mild ($0 < I^2 \leq 25\%$). The funnel plot of the IV indicators is shown in Figure 5, which displays only the results with more than two SNPs. The scatter plot showing the causal association effects was symmetrically distributed on both sides of the IVW model line, indicating that there was no potential bias in the results. Indicators with fewer than three SNPs were not subjected to subsequent horizontal pleiotropy or individual exclusion tests, which might limit the comprehensiveness of our assessment for these specific cases.

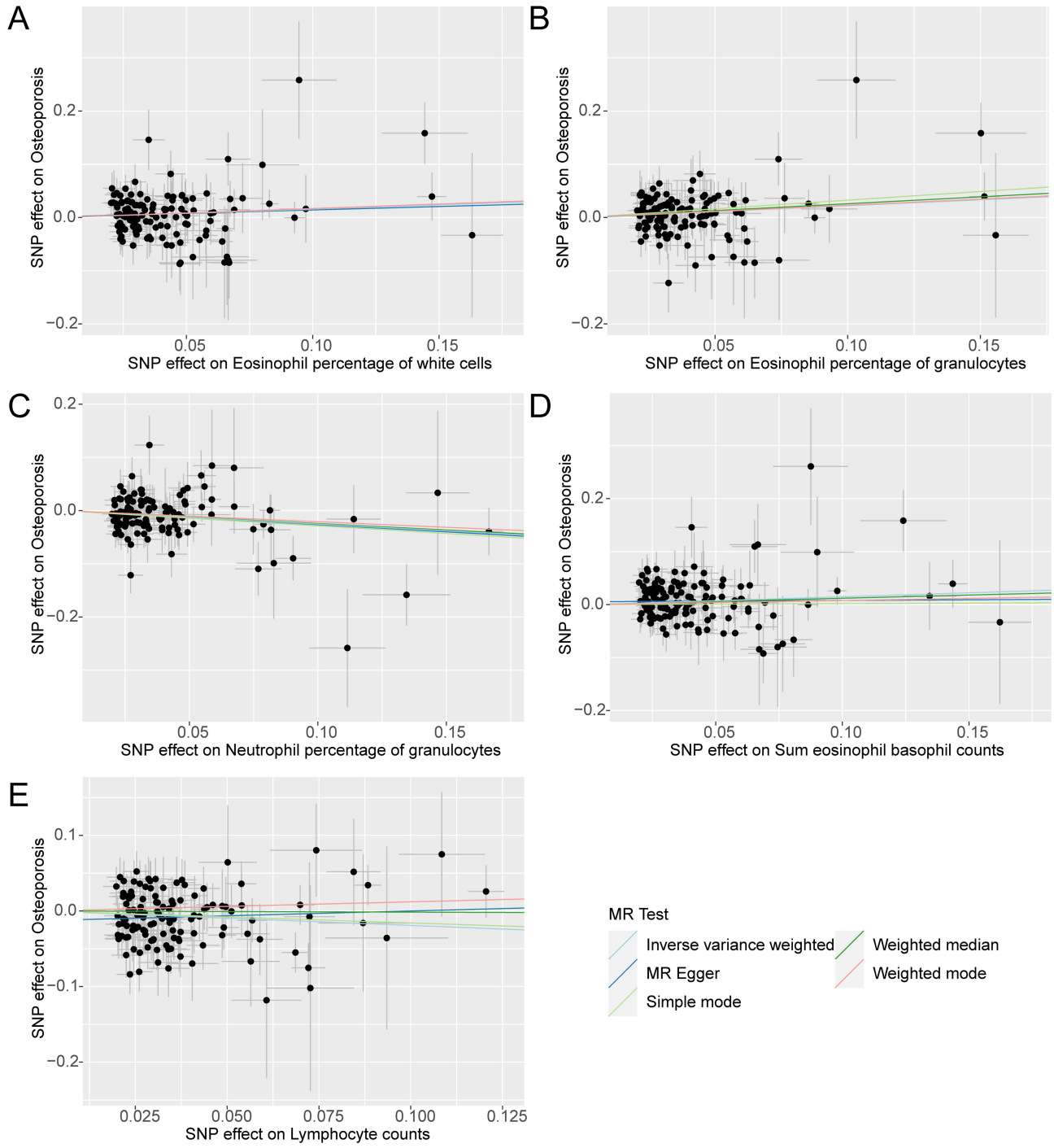


Figure 3 Scatter plot of the causality of immune cell characteristics on osteoporosis. (**A**) eosinophil percentage of white cells, (**B**) eosinophil percentage of granulocytes, (**C**) neutrophil percentage of granulocytes, (**D**) sum eosinophil basophil counts, and (**E**) lymphocyte counts. Black dots indicate SNPs, vertical and horizontal line segments indicate the range of SNP effects, the slope of each line corresponds to the estimation of the MR effect in different models. If the diagonal line is upward, it indicates a positive causal relationship between the exposure factor and the outcome, whereas the diagonal line is downward, the opposite is true.
Abbreviations: MR-Egger, Mendelian randomization-Egger; MR, Mendelian randomization; SNPs, single-nucleotide polymorphisms.

MR-Egger regression was adopted to conduct the horizontal pleiotropy of the IVs. All P-values of the intercept statistical hypothesis tests for each indicator were >0.05, and the intercepts were close to zero, indicating that the causal inference was not affected by horizontal pleiotropy (Table 4).

Table 2 Causal Effect Analysis Results of Multiple Models of Mendelian Randomization for Immune Cell Characteristics and Osteoporosis

Exposure	Method	Number of SNPs	Beta	Standard Error	P-value	OR (95% CI)
Eosinophil percentage of granulocytes	Inverse-variance weighted	127	0.220986	0.072582	0.002	1.247306 [1.081907–1.437991]
Eosinophil percentage of granulocytes	MR-Egger	127	0.231954	0.170390	0.176	1.261062 [0.90302–1.761066]
Eosinophil percentage of granulocytes	Simple mode	127	0.325086	0.268432	0.228	1.38415 [0.81788–2.342486]
Eosinophil percentage of granulocytes	Weighted median	127	0.258089	0.115033	0.025	1.294454 [1.033161–1.621829]
Eosinophil percentage of granulocytes	Weighted mode	127	0.230308	0.175352	0.191	1.258988 [0.89281–1.77535]
Eosinophil percentage of white cells	Inverse-variance weighted	125	0.157557	0.071297	0.027	1.170647 [1.017975–1.346216]
Eosinophil percentage of white cells	MR-Egger	125	0.128512	0.167682	0.445	1.137135 [0.818613–1.579595]
Eosinophil percentage of white cells	Simple mode	125	0.168364	0.241252	0.487	1.183367 [0.737499–1.898793]
Eosinophil percentage of white cells	Weighted median	125	0.168245	0.113873	0.140	1.183226 [0.946536–1.479103]
Eosinophil percentage of white cells	Weighted mode	125	0.168364	0.177955	0.346	1.183367 [0.834912–1.677251]
Lymphocyte counts	Inverse-variance weighted	126	0.191644	0.079679	0.016	0.8256 [0.706229–0.965149]
Lymphocyte counts	MR-Egger	126	0.126856	0.196538	0.520	1.135254 [0.772319–1.668742]
Lymphocyte counts	Simple mode	126	0.158731	0.317116	0.618	0.853226 [0.458279–1.58854]
Lymphocyte counts	Weighted median	126	0.015872	0.120518	0.895	0.984253 [0.777177–1.246504]
Lymphocyte counts	Weighted mode	126	0.120565	0.186837	0.520	1.128134 [0.782207–1.627046]
Neutrophil percentage of granulocytes	Inverse-variance weighted	122	0.254120	0.074578	< 0.001	0.775599 [0.670124–0.897675]
Neutrophil percentage of granulocytes	MR-Egger	122	0.271435	0.166580	0.106	0.762285 [0.549948–1.056606]
Neutrophil percentage of granulocytes	Simple mode	122	0.286791	0.287022	0.320	0.750668 [0.427691–1.317548]
Neutrophil percentage of granulocytes	Weighted median	122	0.243075	0.121309	0.045	0.784212 [0.618263–0.994705]
Neutrophil percentage of granulocytes	Weighted mode	122	0.209646	0.192856	0.279	0.810871 [0.555635–1.183353]
Sum eosinophil basophil counts	Inverse-variance weighted	143	0.147670	0.066734	0.027	1.15913 [1.017015–1.321104]
Sum eosinophil basophil counts	MR-Egger	143	0.024087	0.153491	0.876	1.024379 [0.758239–1.383933]
Sum eosinophil basophil counts	Simple mode	143	0.018483	0.240845	0.939	1.018655 [0.635354–1.633197]
Sum eosinophil basophil counts	Weighted median	143	0.119682	0.109188	0.273	1.127138 [0.909985–1.396111]
Sum eosinophil basophil counts	Weighted mode	143	0.077169	0.157206	0.624	1.080225 [0.793775–1.470046]

Abbreviations: SNPs, single-nucleotide polymorphisms; Beta, Mendelian randomization analysis effect coefficient; OR, odds ratio; CI, confidence interval; MR-Egger, Mendelian randomization-Egger.

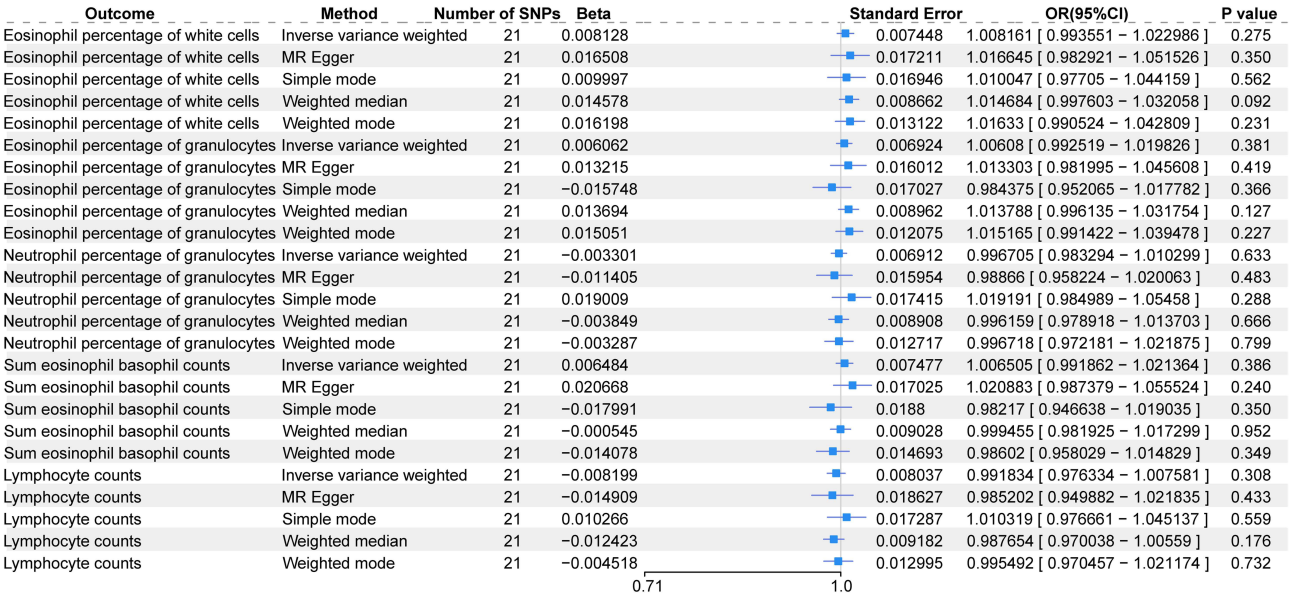


Figure 4 Forest plot of reverse causality of osteoporosis on immune cell characteristics. The effect sizes are presented as ORs with 95% CIs.
Abbreviations: SNPs, single-nucleotide polymorphisms; OR, odds ratio; CI, confidence interval; MR-Egger, Mendelian randomization-Egger; Beta, Mendelian randomization analysis effect coefficient.

Results of the leave-one-out sensitivity analysis to determine the stability of the results are shown in Figure 6. Each line in the figure represents the effect size and 95% CI of the indicator after removal of the corresponding SNP, and the red line represents the reference effect interval. The degree of overlap between each line and the red line interval was high, indicating that the effect estimate will not change significantly with the removal of a single SNP and that the results were stable.

Discussion

Recently, immunotherapy has become a new approach for the treatment of osteoporosis and could lead to the reversal of this potentially devastating aging-related disease. To our knowledge, this is the first study utilizing a bidirectional two-sample MR approach to investigate the causality between immune cell characteristics and the incidence of osteoporosis. This method reduced the bias caused by confounding factors and reverse causality. We found that the eosinophil

Table 3 Heterogeneity Results of Immune Cell Characteristics According to the Mendelian Randomization Analysis of Osteoporosis

Exposure	Method	Q	Q_df	Q_pval	I^2 (%)
Eosinophil percentage of white cells	MR-Egger	118.8964	123	0.588	0
Eosinophil percentage of white cells	Inverse-variance weighted	118.9330	124	0.612	0
Eosinophil percentage of granulocytes	MR-Egger	109.2287	125	0.841	0
Eosinophil percentage of granulocytes	Inverse-variance weighted	109.2338	126	0.857	0
Neutrophil percentage of granulocytes	MR-Egger	115.9135	120	0.589	0
Neutrophil percentage of granulocytes	Inverse-variance weighted	115.9270	121	0.613	0
Sum eosinophil basophil counts	MR-Egger	138.6833	141	0.540	0
Sum eosinophil basophil counts	Inverse-variance weighted	139.4827	142	0.544	0
Lymphocyte counts	MR-Egger	140.9824	124	0.141	12%
Lymphocyte counts	Inverse-variance weighted	144.5438	125	0.112	14%

Abbreviation: MR-Egger, Mendelian randomization-Egger.

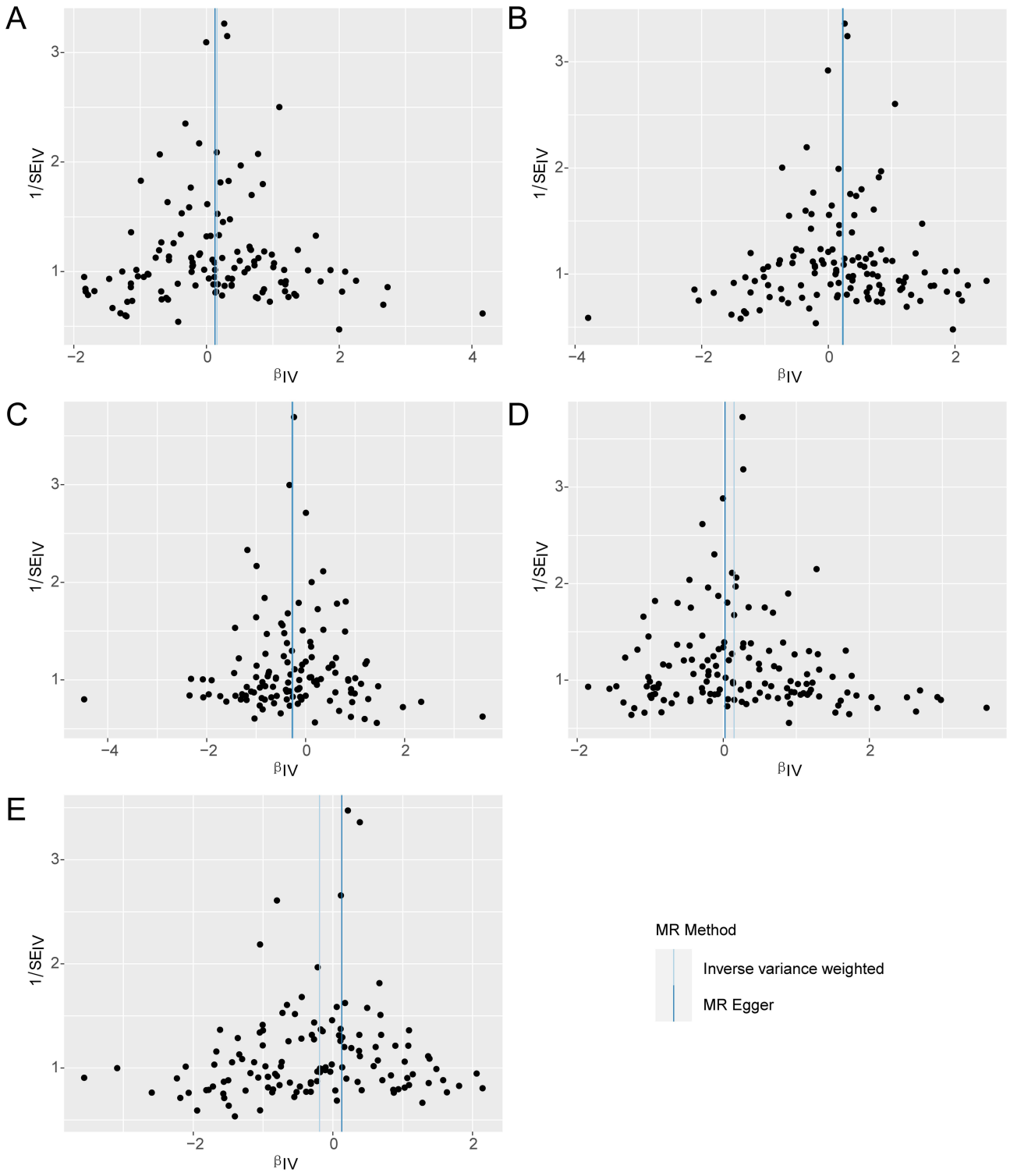


Figure 5 Funnel plot of heterogeneity results of the effect of immune cell characteristics on osteoporosis. (**A**) eosinophil percentage of white cells, (**B**) eosinophil percentage of granulocytes, (**C**) neutrophil percentage of granulocytes, (**D**) sum eosinophil basophil counts, and (**E**) lymphocyte counts. The black dot represents the SNP. The X-axis represents the beta value of the instrumental variable SNP, and the Y-axis represents the standard error of the instrumental variable SNP. Heterogeneity is not considered in a MR analysis when two vertical lines are close to the central line and symmetrical points are observed on both sides.
Abbreviation: MR-Egger, Mendelian randomization-Egger.

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Table 4 Horizontal Pleiotropy of Immune Cell Characteristics and Osteoporosis According to Mendelian Randomization Analysis

Exposure	MR-Egger Intercept	Standard Error	P-value
Eosinophil percentage of white cells	0.001298	0.006781	0.849
Eosinophil percentage of granulocytes	0.000472	0.006635	0.943
Neutrophil percentage of granulocytes	0.000738	0.006348	0.908
Sum eosinophil basophil counts	0.005410	0.006051	0.373
Lymphocyte counts	0.012741	0.007199	0.079

Abbreviation: MR-Egger, Mendelian randomization-Egger.

percentage of granulocytes, eosinophil percentage of white cells, and sum eosinophil basophil counts had significant positive causal associations with the incidence of osteoporosis while lymphocyte counts and neutrophil percentage of granulocytes played negative effect on osteoporosis. The reverse MR was non-significant effect of osteoporosis on 36 immune cell characteristics.

Previous observational studies confirmed the importance of immune cells in the regulation of bone metabolism. Eosinophils and basophils are the primary cells involved in the pathogenesis of allergic inflammation. Eosinophils secrete various inflammatory mediators, including IL-31, which regulates bone metabolism by promoting bone resorption and inhibiting bone formation. It has been observed that post-menopausal women exhibit an increase in serum IL-31 levels alongside a decrease in BMD.[16,17] However, recent research has discovered that eosinophils maintain bone homeostasis by inhibiting excessive osteoclast formation and activity via eosinophil peroxidase.[36] Consequently, eosinophils may play a dual role in either bone formation or bone resorption within human skeletal homeostasis. IL-3 induces RANKL expression in human basophils,[37] suggesting them to interact with bone physiology and activation of immune cells. The eosinophil percentage of granulocytes, eosinophil percentage of white cells, and sum eosinophil basophil counts had significant positive causal associations with the incidence of osteoporosis in this study. Other scholars also found a causal relationship between eosinophil counts and osteoporosis.[38] This positive causal relationship holds promise for immunotherapy targeting. Clinical observations found patients have a significantly increased risk of osteoporosis and fractures in immune-mediated diseases.[20,39] The elevated levels of IgM rheumatoid factor significantly predict an increased risk of long-term fractures in RA patients.[20] Furthermore, studies have found that the risk of osteoporosis and fractures is significantly increased in autoimmune inflammatory myopathy.[39] Although MR studies have not found a causal relationship between RA and osteoporosis,[24] targeted therapy can increase bone density and reduce fracture risk compared to traditional glucocorticoid treatment.[40] Monoclonal antibodies targeting eosinophil-secreted IL-5 offer a direction for treating patients with immune-mediated osteoporosis due to the multifaceted role of eosinophils in immunology.[41]

Lymphocyte also play a role in bone homeostasis and the onset of osteoporosis. B lymphocytes act as active regulators of the RANKL-osteoprotegerin (OPG) axis and play a key role in inhibiting osteoclasts by secreting the decoy receptor OPG, which can prevent bone resorption caused by osteoclasts.[42] Interestingly, B lymphocytes also produce RANKL to further exacerbate bone loss induced by ovariectomy in ovariectomized mice.[43] Frase et al reported that B cells interact with osteoclasts and osteoblasts to regulate the bone remodeling process by secreting cytokines, such as IL-7, RANK, and OPG,[11] which depend on the inflammatory environment. T lymphocytes also play a dual role in regulating bone metabolism,[12] and activated T cells are the main sources of RANKL[44] and tumor necrosis factor-α,[45] which mediate the production of osteoclasts and thus enhance bone resorption. Additionally, T cells have anti-osteoclastogenic properties. Cytokines (including IL-4 and IL-10) secreted by T cells inhibit the differentiation and bone resorption of osteoclasts.[46,47] Moreover, it was found that there was a causal relationship between IL-27 levels and osteoporosis in the same osteoporosis database selected for this study, and SNPs related to IL-27 levels mainly involved immune and inflammatory pathways,[48] which plays a major role in T cell regulation. Lymphocyte subpopulations are equally important in bone homeostasis and osteoporosis.[49] The imbalance of Th17/Tregs ratio caused by osteoporosis environment leads to the imbalance of immune bone niche, which might be the most direct and main factor in for preventing RA and osteoporosis.[14] In contrast, CD8+ T cells are amplified in an auto-regulating manner under osteoclast

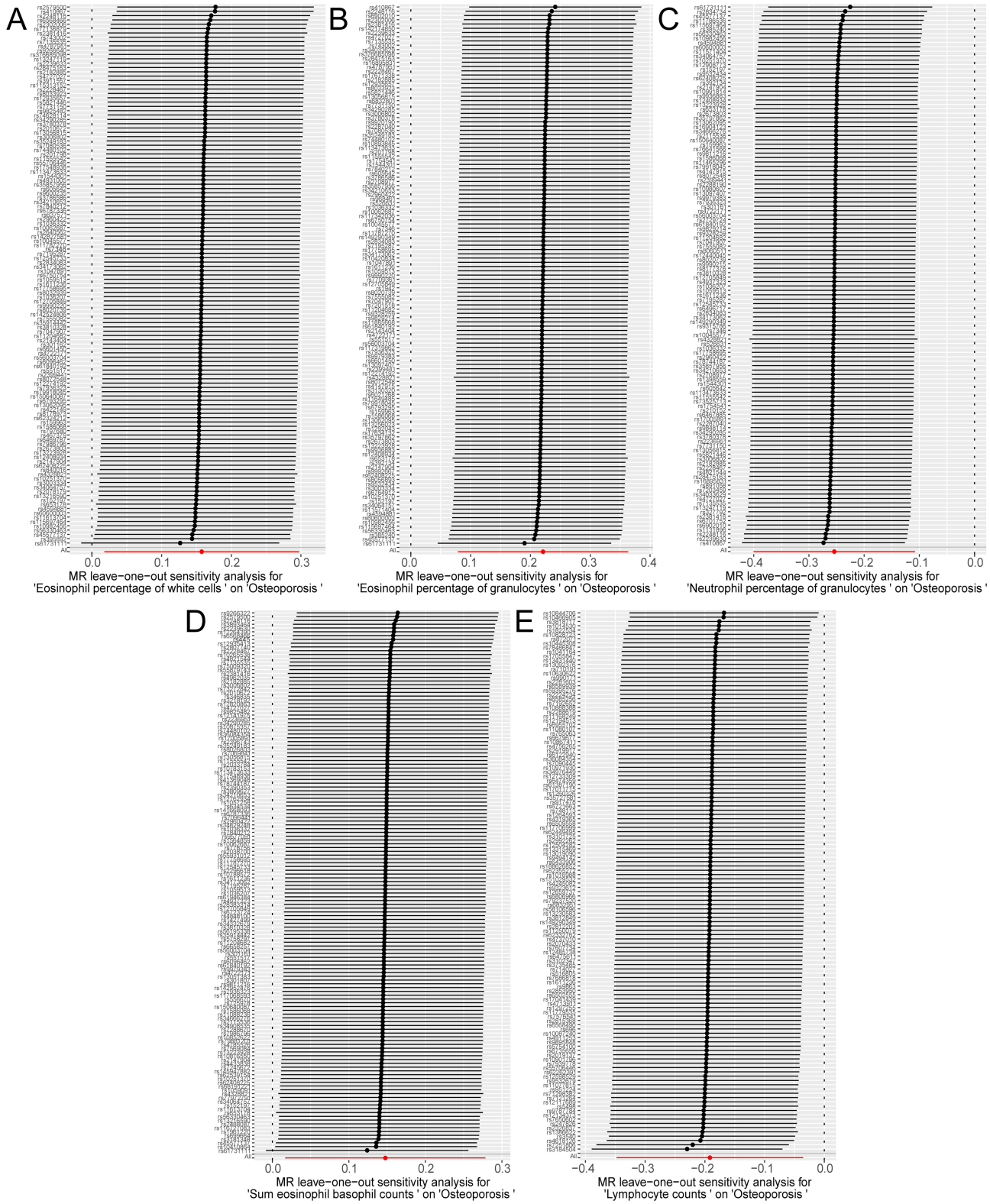

Figure 6 Leave-one-out forest plot on the effect of immune cells characteristics on osteoporosis. (**A**) eosinophil percentage of white cells, (**B**) eosinophil percentage of granulocytes, (**C**) neutrophil percentage of granulocytes, (**D**) sum eosinophil basophil counts, and (**E**) lymphocyte counts on osteoporosis. The red line represents the reference effect range, and each black line represents the effect size and 95% confidence interval of the corresponding SNP after removal.
Abbreviation: MR, Mendelian randomization.

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activity to inhibit bone resorption.[18] Although previous observational studies have demonstrated the dual role of lymphocytes in osteoimmunology, our study has established for the first time a causal relationship between peripheral blood lymphocyte count and osteoporosis, identifying lymphocytes as a protective factor against osteoporosis. Currently, T cell-targeted therapies have become a research hotspot. IL-17 produced by Th17 cells is elevated in the serum of postmenopausal osteoporosis patients,[50] and IL-17-targeted therapy has shown more significant effects than RANKL blockade or TNF-α inhibition.[51] Studies have found that disturbances in the level of short-chain fatty acids (SCFAs) produced by the gut microbiome disrupts the Th17/Treg balance,[52] which are closely related to the pathogenesis of RA and postmenopausal osteoporosis. Certain probiotic treatments have been shown to ameliorate Th17/Treg dysregulation,[53] showing therapeutic potential for conditions such as RA and postmenopausal osteoporosis. Our research further confirms the causal role of lymphocytes in osteoporosis. Future studies could be conducted to explore the mechanisms and potential therapeutic implications of the gut microbiota on osteoporosis.

Neutrophils are the first cells recruited in inflammatory responses and can directly or indirectly affect bone loss and promote the development of osteoporosis by releasing proinflammatory cytokines and reactive oxygen species.[11] Activated neutrophils can secrete the receptor activator of nuclear factor kappa beta ligand (RANKL) to activate osteoclasts and promote bone resorption,[54] or secrete chemokines to recruit pro-osteoporotic cells, such as T helper 17 cells.[55] Our study found a negative causal relationship between neutrophils/grains and osteoporosis. The ratios of neutrophils, monocytes, and lymphocytes have been emphasized as predictive factors for the risk of osteoporosis in previous studies.[56,57] The neutrophil-to-lymphocyte ratio has been found to be positively correlated with lumbar bone BMD.[57] The reduction in estrogen results in a decrease in activated neutrophils, which in turn leads to osteoclast bone resorption.[58] However, the association between neutrophil-mediated inflammation and osteoporosis warrants further investigation. Clinicians need to be aware of neutrophil alterations in osteoporosis patients receiving immunotherapy.

The possibility of reverse causation where osteoporosis alters immune cell counts has been excluded by reverse MR analysis further, strengthening the causal inference from immune traits to osteoporosis. Reverse MR further helps distinguish between causality and genetic correlations. In a causal study of immune cell characteristics and osteomyelitis, reverse MR indicated that osteomyelitis had no significant causal effect on these immune phenotypes.[59] Besides, in the same osteoporosis database selected for this study, they found no significant causal effect of osteoporosis on these immune phenotypes.[48] A similar finding was identified for the incidence of osteoporosis in the present study, confirming that immune cell characteristics have a causal effect on the incidence of osteoporosis rather than a genetic correlation. This finding indicates that immune cell characterises drive osteoporotic pathogenesis rather than secondary effects of progressive bone loss, providing a theoretical basis for the next immunotherapy.

Our MR study further demonstrated a causal relationship between different peripheral blood immune cell counts and the incidence of osteoporosis. The significant advantages of large-sample GWAS data, coupled with an F-statistic greater than 10, indicate that the strength of the instrumental variables is sufficient to ensure the effectiveness of MR analysis. Additionally, the low heterogeneity indicated that the consistency of the selected variables was valid. We used MR-Egger regression to limit the impact of horizontal pleiotropy.[60] The robustness of the MR-Egger method stems from its ability to provide unbiased causal estimates. However, this violates the core assumption of MR and leads to a biased causal effect assessment when horizontal pleiotropy occurs. To satisfy the second assumption of MR, we removed confounded SNPs, and the conclusion remained unchanged and valid. Therefore, we believe that the causal inference made in this study was not affected by horizontal pleiotropy.

Limitations

This study has some limitations. First, as a data mining study based on publicly available GWAS datasets, our findings require replication in independent cohorts or prospective clinical studies to confirm generalizability. The sample size was limited by the database and requires further validation through clinical observational studies. Second, our results only apply to individuals with European lineage, as the cases of osteoporosis were sourced from Finland, which may have affected the generalizability of the results. The effect of immune cell characteristics on the incidence of osteoporosis in other lineages has not yet been investigated. Third, although we have rigorously controlled for horizontal pleiotropy and reverse causality, unmeasured confounders (such as drugs or gene-environment interactions) might still influence the

causal estimates. These confounders could be managed by subtyping patients, including those with postmenopausal osteoporosis, senile osteoporosis, and drug-induced osteoporosis. Further analysis of these subtypes would allow for more accurate causal inferences and enhance our ability to control potential confounding factors.

Conclusions

This study revealed a significant causal effect of five peripheral blood immune cell characteristics on osteoporosis, with no effect of osteoporosis on immune cell characteristics. Forward MR indicated the importance of the immune microenvironment in osteoporosis, and reverse MR suggested that immune cell characterises drive osteoporotic pathogenesis rather than secondary effects of progressive bone loss. These findings offer new insights into targeted immunotherapy, particularly concerning the targeting of eosinophils and T lymphocytes. They suggest that examinations related to osteoporosis are necessary for patients with immune deficiencies or those undergoing immunotherapy. Although this study suggests a weak level of pleiotropy, there are inevitably some confounding factors, such as the selection of only the European population, the lack of subgroup analysis, and the failure to consider drug factors. In the future, these aspects should be subjected to in-depth research on the mechanism.

Data Sharing Statement

All data analyzed in this study were acquired from the publicly accessible GWAS Catalog database via the MRC IEU OpenGWAS database at https://www.ebi.ac.uk/gwas/home. The GWAS IDs for 36 indicators associated with immune cell characteristics are listed in a Table S1 in the Supplementary Materials File. The GWAS ID for osteoporosis is finn-b-M13_OSTEOPOROSIS.

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Author Contributions

All listed authors meet all four ICMJE authorship criteria and made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors have disclosed all potential conflicts of interest related to this study truthfully and completely. The design, data analysis, result interpretation, and conclusion of this study strictly followed academic norms and has not been influenced by any interests, ensuring the objectivity and reliability of the study.

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