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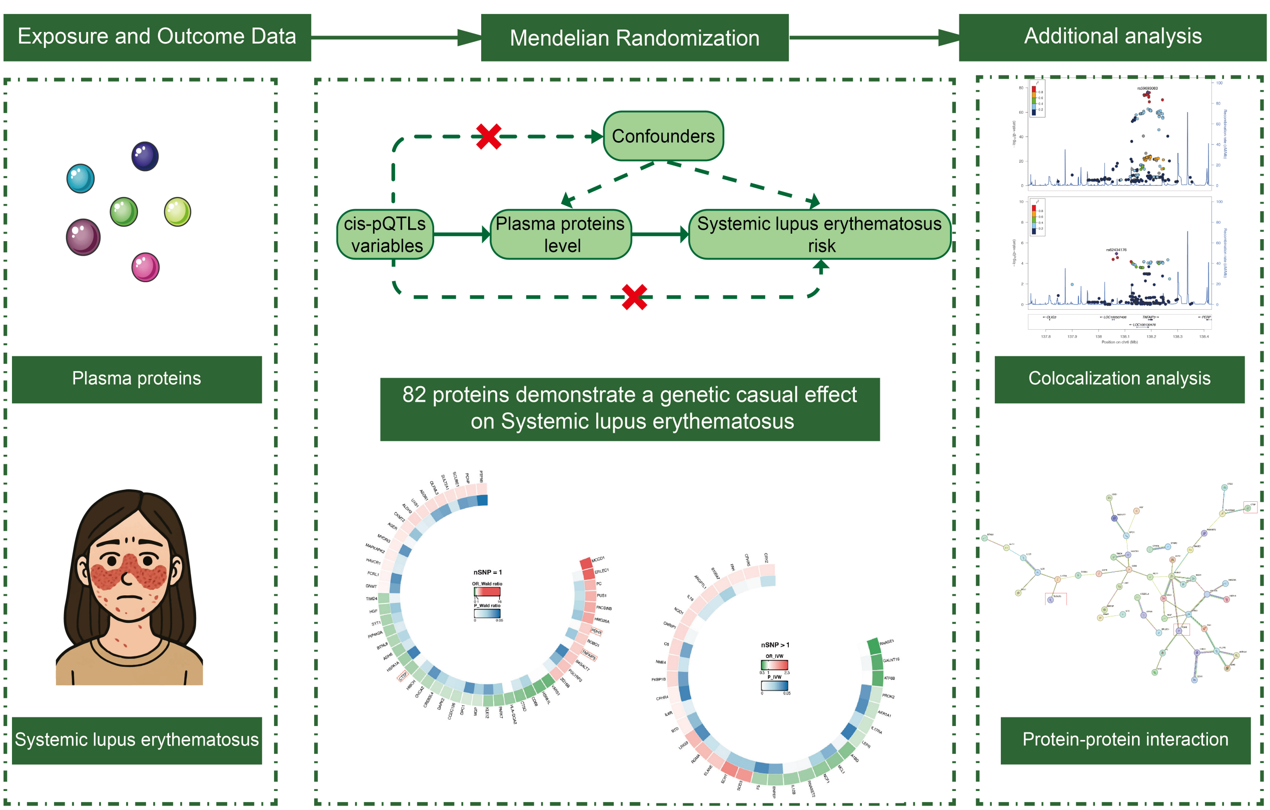
Graphical Abstract



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Research Article

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Exploring the Causal Relationship Between Plasma Proteins and Systemic Lupus Erythematosus: A Mendelian Randomization Study

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Abstract

Background: Systemic lupus erythematosus (SLE) is a complex autoimmune disease that severely impacts patient quality of life. Current treatments primarily manage symptoms rather than cure the disease, emphasizing the need for a deeper understanding of its pathogenesis and the discovery of novel therapeutic targets. Circulating proteins are thought to play a critical role in SLE risk, but their causal relationships remain underexplored.

Methods: This study used pQTL and genome-wide association study (GWAS) data to perform a two-sample Mendelian randomization (MR) analysis to investigate the genetic causal relationships between circulating proteins and SLE. We identified proteins potentially associated with SLE risk and further analyzed their roles in immune regulation and inflammation using Protein-Protein Interaction (PPI) networks. Colocalization analysis was conducted to validate the associations of key proteins with SLE.

Results: Our analysis identified 82 plasma proteins potentially causally linked to SLE risk ($p < 0.05$). Colocalization analysis confirmed the association of proteins such as TNFAIP3, PDHX, and CTSF with SLE, underscoring their critical role in disease pathogenesis. Additionally, PPI network analysis revealed that these proteins are involved in immune modulation and inflammatory pathways, further supporting their relevance as therapeutic targets.

Conclusion: This study identifies 82 plasma proteins that may play a causal role in SLE, with TNFAIP3, PDHX, and CTSF emerging as promising therapeutic targets. These findings provide a foundation for future research aimed at developing precision therapies for SLE.

Keywords: Systemic Lupus Erythematosus; Mendelian Randomization; Circulating Proteins; Colocalization Analysis; Protein-Protein Interaction Network

Introduction

Systemic lupus erythematosus (SLE) is a chronic, multi-system autoimmune disorder characterized by widespread immune dysregulation, autoantibody production, and tissue inflammation that profoundly affects the quality of life and imposes significant socio-economic burdens [1, 2]. The global annual incidence of SLE is approximately 5.14 per 100,000 (1.4 to 15.1 per 100,000), while the prevalence is 43.7 per 100,000 (15.9 to 108.9 per 100,000). The incidence and prevalence rates are significantly higher in females than in males, particularly among women of reproductive age. Additionally, the incidence and prevalence are higher in high-income countries and regions [3]. The conventional treatment regimen for SLE includes nonsteroidal anti-inflammatory drugs (NSAIDs), antimalarials, corticosteroids, and immunosuppressants [4]. Though effective in many cases, these treatments come with limitations in managing long-term disease progression and preventing irreversible organ damage, leading to ongoing research for better management strategies. Recent advancements have introduced biologic agents, offering new therapeutic avenues for SLE management. For instance, belimumab, approved by the U.S. Food and Drug Administration (FDA), targets the B-lymphocyte stimulator (BLyS) to curtail autoantibody production [5]. Despite the diversity of treatment options, therapeutic outcomes for SLE vary considerably among individuals, and most strategies prioritize symptom management over disease eradication [6]. Moreover, long-term use of existing medications

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can lead to serious adverse effects, including infections, osteoporosis, and retinal disorders [6]. This underscores the need for therapeutic strategies that not only manage symptoms but also target the underlying pathophysiology of SLE, aiming for disease remission and preventing flare-ups.

Proteins play a crucial role in immune regulation and inflammatory responses, both of which are closely associated with the pathophysiological processes of SLE [7]. For example, complement activation is one of the key pathological mechanisms leading to tissue inflammation and damage in SLE [8]. Furthermore, proteins are primary targets for pharmacological interventions [9]. In two Phase III trials, anifrolumab, which blocks the type I interferon receptor 1 (IFNAR1), received approval for treating SLE [10]. This represents a shift toward targeting specific molecular pathways to control disease activity in SLE, highlighting the potential of precision medicine in autoimmune disorders. The circulating proteome, comprising proteins released both actively and passively into the bloods from various tissues and cells [11], has been confirmed its correlation with SLE. Yong Dai's team utilized data-dependent acquisition (DDA) and data-independent acquisition (DIA) proteomics techniques to identify three proteins as potential biomarkers for diagnosing SLE [12]. Liu et al. applied TMT-labeled quantitative proteomics alongside enzyme-linked immunosorbent assays (ELISA) to demonstrate notable differences in the serum levels of SAA1 and CD248 between SLE patients and healthy controls [13]. Nonetheless, current research is constrained by small sample sizes, a limited protein range, and potential confounding factors. Additionally, the variability in patient populations and the complexity of autoimmune diseases like SLE make it challenging to pinpoint universal biomarkers or therapeutic targets. Conducting randomized controlled trials to investigate the potential causal relationships between numerous proteins and SLE is also challenging.

Mendelian Randomization (MR) employs genetic variations as instrumental variables (IVs) to establish causal relationships between exposures and outcomes, effectively mitigating confounding factors and preventing reverse causality [14, 15]. The power of MR lies in its ability to discern causal relationships through genetic instruments, and it has emerged as a robust tool in autoimmune disease research. In this study, we conducted a two-sample MR analysis using pQTL data derived from extensive proteomics studies and genome-wide association study (GWAS) data for SLE to explore the genetic causal relationship between these elements. Additionally, we constructed a protein-protein interaction (PPI) network and performed colocalization analysis for proteins statistically significant in the MR analysis. These analyses enable a more comprehensive understanding of the biological pathways involved in SLE and help to identify proteins that could be targeted for therapeutic interventions. This comprehensive approach is aimed at identifying potential therapeutic targets for SLE and providing valuable insights for future clinical applications (Figure 1).

Methods

Source of Exposure Data

We obtained single nucleotide polymorphisms (SNPs) data associated with plasma protein levels from the Fenland study. The Fenland study provided a robust dataset for understanding the genetic underpinnings of complex diseases, with a large sample size and comprehensive data on genetic variations. This research carried out a genome-wide proteomics association study involving 10,708 participants of European ancestry, assessing 4,775 plasma proteins utilizing the SomaScan v4 assay (http://www.omicscience.org/apps/pgwas) [16].

Source of Outcome Data

The outcome of interest, SLE, was studied using GWAS data obtained from the Finnish Genetic Study (FinnGen), a collaborative research initiative that links genetic data from the Finnish biobank with detailed health records from national registries. This study extracted over 500,000 samples from the Finnish biobank, integrating longitudinal phenotypic data and digital health records from the national health registry [17]. We accessed the publicly available FinnGen R10 dataset (https://r10.finngen.fi/), which included data on 1,083 SLE cases and 306,504 controls [17]. The inclusion of longitudinal data allows for the identification of incident SLE cases, reducing the risk of misclassification bias compared to cross-sectional studies.

Study Design

In this research, we performed a comprehensive two-sample MR analysis to evaluate the causal relationship between circulating proteins and SLE. MR analysis has become increasingly popular in exploring the causal pathways involved in autoimmune diseases due to its ability to minimize biases inherent in observational studies. To ensure the validity of the results, the MR analysis mandated that the selected IVs satisfy three critical criteria: (1) the IVs must demonstrate a significant direct association with the exposure variable (circulating proteins); (2) the IVs must be independent of any confounders that could affect the exposure-outcome relationship; (3) the influence of the IVs on the outcome must be mediated exclusively through the exposure, with no alternative causal pathways [18].

Selection of Instrumental Variables

Informed by the three assumptions outlined previously and recent research findings, stringent criteria for SNP selection were applied: (1) SNPs must be significantly associated with circulating proteins, adhering to a stringent significance threshold ($P < 5 \times 10^{-8}$) [19]; (2) To ensure the independence of the selected IVs and mitigate the effects of linkage disequilibrium (LD), SNP clustering methods were utilized (r^2 = 0.001, kb = 10,000) [20]; (3) IVs with an F-statistic less than 10, generally considered weak, were excluded to avoid instability and bias in effect estimation [21]; (4) SNPs strongly associated with the ou tcome variable ($P < 5 \times 10^{-8}$) were also excluded to prevent direct causal interference with the outcome [22]; (5) Cis-protein quantitative trait loci (cis-pQTLs) spanning a gene range of ±1Mb were selected. Located at or near the gene encoding the target proteins, cis-pQTLs are favored for their substantial contribution to explaining protein expression by directly regulating transcription, mRNA splicing, or translation, compared to trans-pQTLs, which act at a distance and may have more complex or indirect effects [23]. This selection criterion enhances the power of our study by

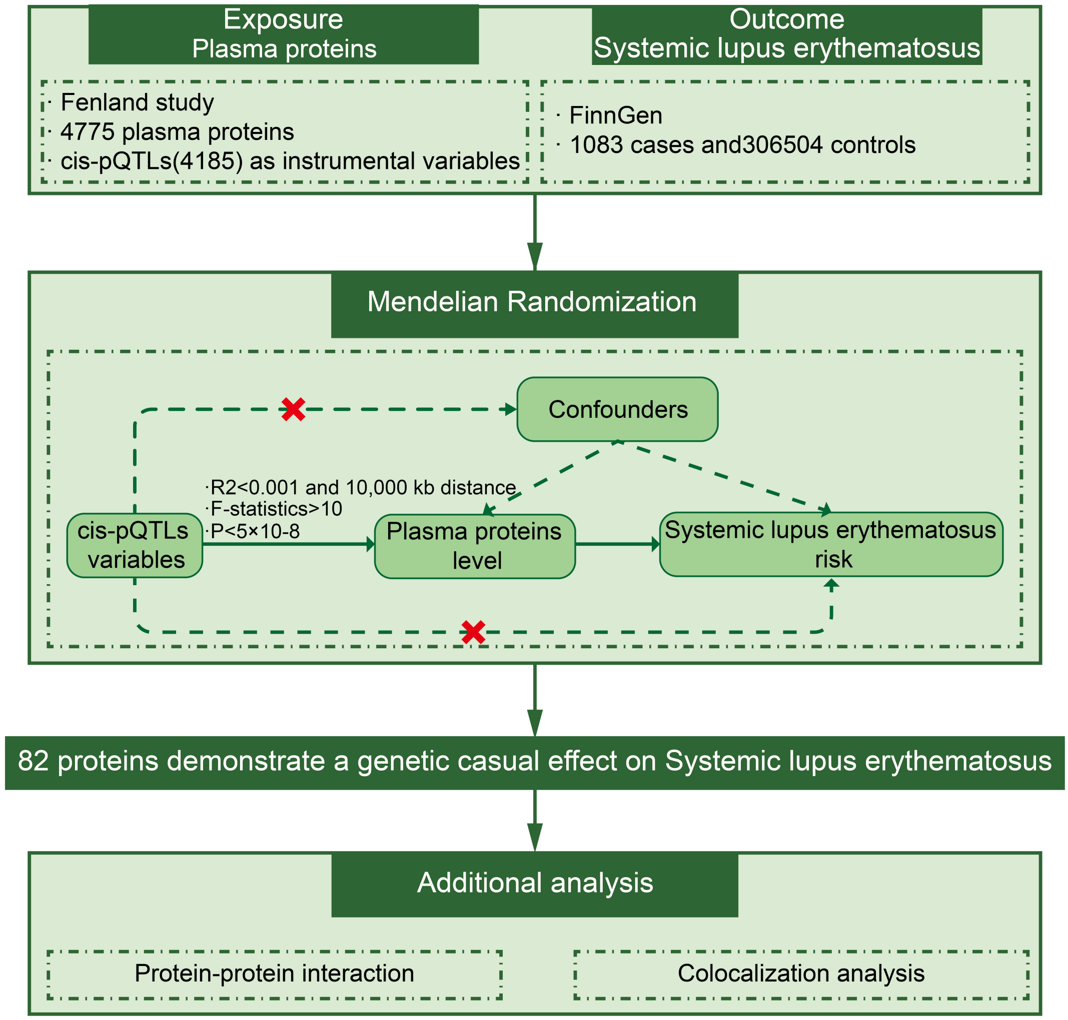

Figure 1. Flow Chart of the Overall Study Design. This study examines the potential genetic causal effects of plasma proteins on the risk of SLE using MR. The exposure (plasma proteins) was assessed using data from the Fenland study, comprising 4,775 plasma proteins. The outcome (SLE) was analyzed using data from the FinnGen study, which included 1,083 cases and 306,504 controls. Cis-pQTLs variables with specific criteria were used to assess the causal relationship. The analysis revealed 82 proteins demonstrating a genetic causal effect on SLE risk. Additional analyses were conducted on protein-protein interaction and colocalization to further validate the findings.

ensuring that the genetic variants selected are likely to have a direct and significant impact on protein expression, reducing the likelihood of bias due to unrelated genetic effects.

MR Analysis and Sensitivity Analysis

In this study, we conducted two-sample MR analysis using R software (version 4.3.3) and the "TwoSampleMR" package, employing various statistical methods to assess the potential causal relationship between circulating proteins and the risk of SLE. We employed the inverse variance weighted (IVW) method as the primary analytical tool when two or more IVs were available. Additionally, when the number of IVs was three or more, the weighted median (WM) and Mendelian randomization-Egger (MR-Egger) methods were also implemented. For proteins represented by a single IV, the Wald ratio method was applied to estimate the change in the log odds ratio of SLE risk per one standard deviation (SD) increase in protein levels [24]. Additionally, we performed sensitivity analysis using Cochran's Q test, MR-Egger, and MR-PRESSO methods. The Cochran's Q statistic was used to assess heterogeneity among the selected IVs, with a $P < 0.05$ indicating significant heterogeneity. A significant intercept in the MR-Egger method suggested potential pleiotropy, which was considered if the $P < 0.05$ [25]. The MR-PRESSO method, utilized via the "MR-PRESSO" package, aimed to identify and remove SNP outliers with horizontal pleiotropy. $P < 0.05$ in the MR-PRESSO global test typically indicates the presence of horizontal pleiotropy in the IVs. However, when the number of SNPs is small (nSNP < 3), this method may be insufficient for effective heterogeneity and pleiotropy analysis [26]. Lastly, the MR-Steiger test was applied to evaluate directional causality by comparing the proportion of variance explained by the IVs in relation to both the exposure and outcome variables, thus assessing the suitability of the IVs [27]. In our analysis, $P < 0.05$ for the MR-Steiger test was considered indicative of a valid causal direction. These multiple sensitivity analyses ensure that our findings are robust to violations of the MR assumptions, minimizing the risk of false-positive causal associations.

pQTL-GWAS Co-localization Analysis

To determine whether protein expression levels and SLE risk share causal variants within the same genomic region, we performed a colocalization analysis using the "coloc" R package with default prior probabilities [28]. Bayesian methods were applied to each cis-gene locus of the proteins to evaluate five mutually exclusive hypotheses: (1) No significant association with either trait (H0); (2) Associated only with protein levels (H1); (3) Associated only with SLE risk (H2); (4) Association

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with both traits, driven by distinct causal variants (H3); (5) Both traits driven by the same causal variant (H4). In our analysis, co-localization was deemed supported when the posterior probability of sharing pathogenic variants (pph4) exceeded 0.6 [29].

PPI Network

To enhance our understanding of the interactions between proteins, we constructed a PPI network using the STRING database (version 11.5, https://cn.string-db.org/), a comprehensive resource that integrates experimental data, computational predictions, and text mining to catalog protein-protein interactions [30]. We developed the PPI network with a minimum interaction threshold of 0.4 (medium confidence), maintaining other parameters at their default settings. The PPI network is essential for visualizing protein interactions and uncovering potential signaling pathways involved in SLE.

Results

Circulating proteins and SLE risk mendelian analysis results

In this study, due to the stringent selection criteria for IVs, only 1581 of the 4,775 circulating proteins assessed in the Fenland study were included in the analysis. This rigorous filtering ensures that the remaining proteins are associated with high-quality, independent genetic instruments, minimizing the risk of bias. MR analysis revealed that 82 plasma proteins were potentially causally associated with SLE risk ($P < 0.05$) as shown in Figure 2. This substantial number of proteins offers valuable insights into the complex interplay of molecular factors contributing to SLE. Specifically, high expression of 46 proteins and low expression of 36 proteins are positively correlated with an increased risk of SLE. Additionally, for proteins with at least three SNPs (nSNP ≥ 3), 13 proteins were further validated by the WM method as being associated with SLE risk ($P < 0.05$) (Table S1).

The minimum F-statistic for the selected IVs for each protein was 23.926, far exceeding the threshold of 10, confirming their strength as robust instruments. The p-values of the Steiger test ranged from 0 to 9.01E-06, affirming that the directionality of the IVs is consistent with the fundamental assumptions of the MR analysis. Additionally, the MR-Egger intercept tests for each protein indicated no evidence of horizontal pleiotropy ($P > 0.05$), with further details available in Table S1, supporting the validity of our causal inferences.

Co-localization Analysis

Bayesian co-localization analysis was conducted on 82 circulating proteins that showed statistically significant associations, encompassing both upstream and downstream regions (detailed results are presented in Table S2). Co-localization analysis is a crucial step in understanding the genetic mechanisms that may underlie both the exposure (protein levels) and the outcome (SLE). The analysis revealed co-localization between SLE and several proteins, namely PDHX (pph4 = 0.67), CTSF (pph4 = 0.64), and TNFAIP3 (pph4 = 0.66), suggesting that these proteins may share common genetic variants with SLE (Figure 3).

PPI networks

A total of 82 proteins ($P < 0.05$) were entered into the STRING database to construct a protein network. Given the study's threshold for minimum interaction strength of 0.4, only 50 imported proteins successfully formed a network with other supplementary proteins, collectively comprising 82 nodes and 54

Figure 2. Mendelian Randomization Analysis Identified 82 Plasma Proteins Potentially Causally Linked to SLE Risk ($p < 0.05$). (A) Presents the analysis for proteins with a single associated SNP (nSNP = 1) using the Wald ratio test in the Mendelian Randomization (MR) analysis. (B) Depicts proteins with multiple associated SNPs (nSNP > 1) using the Inverse Variance Weighted (IVW) method in the MR analysis.

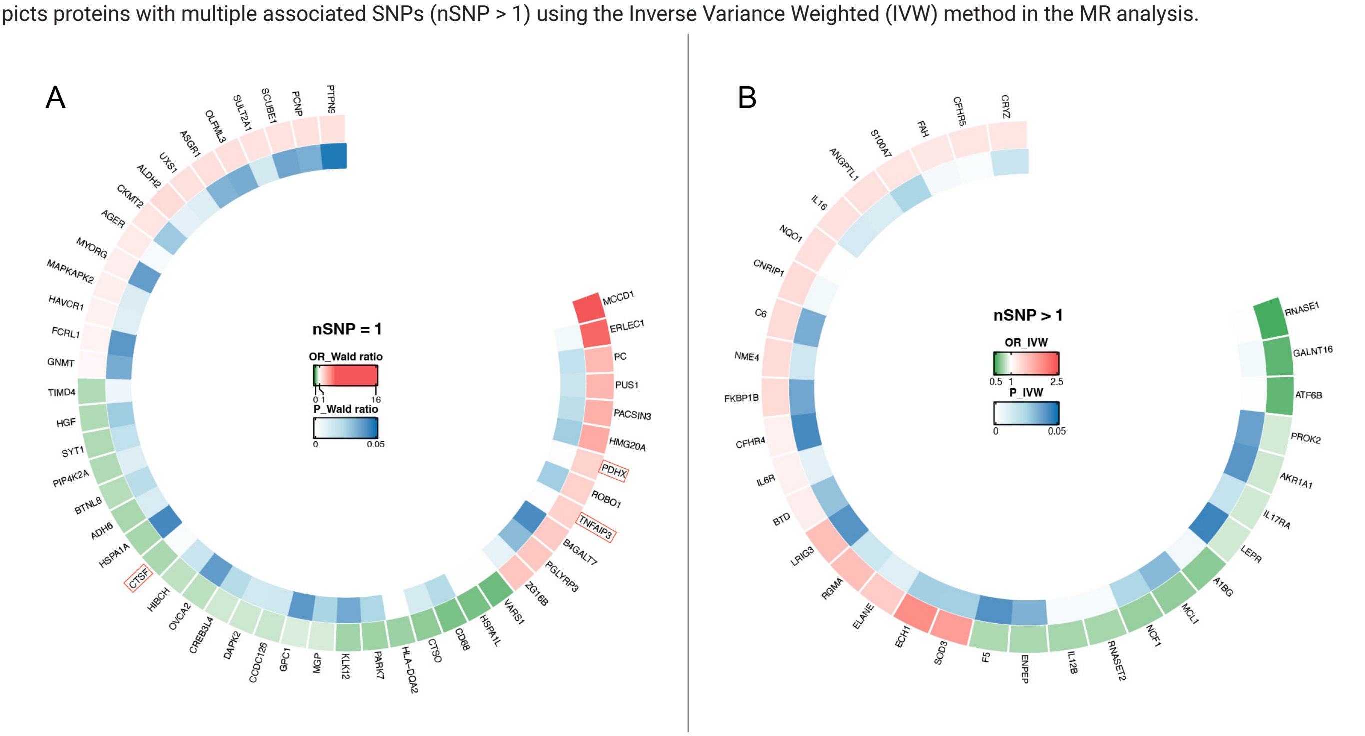
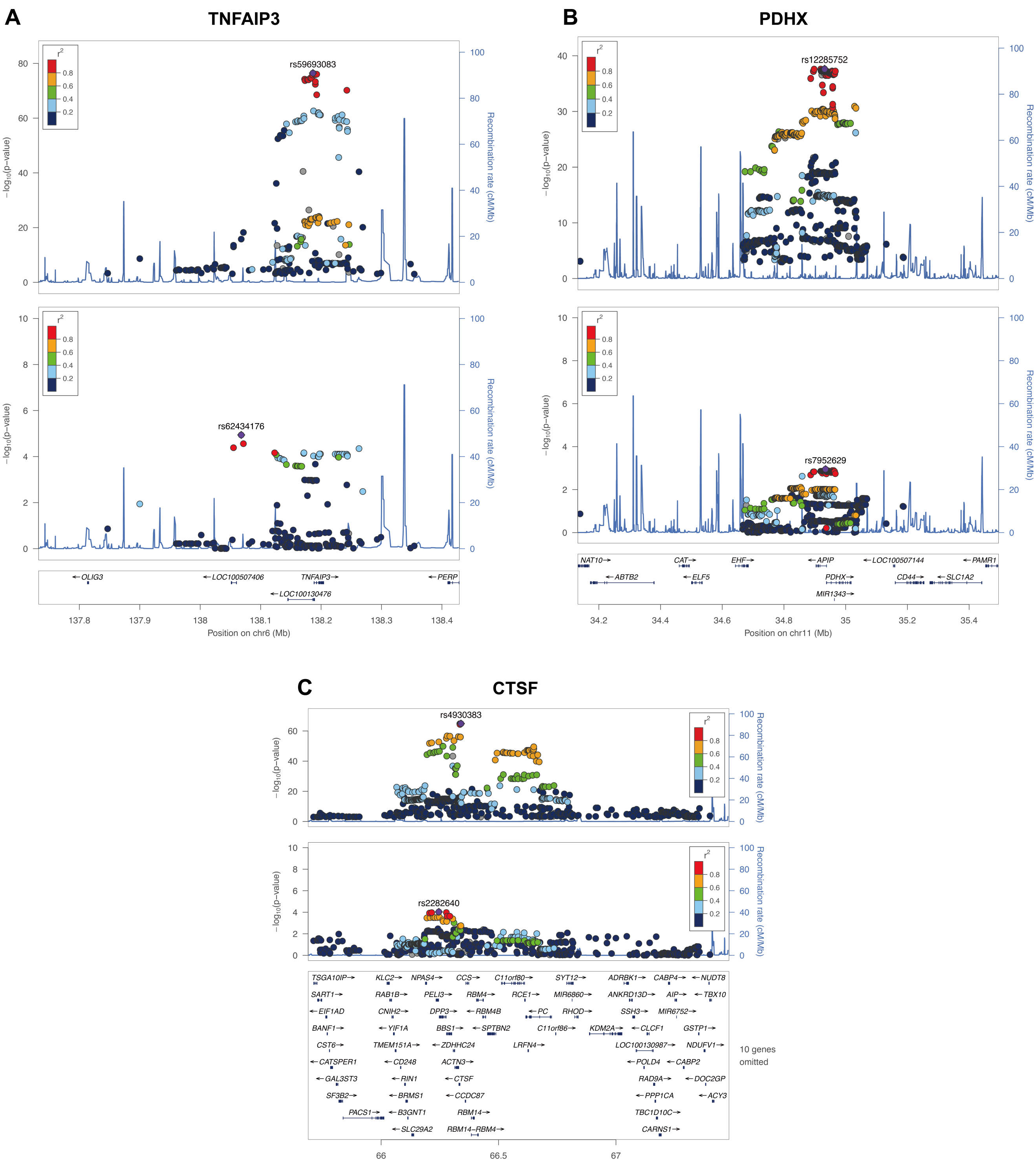


Figure 3. Colocalization Analysis of TNFAIP3, PDHX, and CTSF with SLE Risk. (**A**) Illustrates the co-localization pattern of TNFAIP3 with SLE; the upper image shows pQTLs for TNFAIP3 plasma protein levels, and the lower image displays genetic associations with SLE, with key variant rs59693083 exhibiting significant colocalization. (**B**) Depicts the co-localization pattern of PDHX with SLE; the upper image presents pQTLs for PDHX plasma protein levels, and the lower image shows genetic associations with SLE, with key variant rs12289762 demonstrating significant colocalization. (**C**) Presents the co-localization pattern of CTSF with SLE; the upper image displays pQTLs for CTSF plasma protein levels, and the lower image illustrates genetic associations with SLE, with key variant rs4920540 showing significant colocalization, indicating shared causal variants for protein expression and SLE across all panels.



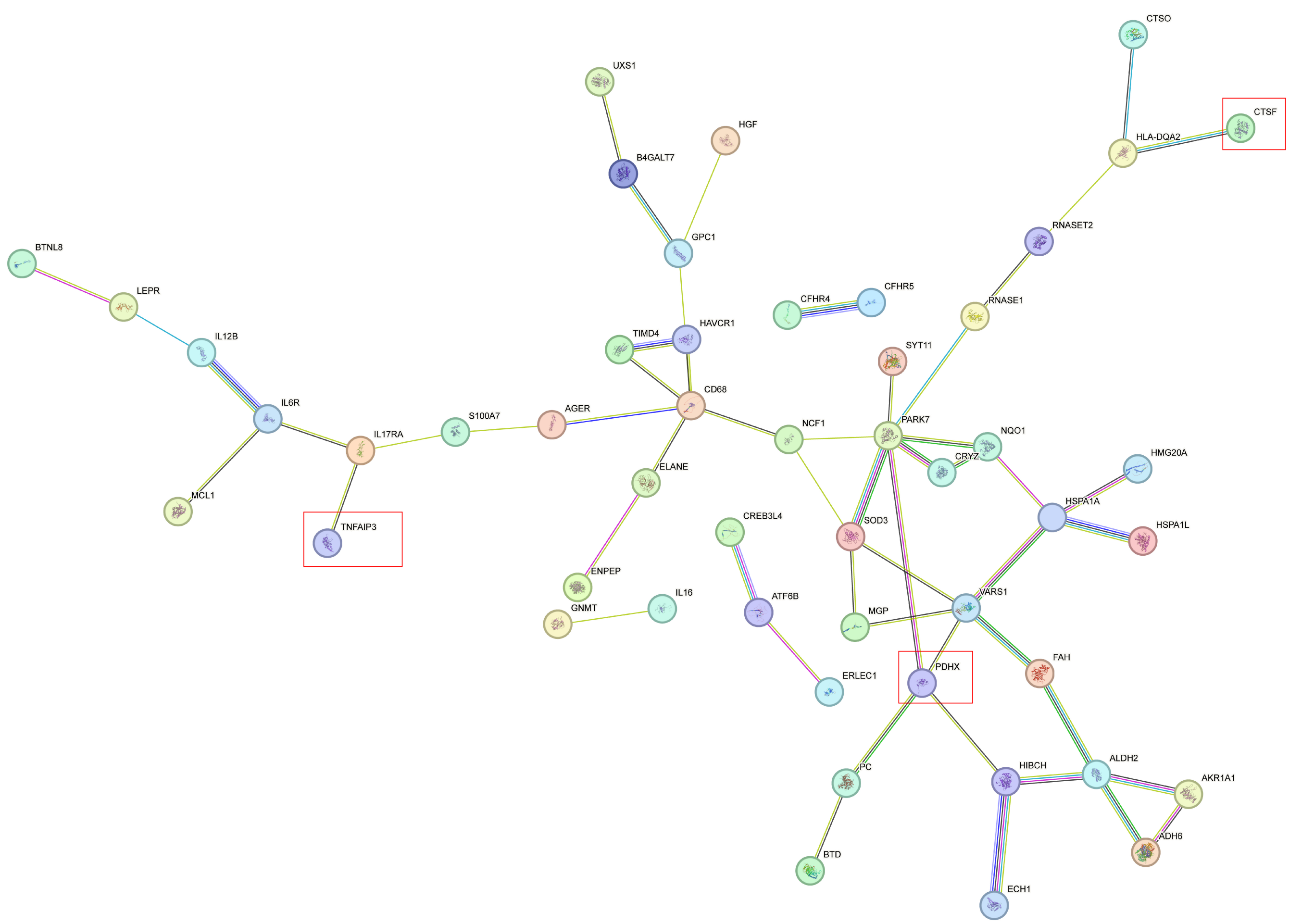
edges (Figure 4). Subsequent network functional enrichment analysis (Figure S1) identified several proteins significantly involved in biological processes such as inflammatory response and immune system regulation, with PFDR values of 1.1e-04 and 4.8e-04, respectively. These processes are intimately connected to the pathophysiology of SLE [31, 32], as chronic inflammation and immune dysregulation are hallmarks of the disease.

Discussion

This proteomics MR study investigated the causal relationships between 4775 plasma proteins and the risk of SLE, leveraging large-scale pQTL and GWAS datasets to overcome limitations of previous observational studies. Employing stringent selection criteria, we identified 82 plasma proteins significantly associated with SLE risk. Notably, proteins such as TNFAIP3, PDHX, and CTSF appear to share the same causal variants as the disease, as evidenced by colocalization analysis. The identification of these proteins highlights their potential as novel therapeutic targets and biomarkers for early diagnosis of SLE, addressing the unmet need for more effective and personalized treatments.

TNFAIP3 is an ubiquitin-editing enzyme extensively documented to act as an endogenous negative feedback regulator of inflammatory responses by inhibiting NF-κB signaling pathway activity [33, 34]. However, TNFAIP3 also promotes the phosphorylation of receptor-interacting protein 3 (RIP3) through deubiquitination [35], thereby activating the NLRP3 inflammasome pathway, which contributing to the development of lupus nephritis [36]. This dual role of TNFAIP3 as both an anti-inflammatory and pro-inflammatory mediator highlights its complex role in SLE pathogenesis. Additionally, various GWAS studies across different populations have demonstrated that SNPs at the TNFAIP3 locus are associated with susceptibility to SLE [37]. For instance, the rs2230926 SNP alters the amino acid sequence of the A20 protein (from phenylalanine to cysteine), diminishing its capacity to inhibit TNF-induced NF-κB activation. This alteration compromises the inflammatory control in individuals carrying this risk allele, thereby heightening their susceptibility to SLE [38, 39]. Additionally, cohort studies have associated the TNFAIP3 rs5029939 genetic polymorphism with SLE susceptibility and potential impacts on its clinical phenotype [39]. Notably, this study identifies for the first time that rs59693083 (located in the TNFAIP3 promoter [40]) escalates the risk of SLE, offering new insights into the pathogenesis and clinical treatment of the disease.

Figure 4. Protein-Protein Interaction (PPI) Network Illustrating Relationships Between Plasma Proteins ($p < 0.05$) Associated with Systemic Lupus Erythematosus (SLE) Risk. Each node represents a plasma protein, and edges between the nodes indicate direct interactions. The greater the number of edges, the stronger the interactions between the proteins. Notable proteins such as TNFAIP3, PDHX, and CTSF are highlighted in red.



PDHX, a crucial component of the pyruvate dehydrogenase complex (PDC), primarily facilitates the conversion of pyruvate into acetyl-CoA, bridging glycolysis to the Krebs cycle [41]. Research indicates that the 11p13 locus, situated between PDHX and CD44, is linked to genetic susceptibility to SLE. This region contains multiple regulatory sites, which potentially affect the expression and function of both PDHX and CD44, consequently influencing immune regulation and inflammatory responses in SLE [42]. This aligns with our MR results. Intriguingly, high PDHX expression has also been associated with diminished immune cell infiltration in the tumor microenvironment [43], suggesting that PDHX's role in SLE pathogenesis might be more complex than previously understood. These insights necessitate further basic and clinical studies to elucidate the underlying mechanisms.

CTSF is a lysosomal cysteine protease that plays a role in various physiological processes, including antigen processing [44]. In clear cell renal carcinoma (ccRCC) studies, CTSF expression has been found to inversely correlate with the infiltration of immune cells and the expression of MHC molecules such as TAP1 and TAP2 [45]. Our MR results show a negative correlation between CTSF and SLE risk, suggesting that CTSF may influence SLE susceptibility. Additionally, cysteine cathepsins (Cts) have been implicated in the hydrolysis of the extracellular matrix (ECM), processing cytokines, chemokines, and cell adhesion molecules, which are critical in inflammatory responses [46]. Cts may mitigate inflammation triggered by cellular debris by degrading damaged organelles and proteins. Although the link between CTSF and SLE risk has not been widely studied, the causal relationship identified in this study warrants further investigation into CTSF's specific role and mechanisms in SLE, which could uncover new therapeutic targets.

This study employed a large-scale dataset for MR analysis to enhance result reliability. In the initial phases, we selected cis-pQTLs for inclusion due to their typical proximity to or within genes encoding proteins, thus directly regulating protein expression by affecting transcription, translation, degradation, stability, or activity. This reduces the likelihood of pleiotropy compared to trans-pQTLs, strengthening the validity of our causal inferences. Additionally, our team utilized various statistical methods such as the Wald ratio, IVW, WM, and MR-Egger to bolster the robustness of the MR analysis, with sensitivity analyses confirming no evidence of horizontal pleiotropy or weak instrument bias. Finally, we performed PPI network analysis to validate the links between protein-related biological processes and the disease's pathological processes.

Despite the strengths of our study, several limitations must be acknowledged. Firstly, it relies on population data from individuals of European ancestry, which may limit the generalizability of the findings to other ancestral groups, as genetic associations and protein levels can vary by ethnicity. Future studies should replicate these findings in diverse populations, including African, Asian, and Hispanic cohorts, to ensure global applicability. Secondly, while the Fenland study included a broad range of circulating proteins, our stringent selection criteria for IVs might have excluded other potentially relevant target proteins. Moreover, protein expression and function are influenced by various factors, such as environmental interactions and epigenetic modifications, which were not comprehensively accounted for in our analysis. Furthermore, there is limited research on CTSF and its relationship with SLE, and much of the proposed mechanisms discussed in this paper are primarily based on inference from related cathepsins or other diseases. Therefore, further research —including in vitro studies with SLE patient samples and in vivo murine models—is needed to establish the direct relationship between CTSF and SLE, elucidate its specific mechanisms, and evaluate its potential as a therapeutic target.

Conclusion

Overall, this study offers a comprehensive assessment of the causal relationships between circulating proteins and SLE, further confirming their critical roles in the initiation and progression of the disease. By combining MR, colocalization, and PPI network analyses, we identified 82 proteins with potential causal associations with SLE. Notably, proteins such as TNFAIP3, PDHX, and CTSF are identified as likely candidates for new therapeutic targets. These findings contribute to the growing body of knowledge on the molecular mechanisms underlying autoimmune diseases, offering promising directions for the development of precision therapies for SLE. However, additional research is essential to unravel the complex mechanisms linking these candidate proteins with SLE risk, a crucial step in validating their potential clinical relevance.

Abbreviations

B-lymphocyte stimulator: BLyS; cysteine cathepsins: Cts; Cis-protein Quantitative Trait Loci: cis-pQTLs; data-dependent acquisition: DDA; data-independent acquisition: DIA; Extracellular Matrix: ECM; enzyme-linked immunosorbent assays: ELISA; Food and Drug Administration: FDA; Genome - Wide Association Study: GWAS; I interferon receptor 1: IFNAR1; Instrumental Variables: IVs; Inverse Variance Weighted: IVW; Linkage Disequilibrium: LD; Mendelian Randomization: MR; Mendelian Randomization-Egger: MR-Egger; protein Quantitative Trait Loci: pQTL; Protein-Protein Interaction: PPI; Receptor-Interacting Protein 3: RIP3; Single Nucleotide Polymorphisms: SNPs; Systemic Lupus Erythematosus: SLE; Trans-protein Quantitative Trait Loci: trans-pQTLs; Weighted Median: WM.

Author Contributions

Xinzhen Zhao: Writing – review & editing, Writing – original draft, Conceptualization, Visualization, Formal analysis. Yinying Chai: Writing – review & editing, Validation, Supervision, Data curation, Formal analysis. Qianran Hong: Writing – review & editing, Writing – original draft, Validation, Funding acquisition, Formal analysis. Yuxuan Song: Writing – review & editing, Validation, Funding acquisition, Data curation. Yibo He: Writing – review & editing, Conceptualization, Supervision. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

The data that supports the findings of this study are available in the supplementary material of this article.

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Review Article

Life Conflux

Multidimensional Roles of EZH2 and Its Therapeutic Potential in Cancer Therapy

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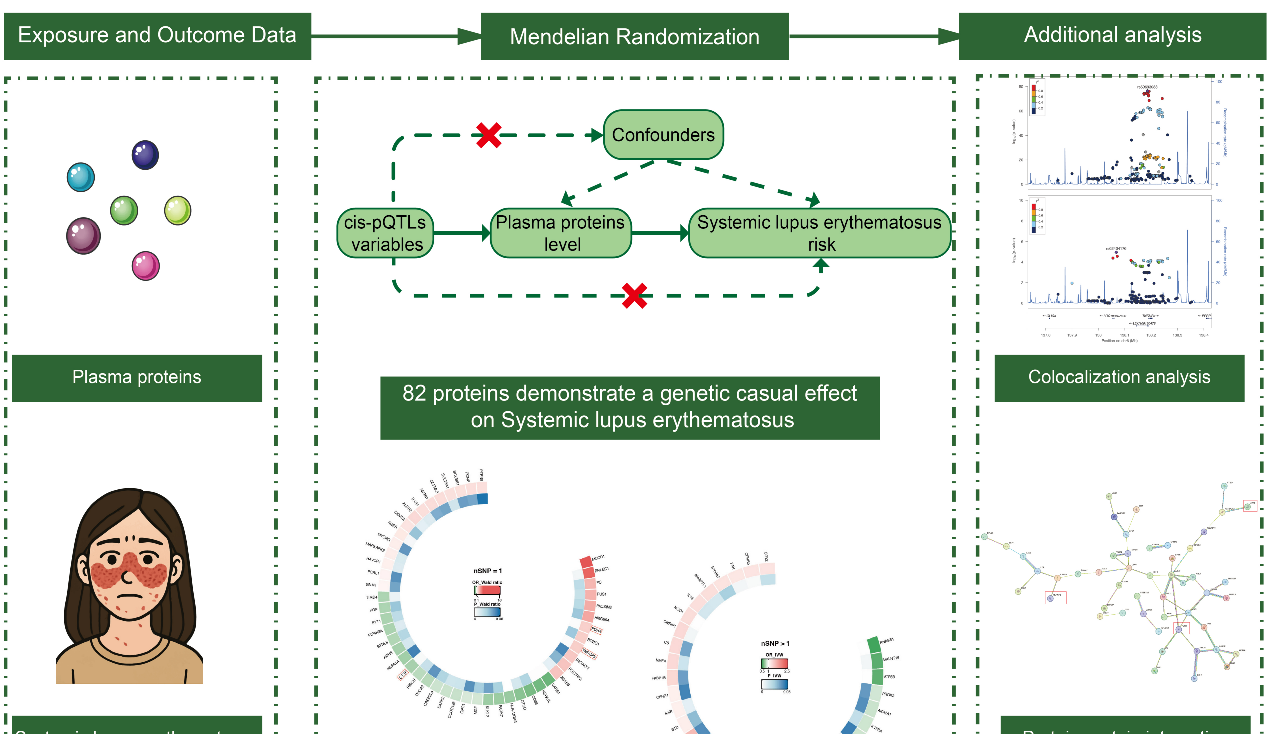
Graphical Abstract



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Review Article

Life Conflux

Multidimensional Roles of EZH2 and Its Therapeutic Potential in Cancer Therapy

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Abstract

Enhancer of zeste homolog 2 (EZH2), a core member of the Polycomb Group (PcG) family, is a pivotal epigenetic regulator. As the catalytic subunit of Polycomb Repressive Complex 2 (PRC2), EZH2 mediates trimethylation of histone H3 lysine 27 (H3K27me3), leading to chromatin condensation and altered expression of downstream genes. This mechanism enables EZH2 to exert multidimensional roles in development, tumors, immunity, and the nervous system. Given its critical role in epigenetic regulation and multidimensional oncogenesis, EZH2 has emerged as a hot target for cancer therapy. This review summarizes EZH2's regulatory functions and specific pro-tumorigenic mechanisms, detailing its roles in epigenetic regulation, tumor proliferation and metastasis, tumor microenvironment, stemness maintenance, drug resistance, metabolic reprogramming, and dysregulated signaling pathways, aiming to inspire new perspectives in cancer treatment research.

Keywords: EZH2; epigenetic modification; methylation; H3K27me3; cancer.

Background

Cancer occurrence is highly correlated not only with genetics, diet, infection, microbiota, smoking, alcohol consumption, and other factors [1-4], but also with epigenetic dysregulation (such as DNA methylation, histone modifications, and non-coding RNA regulation), which silences tumor suppressor genes and activates oncogenes, thereby playing a key role in driving cancer formation [5-7].

Histone modification, as one of the core mechanisms in epigenetics for regulating gene expression, alters chromatin structure through chemical modifications, thereby affecting DNA accessibility and gene transcriptional activity [3, 8]. Among these, trimethylation of histone H3 lysine 27 (H3K27me3), catalyzed by Polycomb Repressive Complex 2 (PRC2), is a key type of histone modification and serves as a critical repressive mark in epigenetic regulation [9, 10].

Enhancer of zeste homolog 2 (EZH2), as the core subunit of PRC2, exerts its oncogenic effects mainly via two modes: the PRC2-dependent classical function and the PRC2-independent non-classical function (Figure 1). The classical function involves EZH2 participating in the formation of the PRC2 complex and binding to the promoter regions of target genes, where it catalyzes H3K27me3 to create a condensed chromatin structure, thereby hindering the binding of RNA polymerase II and transcription factors and affecting downstream gene expression [11, 12]. The non-classical function involves EZH2 directly methylating non-histone proteins, resulting in downstream gene activation or ubiquitin-mediated degradation without the need to form the PRC2 complex [13, 14]. However, recent studies have shown that EZH2 can even function independently of its methyltransferase activity by acting as a transcriptional co-activator that directly binds to target proteins to activate downstream targets, or by directly binding to the promoters of metabolic genes to regulate gene activation [15-17]; thus, these processes are not blocked by enzymatic EZH2 inhibitors.

Epigenetic Regulation of EZH2 in Tumors

EZH2 Catalyzes Methylation of Histone

Histone methylation, as the most central catalytic function of EZH2, drives gene silencing by mediating H3K27 trimethylation, thereby suppressing the expression of tumor suppressor genes and promoting cycle progression. In a controlled RT-

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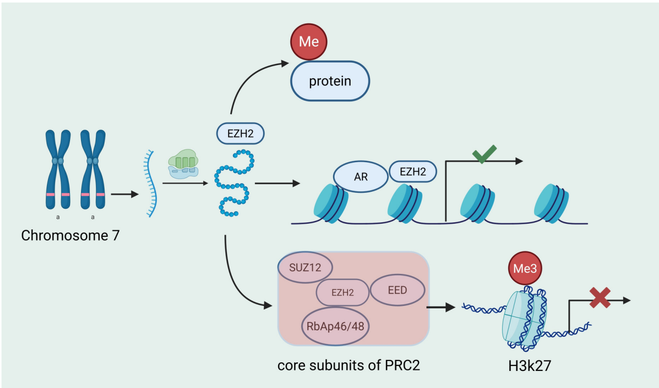
Figure 1. The main mechanisms through which EZH2 exerts its effects are: histone methylation, non-histone methylation, and transcriptional co-activation.



PCR study comparing cholangiocarcinoma tissues with or without EZH2 depletion, it was found that EZH2 knockdown led to significant upregulation of 12 key tumor suppressor genes (such as PAX3, SFRP1, CDKN1A, and GAS1), suggesting that EZH2 may promote cholangiocarcinoma cell growth through epigenetic silencing of tumor suppressor genes [11]. Overexpression of miR-139-5p can induce cellular senescence and inhibit hepatocellular carcinoma cell proliferation. EZH2 promotes TOP2A expression by epigenetically silencing miR-139-5p via H3K27me3, thereby driving hepatocellular carcinoma cell proliferation and malignant progression [12]. Regarding dysregulated cell cycle control in cancer cells, a study found that inhibition of EZH2 expression in multiple myeloma cells increased the levels of cyclin-dependent kinase inhibitors p15 and p21, and this upregulation was associated with the CDKN2B and IFIT3/MYC/CDKN1A pathways [18]. Pan-cancer analyses have shown that PRC2 is enriched at the promoter regions of EMT-related genes, directly suppressing the expression of epithelial markers such as E-cadherin [19-21].

Non-Coding RNA Regulates EZH2 Stability

Studies have revealed that in hepatocellular carcinoma cells, non-coding RNA such as miR-26a, miR-138-5p, and miR-144/451a form a negative feedback loop with EZH2 to regulate cancer cell growth: EZH2-mediated H3K27me3 modification at the promoter regions suppresses the transcription of these miRNAs, while luciferase reporter assays demonstrate that these miRNAs directly target the 3'UTR of EZH2 mRNA, directing post-transcriptional repression and thus modulating gene expression [22-24]. FUS protein is a multifunctional nucleic acid-binding protein that can recognize and bind specific sequence elements within the 3'UTR of target mRNA in cells, thereby preventing the binding of other factors that promote mRNA degradation or structurally enhancing mRNA stability; it thus exerts important biological functions [25, 26]. Non-coding RNA such as hsa_circ_0006232, LINC00313, and LINC SNHG14 have been found to promote the interaction between FUS protein and EZH2, prolong the half-life of EZH2 mRNA, increase its stability, and consequently enhance EZH2-mediated epigenetic silencing of downstream factors (e.g., PTEN and EPHA7), thereby facilitating tumor progression [27-29].

Dynamic Assembly of PRC2 Complex

EZH2 Y641 mutation promotes multiround H3K27 methylation by altering H-bonding patterns and/or steric crowding within the enzyme–bisubstrate ternary complex active site; in other words, the Y641-mutant EZH2 changes substrate preference, preferentially catalyzing the conversion of H3K27me2 to H3K27me3, leading to the formation of lymphoma-specific H3K27me3 hyperdomains [30, 31]. Mutations in SUZ12 and EED are highly conserved; among them, the Suz12 D605V and Eed G255D mutations markedly reduce the histone methyltransferase activity of the PRC2 complex and impair the catalytic formation of H3K27me3 [32].

The Role of EZH2 in Tumor Proliferation,Invasion and Metastasis

EZH2-Related Signaling Axes and Non-Coding RNA Regulatory Networks Influence EMT Process

Epithelial-mesenchymal transition (EMT) in tumor cells is crucial for promoting metastasis and invasion, and this process can be regulated by multiple signaling axes, such as EZH2-MIR-293A-SNAI [33], Snail/Slug-MIR-101-EZH2 [34], MIR-217-MALAT1-EZH2-H3K27me3 [35], and TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 [36]. Long non-coding RNA can recruit EZH2 to exert targeted repression of epithelial markers, thereby facilitating EMT; for example, LINC01133 brings EZH2 and LSD1 to the promoters of KLF2, P21 and E-cadherin to silence their transcription and enhance tumor invasion and metastasis [37]; LINC00978 recruits EZH2 to the promoters of P21 and E-cadherin to promote EMT [19]. In addition to recruitment, LINC00152 can release EZH2 after binding to the PRC2 complex, thereby reducing H3K27me3 enrichment at the ZEB1 promoter and blocking its transcriptional repression, which promotes EMT and oxaliplatin resistance in esophageal cancer cells [38].

Non-Canonical Pathways Promote Tumor Invasion and Metastasis

In addition to epigenetic regulation of histones, splicing-dependent mechanisms also play important roles in cancer cell EMT. Studies have shown that LINC01348 competitively binds to splicing factor 3B subunit 3 (SF3B3), blocking efficient splicing of EZH2 pre-mRNA and leading to loss of functional EZH2 protein, thereby inhibiting hepatocellular carcinoma invasion [39]. Moreover, the EZH2-catalyzed product H3K27me3 has been found to act as an atypical repressor driving peritoneal metastasis in triple-negative breast cancer. Specifically, H3K27me3 is markedly enriched at the promoter of the KRT14 gene; it compresses the GC-box region within the KRT14 promoter to prevent binding of the repressor SP1 and permits RNA polymerase II to initiate transcription of KRT14, thereby promoting triple-negative breast cancer migration, invasion, and peritoneal metastasis. In this study, enrichment of H3K27me3 at the KRT14 promoter did not repress but instead enhanced its transcription [40]. In addition, post-translational modifications of non-histone proteins—such as phosphorylation, methylation, acetylation, and ubiquitination—closely influence protein stability, activity modulation, and protein–protein interactions [3, 41-45]. It has been reported that EZH2, acting as a methyltransferase, directly catalyzes trimethylation of lysines K53 and K333 on SMAD3 under stimulation by the tumor suppressor TGF-β; subsequently, methylated SMAD3 modulates SMAD3 S423/S425 phosphorylation through regulated membrane recruitment, thereby promoting tumor metastasis [46].

EZH2-Regulated Tumor Proliferation and Metastasis via Wnt/β-catenin Signaling Pathway
According to the FpClass database, EZH2 can interact with metal-response element-binding transcription factor 2 (MTF2), and the Wnt pathway antagonist secreted frizzled-related protein 1 (SFRP1) has been identified as a target gene of EZH2. Subsequent studies demonstrated that MTF2 promotes EMT progression in osteosarcoma via the EZH2/SFRP1/Wnt signaling axis [47, 48]. Beyond osteosarcoma, EZH2-mediated regulation of the Wnt/β-catenin pathway in tumor proliferation and metastasis has been validated in multiple cancer types, including colorectal cancer [49, 50], gastric cancer [51], melanoma [52, 53], glioma [54], laryngeal cancer [55], cervical cancer [56], and liver cancer [57], establishing a pan-cancer theoretical and practical basis for EZH2-targeted therapy to suppress the Wnt/β-catenin pathway driving malignant progression. Nevertheless, the precise mechanisms may vary according to tumor heterogeneity and tissue specificity.

Tumor Microenvironment Remodeling

Studies have found that EZH2 deletion or inhibition causes opposite CCL2 expression and thus influences different polarization states of tumor-associated macrophages (TAMs). Whether this depends on histone methyltransferase activity can explain the up-regulation: after treatment with the EZH2 inhibitor EPZ-6438, increased CCL2 levels lead to elevated M2-type TAMs and higher tumor vascular density, further promoting tumor metastasis. Meanwhile, EZH2 can recruit DNMT1 to the miR-124-3p promoter that targets CCL2, forming a hypermethylated structure; this explains why EZH2 loss results in CCL2 down-regulation [58]. Therefore, the above studies indicate that EZH2 exerts opposite regulatory effects on TAMs polarization in breast cancer through its enzymatic or non-enzymatic activities. In addition, contradictory findings show that EZH2 inhibitors can reprogram tumor cells into a more immunogenic state. Specifically, EZH2 inhibitors up-regulate genes related to adhesion, inflammatory response, and B-cell activation in the tumor microenvironment, thereby inhibiting tumor progression and metastasis [59, 60]. From these studies we can see that EZH2-mediated regulation of the tumor immune microenvironment involves highly complex mechanisms with dual roles, reminding us to be especially cautious when choosing EZH2 inhibitors in different tumor microenvironments; otherwise, not only may the expected therapeutic effect not be achieved, but tumor progression may even be exacerbated. Beyond differences in immune cell types and distribution, the hypoxic tumor microenvironment is also a major challenge in solid tumor therapy; in hepatocellular carcinoma cells, hypoxia-induced elevation of LINC00839 is closely associated with a series of malignant phenotypes [61]. Related studies have shown that under hypoxic conditions EZH2 expression and activity increase, thereby affecting tumor cell proliferation, invasion, and metastasis. Moreover, EZH2 can actively participate in remodeling the tumor microenvironment by regulating hypoxia-related factors such as HIF-1α, promoting tumor EMT and malignant progression [62–64].

The Role of EZH2 in Tumor Drug Resistance

EZH2 Affects Drug Resistance through Classical Pathways
In ovarian cancer, c-Myc enhances EZH2 expression by directly repressing miR-137, which targets EZH2 mRNA; conversely, EZH2 can silence miR-137, further strengthening the negative-feedback loop that modulates tumor resistance to cisplatin [65]. Similarly, in esophageal squamous carcinoma cells, CTCF recruits EZH2 and PXN proteins to the miR-137 promoter to inhibit its expression, while EZH2 and PXN mRNAs are downstream targets of miR-137, thereby regulating cellular radiosensitivity through this negative-feedback mechanism [66]. In non-small-cell lung cancer, EZH2 catalyzes H3K27me3 at the puma promoter to silence the apoptosis regulator puma and influence sensitivity to platinum-based agents [67]. In glioblastoma, a circular EZH2-encoded EZH2-92aa protein suppresses NKG2D-ligand expression, allowing tumor cells to evade NK-cell cytotoxicity; although no immunotherapeutics were directly tested, this finding suggests that cellular immunotherapy, immune-checkpoint inhibitors, and cytokine-based drugs (e.g., IL-2, IL-15) may be less effective in glioblastoma [68]. In chronic myeloid leukemia, the lncRNA HOTTIP recruits EZH2 to the PTEN promoter, causing hypermethylation and PTEN down-regulation, which induces resistance to imatinib mesylate [69]. Collectively, these studies demonstrate that EZH2-mediated epigenetic resistance mechanisms critically influence resistance to traditional platinum cytotoxics, immunotherapeutics, radiotherapy, and small-molecule targeted agents across both solid and hematologic malignancies, underscoring EZH2's broad significance in tumor drug resistance. While the precise mechanisms and hierarchical roles of EZH2 in resistance remain incompletely understood, combining EZH2 inhibitors with anticancer drugs holds promise for enhancing drug sensitivity and reducing resistance. To date, such combination studies are largely confined to preclinical models and have shown improved therapeutic outcomes; however, corresponding clinical data are scarce, highlighting the need for expanded translational and clinical investigations.

EZH2 Affects Drug Resistance through Non-Classical Pathways
BRAF mutation activation is the most frequent alteration in cutaneous malignant melanoma [70–72], and BRAF-targeted agents have been highly anticipated for melanoma therapy. Nevertheless, clinical data show that approximately 50% of patients develop varying degrees of resistance after 6–8 months of BRAF-inhibitor treatment [73]. One study found that adding the EZH2 inhibitor EPZ-6438 to vemurafenib markedly improves the response in BRAF-resistant melanoma cells (A375R), as evidenced by decreased viability, cell-cycle arrest, and increased apoptosis. The authors hypothesized that EZH2 may act as a transcriptional co-activator that stimulates transcription of the proto-oncogene PLK1 by associating with E2F1, thereby enhancing PLK1 expression. An alternative mechanism proposes that EZH2 directly methylates STAT3, increasing its phosphorylation; STAT3 then functions as a PLK1 transcriptional activator [74], ultimately leading to PLK1

overexpression [75]. However, several questions surround these mechanistic assumptions. First, if EZH2 indeed serves as a transcriptional co-activator that partners with E2F1 to drive PLK1 transcription, its effect should not be sensitive to methyltransferase inhibitors. Second, the study observed that EZH2 inhibition alone had no impact on PLK1 expression in melanoma cells, indicating that EZH2 inhibitors merely act synergistically with vemurafenib and do not directly suppress PLK1—an outcome that contradicts the proposed mechanism. Therefore, more complex regulatory networks likely remain to be uncovered.

EZH2-Regulated Tumor Drug Resistance via PI3K/AKT Pathway

The PI3K/AKT pathway is the most frequently reported signaling axis through which EZH2 influences tumor drug resistance. In most studies EZH2 acts as an activator of this pathway; however, owing to tissue- and tumor-specific contexts, the exact activation modes differ across cancer types. In acute myeloid leukemia, combined use of the EZH2 inhibitor DZNep and the selective BCL-2 inhibitor venetoclax achieved synergistic efficacy and markedly increased venetoclax sensitivity. Whole-transcriptome analysis revealed that PIK3IP1 (a negative regulator of PI3K/AKT/mTOR signaling) is inversely correlated with EZH2 expression, indicating that EZH2 epigenetically up-regulates the PI3K/AKT pathway to mediate cellular resistance [76]. In chronic myeloid leukemia, LINC-HOTTIP recruits EZH2 to the PTEN promoter, inducing hypermethylation and down-regulating PTEN, thereby driving imatinib resistance [69]. Although the study did not explicitly address PI3K/AKT activation, PTEN is a well-established lipid phosphatase that dephosphorylates PIP3 to PIP2 and suppresses AKT activity across numerous cancers [69, 77, 78]. While EZH2 generally functions as a PI3K/AKT activator, it can also inhibit this pathway in specific settings. In colorectal cancer, LINC-PiHL binds EZH2 and reduces its occupancy at the HMGA2 promoter, lowering H3K27me3 levels at this locus. Up-regulated HMGA2 then enhances PI3K/AKT phosphorylation, leading to oxaliplatin resistance [79]; here, EZH2 behaves as a PI3K/AKT inhibitor. Collectively, these findings illustrate EZH2's multi-target, cell-type, and microenvironment-dependent functions.

Stemness Maintenance

A large body of research has confirmed that EZH2 can act as a cancer stem cell marker and is highly expressed in neuroblastoma, epidermal carcinoma, high-risk cytomegalovirus-infected breast cancer, medulloblastoma, neural stem cells, pancreatic cancer, and other tumor types, thereby promoting tumor self-renewal and metastatic capacity [80-86]. Correspondingly, depletion of EZH2 suppresses the self-renewal ability of tumor cells and reduces their proliferation and invasive potential [87-89].

Non-coding RNA Regulatory Networks

Similarly, EZH2 sustains tumor cell stemness through multiple mechanisms. Most commonly, lncRNA recruits EZH2 to the promoters of tumor-suppressor genes to catalyze H3K27me3 formation, thereby promoting CRC cell proliferation or stemness maintenance. For example, LINC01419 recruits EZH2 to the FBP1 promoter to enhance lung adenocarcinoma cell proliferation and stemness [90]; LINC-HOXB-AS3 recruits EZH2 to the Dicer promoter, epigenetically repressing its transcription to foster sorafenib resistance and stemness maintenance in hepatocellular carcinoma cells [91]. Beyond recruitment, lncRNA can also serve as molecular "sponges" to influence EZH2 expression. LINC-PDZD7, for instance, acts as a miR-101 sponge, preventing the latter from binding to EZH2 mRNA, thereby increasing EZH2 levels and suppressing the stemness regulator ATOH8 [92]. Intuitively, stronger tumor stemness should correlate with stronger immune evasion, and studies have already shown that certain lncRNA are closely linked to tumor immune features [93]. Therefore, it is reasonable to hypothesize that lncRNA may modulate tumor stemness via EZH2, in turn affecting immune responsiveness—a mechanism that clearly warrants further investigation.

Synergistic DNA Methylation Effect

Co-immunoprecipitation revealed that TRIM37 interacts with EZH2 to maintain glioma cell growth and stemness; the underlying mechanism involves down-regulation of the Sonic Hedgehog pathway inhibitor PTCH1, thereby activating the Sonic Hedgehog stem cell signaling pathway [94].

Metabolic Reprogramming

Glycolysis

Tumor cells reprogram metabolism to increase glucose uptake and accelerate glycolysis, producing energy mainly via glycolysis even under abundant oxygen—a phenomenon termed the "Warburg Effect" [95, 96]. Although this mode is energetically inefficient, it supplies proliferating cells with metabolic intermediates. Numerous studies have confirmed the close relationship between EZH2 and tumor metabolic abnormalities [97]. In glioblastoma, an EZH2-EAF2-pVHL-HIF-1α axis drives glycolysis and malignant progression: EAF2 binds and stabilizes pVHL (a tumor suppressor that mediates degradation of the glycolysis master regulator HIF-1α [98, 99]), whereas EZH2 epigenetically silences EAF2, elevating HIF-1α and reprogramming metabolism [100]. Additionally, the EZH2-miR-328-β-catenin axis has been shown to promote glioma glycolysis, indicating that EZH2 can modulate glycolysis via signaling pathways [101]. Intriguingly, in hypoxia-induced HIF-1α-upregulated non-small-cell lung cancer models, EZH2 expression is also increased; EZH2 then epigenetically represses FBXL7, reducing ubiquitin-mediated degradation of its substrate PFKFB4 and ultimately enhancing glucose metabolism and malignant phenotypes [102]. Given the cooperative, complementary oncogenic roles of HIF-1α and EZH2 in diverse tumors, the dual-target compound DYB-03 was screened and found to induce stronger apoptosis and angiogenesis inhibition in lung cancer cells than single-target inhibitors [103]. In bladder cancer, aldehyde oxidase 1 (AOX1) suppresses the tryptophan-kynurenine pathway, lowering NADP levels and decreasing pentose-phosphate-pathway flux and nucleotide synthesis, thereby weakening tumor invasion; EZH2-mediated epigenetic silencing of AOX1 reverses this, increasing pentose-phosphate-pathway flux and nucleotide synthesis [104]. In colorectal cancer, the homeobox transcription factor PROX1 recruits EZH2 to the SIRT3 promoter, repressing SIRT3 and promoting tumor cell proliferation and glycolysis [105]. Non-canonical actions have also been described: in ovarian malignancies, cell-proliferation assays and immunoblotting after treatment with EZH2 inhibitor (DZNep), EZH2 degrader (YM281), or EZH2 catalytic inhibitors (GSK126, EPZ-6438) revealed that EZH2's oncogenic role is independent of its catalytic activity. Integrated ChIP-seq and RNA-seq data showed EZH2 directly binds and transcriptionally activates metabolic genes related to the TCA cycle and OXPHOS, such as IDH2 and OGDHL, thereby fostering metabolic adaptation and malignant progression [17]. In summary, EZH2 markedly promotes tumor-cell glycolysis to drive cancer cell proliferation.

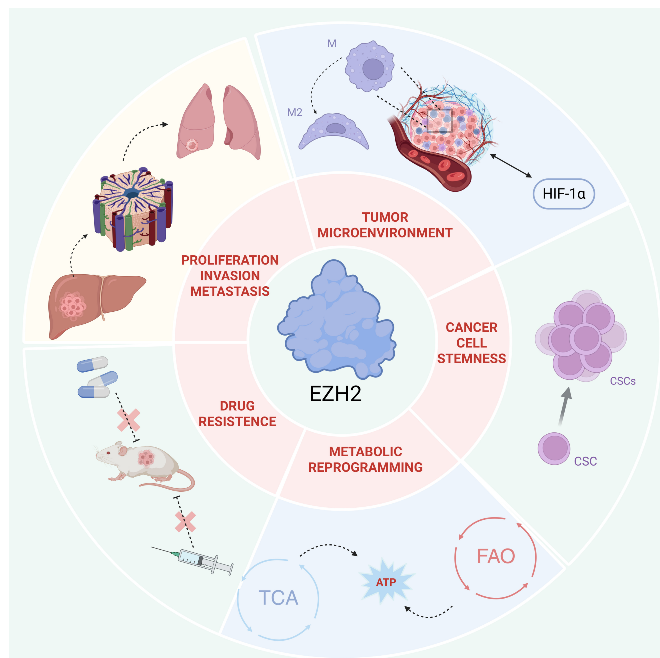
Lipid Metabolism

Rapidly proliferating tumor cells usually enhance fatty acid oxidation (FAO) and synthesis to satisfy the demand for membrane and signaling-molecule production [106]; yet EZH2's influence on tumor lipid levels exhibits contradictory, context-dependent effects across species and organs [107-110]. Thus, a comprehensive dissection of EZH2's specific lipid-metabolic targets and signaling axes—together with cell-type specificity—is urgently needed for precise clinical guidance. In triple-negative breast cancer, EZH2 interacts with the metabolic key enzyme PKM2 to form an epigenetic-regulatory complex that reprograms cellular metabolism from glycolysis toward FAO-β-oxidation, thereby supporting tumor growth [111]. In ARMH1-knockdown leukemia cells (MOLM-14 and HEL92.1.7), supplementation of exogenous lipid mixtures restored proliferation to levels comparable with wild-type controls, indicating ARMH1's involvement in leukocyte fatty acid (FA) synthesis. Subsequent CO-IP confirmed a physical interaction between ARMH1 and EZH2, suggesting functional interdependence linked to lipid metabolism [112]. Moreover, oHSV-infected glioblastoma cells exhibited elevated EZH2 expression and a pronounced increase in FAO, implying that EZH2 plays a key role in lipid-metabolic reprogramming in these tumors [113]. Notably, high intratumoral FA levels not only sustain energy supply and membrane synthesis but also broadly impact the anti-tumor efficacy of EZH2 inhibitors (GSK126, EPZ-6438): EZH2i reduces H3K27me3 at the promoters of lipid-synthesis genes SCD1 and ELOVL2, leading to elevated FA levels and attenuated suppression of melanoma growth. Combining EZH2i with lipid-lowering agents markedly enhanced tumor-growth inhibition, demonstrating that EZH2i profoundly influences tumor lipid metabolism and that lipid levels in turn modulate cellular sensitivity to EZH2i [114]. Accordingly, recent studies on targeted disruption of tumor oxidative defense further confirmed that various EZH2 inhibitors induce dysregulation of lipid-metabolic genes [115].

Amino-acid metabolism

EZH2 expression in colorectal cancer is inversely correlated with glutaminase levels; thus, EZH2 down-regulation accelerates glutamine hydrolysis to glutamate, increasing glutathione synthesis and enhancing cellular antioxidant capacity, thereby attenuating ROS-induced cell death under glucose deprivation—a mechanism potentially linked to tolerance to EZH2 inhibitors [116]. EZH2 influences amino-acid metabolism via two principal routes:(1) suppressing glutamine metabolism as described above, (2) promoting S-adenosyl-methionine (SAM) synthesis. Studies show EZH2 orchestrates a cascade that regulates methionine availability: EZH2 binds the retinoid X receptor α (RXRα) promoter to repress its transcription; diminished RXRα relieves inhibition of LAT1, whose up-regulation then increases methionine import. Elevated methionine satisfies tumor metabolic demand and serves as a methyl donor for DNA and histone methylation [117, 118]. In lung adenocarcinoma, EZH2 inhibition activates Gamma-Aminobutyric Acid (GABA) synthesis because the GAD1 and GAD2 genes are epigenetically silenced by H3K27me3; GABA, a key neurotransmitter, may modulate tumor–stromal cell communication within the tumor microenvironment [119]. Although amino acids act as carbon and nitrogen sources, fueling rapid proliferation and macromolecule synthesis, and their metabolites can act as signaling molecules to modulate oncogenic pathways, the underlying regulatory mechanisms remain poorly understood. Thus, elucidating how EZH2, already implicated in amino-acid metabolism, orchestrates metabolic reprogramming is of paramount importance (Figure 2).

Figure 2. The functions of EZH2 in influencing tumors include: proliferation, invasion and metastasis, drug resistance, metabolism, stemness, and tumor microenvironment.



Signaling Pathways Dysregulation

PI3K/AKT/mTOR Signaling Pathway

The PI3K/AKT/mTOR pathway is a key intracellular signaling axis that widely participates in proliferation, survival, differentiation, angiogenesis, drug resistance, and other biological processes [120–122]. In cancer, activation of this pathway is closely associated with tumor metastasis, invasion, and resistance [123–125]. Among all signaling cascades, the PI3K/AKT/mTOR pathway is the most tightly linked to EZH2: in multiple cancer types, EZH2 is recruited or bound by other biomarkers to epigenetically silence the PI3K/AKT inhibitor PTEN, thereby activating the pathway and influencing cancer-cell proliferation, stemness maintenance, metastasis, invasion, and drug resistance—examples include osteosarcoma [27], leukemia [69], colorectal cancer [126], gastric cancer [127], and prostate cancer [128]. Interestingly, activation of PI3K/AKT can also directly or indirectly mediate phosphorylation of EZH2 at S21; for instance, in ablation cells, PI3K/AKT activity positively correlates with EZH2-S21 phosphorylation. Phosphorylation at S21 is precisely the switch that converts EZH2 from a Polycomb-dependent transcriptional repressor into a transcriptional co-activator that cooperates with factors such as the androgen receptor to activate downstream gene expression [129].

Wnt/β-catenin Signaling Pathway

It is well known that the Wnt/β-catenin pathway plays essential roles in embryonic development [130], cell proliferation, differentiation, and tissue homeostasis. In cancer, this pathway markedly promotes excessive extracellular-matrix degradation, tumor invasion and migration, drug resistance, and stemness maintenance [131]. In normal esophageal epithelial cells, EZH2 binds the WNT2 promoter and, via its histone methyltransferase activity, represses WNT2 expression, preventing aberrant Wnt activation. In contrast, esophageal squamous-cell carcinoma shows reduced EZH2 occupancy at the WNT2 promoter, relieving repression and increasing WNT2 transcription [132]. Similar mechanisms operate in glioblastoma, bladder cancer, and hepatocellular carcinoma, where LINC-H19 recruits EZH2 to the promoters of Wnt inhibitors AXIN2 and NKD1, depositing H3K27me3 and thereby up-regulating Wnt/β-catenin signaling [133, 134]. In glioblastoma, HP1 recruits histone deacetylases (HDACs) to H3K9me2-marked loci to silence gene transcription, but EZH2–HP1BP3 interaction impairs HP1-mediated HDACs recruitment, reducing H3K9me2 and derepressing WNT7B, which influences temozolomide resistance [135]. In colorectal cancer, PAF—an overexpressed translesion DNA-synthesis component—dissociates from PCNA upon Wnt activation, binds β-catenin directly, and recruits EZH2 to form a transcriptional activation complex that boosts β-catenin target gene expression, independent of EZH2 methyltransferase activity [136]. Collectively, EZH2 up- or down-regulates Wnt/β-catenin signaling in different tumors via both canonical and non-canonical mechanisms, thereby influencing proliferation, metastasis, drug resistance, and other malignant phenotypes. The precise mode of action depends on tumor type, cellular microenvironment, and pathway crosstalk.

STAT Signaling Pathway

Studies have shown that EZH2 can exert non-canonical functions by directly binding to and methylating STAT3. After methylation, STAT3 undergoes further phosphorylation at the Y705 site, leading to enhanced transcriptional activity—clearly a direct phosphorylation process mediated by EZH2 on STAT3. In addition to directly phosphorylating STAT3, EZH2 can also indirectly phosphorylate the STAT signaling pathway by regulating JAK2. The STAT3 pathway is widely upregulated in various cancers and is closely associated with malignant phenotypes such as tumor cell apoptosis, invasion, and drug resistance, including in glioblastoma [13, 137], melanoma [75], renal cancer [138], breast cancer [139], and colorectal cancer [140]. Moreover, it has been found that EZH2-mediated regulation of the STAT signaling pathway is involved in the glycolytic process of oral squamous cell carcinoma, providing energy and substrates for rapidly growing tumor cells and facilitating the EMT process [141–143], as well as in regulating the polarization of M1-type macrophages toward the M2 phenotype [144, 145]. Furthermore, after chemotherapy treatment, the EZH2/STAT3 pathway in breast cancer cells is activated, which in turn stimulates the secretion of exosomes carrying miR-378a-3p and miR-378d targeting DKK3 and NUMB, ultimately upregulating the Wnt/β-catenin and Notch pathways to promote tumor drug resistance and stemness maintenance [146]. In summary, the EZH2-regulated STAT signaling pathway plays multiple critical roles in tumor development, metabolic reprogramming, immune microenvironment modulation, invasion, metastasis, and resistance formation. This suggests great potential for the future development of combination therapies using EZH2 inhibitors/degraders and STAT3 inhibitors. However, the complex crosstalk mechanisms within these pathways also indicate that targeting crosstalk nodes may be more effective than inhibiting a single pathway alone.

ERK Signaling Pathway

Studies have shown that the MEK–ERK–Elk-1 signaling axis promotes EZH2 overexpression in triple-negative and ERBB2-overexpressing aggressive breast cancer subtypes by modulating Elk-1 binding sites within the EZH2 promoter. Using promoter assays, cellular experiments, and tissue analyses, the research team demonstrated that MEK inhibitors and Elk-1 siRNA markedly reduce EZH2 mRNA and protein levels. In this context, EZH2 functions as a downstream target gene of the MEK/ERK pathway, influencing breast cancer cell proliferation and invasion [147]. Meanwhile, EZH2 knockdown was found to down-regulate the AKT/ERK signaling cascade, thereby suppressing FSH secretion and inhibiting the development of ovarian cancer in sheep [148]. Additionally, EZH2-mediated silencing of the tumor-suppressor gene SMAD4 activates the ERK/c-Myc pathway, driving resistance in non-small-cell lung cancer [149].

YAP Signaling Pathway

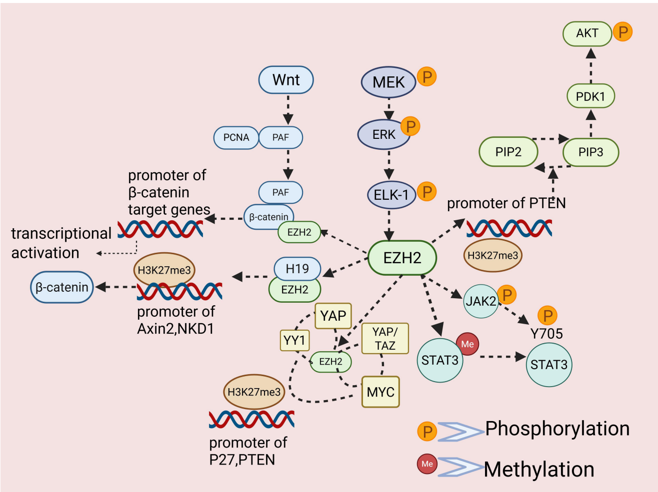
YAP functions as either an oncogene or a tumor suppressor in various cancers. Studies show that EZH2 can form a complex with YAP and the multifunctional transcription factor YY1, facilitating epigenetic modification of the cyclin-dependent kinase inhibitor P27 promoter region and thereby promoting cell proliferation [150]. Similarly, EZH2, together with MYC and YAP/TAZ proteins, can assemble a transcriptional repressor complex that down-regulates the tumor suppressor PTEN [151]. Beyond these transcriptional repressor complexes, the non-canonical Wnt pathway is also critical for maintaining YAP protein levels [152] (Figure 3).

Other pathways

In addition to the major signaling cascades described above, EZH2 participates in the regulation of several non-dominant yet important pathways. For example, in breast cancer the long isoform of NSD3 (NSD3-long) cooperates with EZH2 and RNA polymerase II to stimulate transcription of genes involved in NOTCH receptor cleavage, leading to nuclear accumulation of NICD1. Overexpressed NICD1 then mediates transcriptional repression of E-cadherin and promotes tumor EMT [153]. Other studies have demonstrated that EZH2 can elevate H3K27me3 levels at the GADD45A promoter to suppress its transcription; reduced GADD45A expression subsequently activates the p38/

MAPK pathway [154].

Figure 3. The main signaling pathways regulated or involved by EZH2 are: PI3K/AKT/mTOR, Wnt/β-catenin, STAT, ERK, and YAP Signaling Pathway.



Conclusion

EZH2, a pivotal epigenetic regulator, exerts complex and multidimensional roles in tumorigenesis, progression, metastasis, and drug resistance. Via the canonical PRC2 complex it catalyzes H3K27me3 to silence tumor-suppressor genes, thereby promoting proliferation and invasion, whereas non-canonical actions—direct methylation of non-histone proteins or functioning as a transcriptional co-activator—support stemness maintenance, metabolic reprogramming, and tumor-microenvironment remodeling. During invasion and metastasis, EZH2 drives migration through modulation of EMT-related axes, interference with splicing, and post-translational modification of non-histone proteins; its crosstalk with the Wnt/β-catenin pathway has been validated in multiple cancers, demonstrating both pan-cancer relevance and tissue specificity. In the tumor microenvironment, EZH2 depletion or inhibition can exert opposing effects on TAMs polarization, and its elevated expression and activity under hypoxia further fuel malignant progression. Regarding drug resistance, EZH2 participates in epigenetic silencing as well as non-canonical resistance mechanisms, influencing BRAF-inhibitor resistance and interacting with reprogrammed signaling pathways. As a stemness marker, EZH2 is highly expressed across tumors, fostering self-renewal and metastasis through non-coding RNA networks, cooperative DNA methylation, and signaling circuitries. Metabolically, EZH2 markedly enhances glycolysis, alters fatty-acid synthesis, and modulates amino-acid pathways to supply energy and biosynthetic precursors for rapid proliferation. Pathway-wise, EZH2 broadly regulates PI3K/AKT/mTOR, Wnt/β-catenin, STAT, ERK, YAP, and others, impacting proliferation, stemness, invasion, metastasis, and drug resistance. Despite its prevailing oncogenic image, accumulating evidence indicates context-dependent or even tumor-suppressive roles for EZH2, underscoring its functional complexity and prompting deeper investigation.

Given EZH2's multifaceted significance, it has become a prime therapeutic target. Multiple EZH2 inhibitors are in clinical trials and show favorable tolerability and antitumor activity. However, functional heterogeneity across tumor types and individuals necessitates further dissection of EZH2 mechanisms within distinct microenvironments, development of precision strategies, and mitigation of potential resistance. Combining EZH2-targeted agents with immunotherapy, chemotherapy, or other modalities holds great promise for breakthroughs that could improve patient outcomes and quality of life.

Abbreviations

Aldehyde Oxidase 1: AOX1; Androgen Receptors: AR; Epithelial-Mesenchymal Transition: EMT; Enhancer of Zeste Homolog 2: EZH2; Fatty Acid: FA; Fatty Acid Oxidation: FAO; Gamma-Aminobutyric Acid: GABA; Histone Deacetylases: HDACs; Metal-Response Element-Binding Transcription Factor 2: MTF2; Secreted Frizzled-Related Protein 1: SFRP1; Trimethylation of Histone H3 Lysine 27: H3K27me3; Polycomb Group: PcG; Polycomb Repressive Complex 2: PRC2; Retinoid X Receptor α: RXRα; S-Adenosyl-Methionine: SAM; Tumor-Associated Macrophages: TAMs.

Author Contributions

Mingfen Li and Hongsheng Lin were in charge of the proofreading and design of the manuscript. Hui Yin took responsibility for the writing of the manuscript. Jinna Tan and Jiaqian He were responsible for the collection and organization of literature. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Not Applicable

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Do open access articles have a citation advantage? — a research based on European Urology Family

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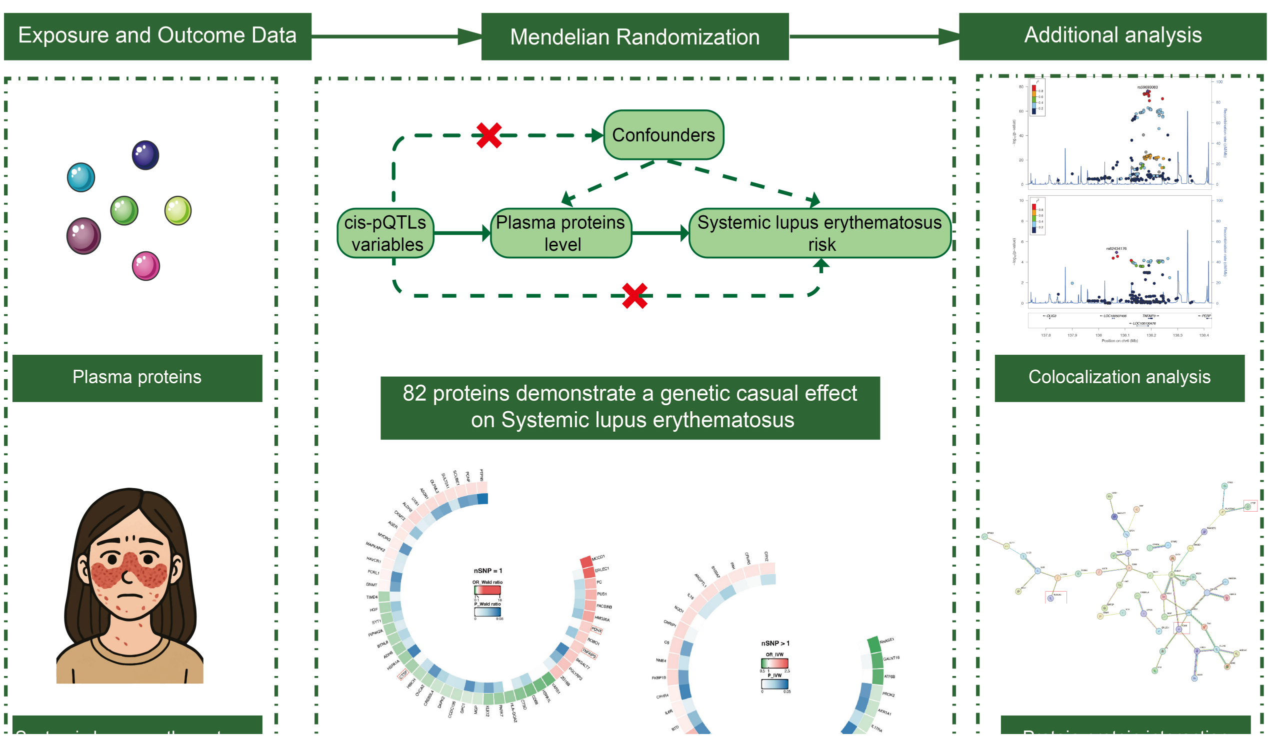
Graphical Abstract



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Do open access articles have a citation advantage? — a research based on European Urology Family

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Abstract

Background: Open access (OA), referring to the practice of providing unrestricted access to scholarly research outputs, has become a main publishing choice for authors. Previous studies have reported OA articles had a citation advantage compared to non-OA articles. Thus, this research aimed to further explore the impact of OA on article citations.

Methods: We searched the Web of Science for research articles and reviews published in European Urology and its sub-journals in 2021. After data extraction, IBM SPSS Statistics 26 was used for statistical analysis, and binary logistic regression analysis was conducted to examine the influence of different variables, particularly OA, on article citation counts.

Results: The study included 135 articles from European Urology, 183 articles from European Urology Focus, and 101 articles from European Urology Oncology. The analysis revealed a significant positive effect of OA on article citations in European Urology (OR = 0.391, 95%CI = 0.189-0.810, p = 0.011). However, OA did not have a statistically significant impact on article citations in European Urology Focus (p = 0.847) or European Urology Oncology (p = 0.83).

Conclusion: Our analysis suggests OA significantly boosts citations in high-impact journals, but shows minimal effect in lower-impact venues.

Keywords: open access; European Urology; regression analysis.

Introduction

Open access (OA) refers to the practice of providing unrestricted access to scholarly research outputs. It is a movement within the academic and research communities aimed at making research findings freely available to anyone with an internet connection, without the need for subscription or payment. Compared with traditional subscription-based access, OA provides unrestricted online access to research, which might lead to wider reach and higher citations. Previous studies in the field of human electrophysiology have reported OA articles had a citation advantage compared to non-OA articles [1], while literature [2] from dentistry field has demonstrated divergent results. However, OA also presents limitations, such as the financial burden of publication fees that some authors cannot afford.

As the important journals in urology, European Urology (Cite Score = 47.2), European Urology Focus (Cite Score = 11.0), European Urology Oncology (Cite Score = 13.0) publish peer-reviewed original articles and topical reviews on a wide range of urological problems, which offers authors two choices to publish their research: Gold OA and Subscription. Gold OA articles are freely available to both subscribers and the wider public with permitted reuse while Subscription articles are made available to subscribers as well as developing countries and patient groups through access programs. Also, these journals are under a under a unified publisher, which could reduce bias. Therefore, this study aimed to discovered the role of OA in citation of OA articles in these journals.

Methods

We selected 135 records of articles published in European Urology, 183 articles in European Urology Focus and 101 articles in European Urology Oncology in 2021 on the Web of Science, which contained 2 study types: research article and review article. The records included OA status, study types,

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number of references, length of article in pages, the continent of origin of the corresponding author, number of authors, number of citations, number of months online and international collaboration status. International collaboration status "yes" was defined as the article containing authors from at least two countries. The deadline for calculating the number of citations and number of months online was October 1th, 2024. If the number of article citations was larger than 30, the article would be categorized as highly cited, otherwise, it would be classified as low-citation group. Fisher's exact test or χ^2 test were used to compare differences in dichotomous variables between OA and subscription access articles. Student's t-test was employed to assess differences in continuous variables. Binary logistic regression was performed to identify independent significant factors for citation. Statistical analyses were performed with IBM SPSS Statistics 26. P values < 0.05 were considered statistically significant.

Results

For European Urology, 135 articles were enrolled, of which 61 exhibited OA articles, while 74 presented as subscription access articles. The specific characteristics of the articles are elucidated in Table 1. Number of citations of OA articles were significantly higher than that of subscription access articles (p = 0.014, Table 1), while other variables between them had no statistically significant difference. Results of logistic regression analyses are displayed in Table 2. Variables showing statistical significance (p <0.05) in univariate analysis were included in multivariate analysis. Among all variables in multivariate analysis, only OA had a statistically significant positive impact on the highly cited group (OR = 0.391, 95%CI = 0.189-0.810, p = 0.011, Table 2).
However, in European Urology Focus, OA articles were not significantly higher than that of subscription access articles (p = 0.847, Table 1), and the same applied to European Urology Oncology (p = 0.83, Table 1).

Discussion

An empirical investigation over multiple disciplines [3] reported that OA had a general positive effect in increasing journal CiteScores. Similarly, a study in the field of hepatology [4] and craniofacial surgery [5] identified a citation benefit from OA. However, a study in ophthalmology field [6] did not found the positive effect of OA on citation. According to Yang's study [3], high-ranking journals realize less benefit from OA because researchers will always cite such journals in their fields, regardless of their access policies, which might explain the inconsistent results of studies on different types of journals.
According to our research, OA was significantly associated with higher citation counts for articles from European Urology in 2021. But in European Urology Focus and European Urology Oncology, OA did not have statistically significant positive impact. We infer that in high-impact journals, OA will lead to more significant citation enhancement. This is because papers published in high-scoring journals tend to have a greater impact factor, whereas papers in relatively low-scoring journals may have limited citation value irrespective of whether they are openly accessible or not, thus failing to produce notable citation enhancement. This explains why we only observed the citation enhancement effect of OA in European Urology. Thus, for authors who plan to publish their articles in high-impact journals, OA merits consideration in order to gain citation advantage.
The limitation of the present study is that we only included articles from European Urology, European Urology Focus, and European Urology Oncology, which restricted the generalizability of our findings to other journals. Additionally, the study was limited to a sample size of 419, and such insufficient sample size may introduce selection bias, potentially compromising the robustness of the conclusions.

Conclusion

Our analysis suggests open access significantly boosts citations in high-impact journals, but shows minimal effect in lower-impact venues. However, further large-sample studies are necessary to support this conclusion.

Abbreviations

OA: open access.

Authors' contributions

Jincong Li and Ruimeng Yue performed data management. Jincong Li, Chengxiang Tian and Shuangying Zhang performed data analysis. Jincong Li, Shuangying Zhang, Yun Peng, Yuxuan Song and Yuyao Jian wrote the manuscript. Rui Chen, Yang Liu, Yun Peng and Yuxuan Song performed conception and design. Jincong Li, Yun Peng and Yuxuan Song performed project development. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Data Availability

The dataset used in the present study could be accessed from Web of Science.

Table 1. The main characteristics of all the articles included.

Variables	European Urology			European Urology Focus			European Urology Oncology		
	Open access n=61	Subscription access n=74	p	Open access n=64	Subscription access n=119	p	Open access n=32	Subscription access n=69	p
Number of months online	35.90 ±3.234	35.77 ±3.602	0.826	37.78 ±3.475	39.06 ±3.521	0.02	38.00 ±3.048	38.04 ±3.440	0.951
Continent of origin of the corresponding author			0.138			0.4			0.595
Asia	0(0%)	5(6.8%)		0(0%)	5(4.2%)		0(0%)	2(2.9%)	
Africa	0(0%)	0(0%)		0(0%)	1(0.8%)		0(0%)	0(0%)	
Australia	4(6.6%)	2(2.7%)		1(1.6%)	3(2.5%)		0(0%)	0(0%)	
Europe	29(47.5%)	39(52.7%)		45(70.3%)	73(61.3%)		22(68.8%)	44(63.8%)	
North America	28(45.9%)	27(36.5%)		18(28.1%)	37(31.1%)		10(31.2%)	23(33.3%)	
South America	0(0%)	1(1.4%)		0(0%)	0(0%)		0(0%)	0(0%)	
Number of authors	16.34 ±9.857	14.78 ±8.725	0.331	12.84 ±8.342	10.24 ±5.418	0.011	17.41 ±11.410	12.07 ±5.364	0.002
International collaboration			0.304			0.003			0.646
Yes	36(59.0%)	50(67.6%)		45(70.3%)	56(47.1%)		21(65.6%)	42(60.9%)	
No	25(41.0%)	24(32.4%)		19(29.7%)	63(52.9%)		11(34.4%)	27(39.1%)	
Number of references	39.21 ±40.934	38.95 ±40.033	0.97	29.13 ±17.893	26.75 ±11.762	0.282	40.66 ±26.374	33.23 ±21.379	0.135
Length of article in pages	9.52 ±3.534	9.50 ±4.162	0.971	7.89 ±2.939	7.22 ±2.108	0.076	9.94 ±4.111	8.75 ±4.384	0.201
Number of citations			0.014			0.847			0.83
>30	36(59.0%)	28(37.8%)		9(14.1%)	18(15.1%)		9(28.1%)	18(26.1%)	
<=30	25(41.0%)	46(62.2%)		55(85.9%)	101(84.9%)		23(71.9%)	51(73.9%)	
Study types			0.714			0.35			0.934
Research article	47(77.0%)	55(74.3%)		42(65.6%)	86(72.3%)		22(68.8%)	48(69.6%)	
Review	14(23.0%)	19(25.7%)		22(34.4%)	33(27.7%)		10(31.2%)	21(30.4%)	

Table 2. Univariate and multivariate logistic regression analysis for European Urology articles.

Variable	Univariate analysis		Multivariate analysis	
	OR (95%CI)	p-value	OR (95%CI)	p-value
Number of months on-line	1.028(0.931-1.135)	0.582		
Continent of origin of the corresponding author		0.493		
Asia	Reference			
Australia	8.0(0.500-127.900)	0.141		
Europe	2.974(0.316-28.034)	0.341		
North America	4.462(0.468-42.514)	0.194		
South America	n.a.			
Number of authors	1.013(0.977-1.052)	0.483		
International collabora-tion		0.659		
No	Reference			
Yes	1.171 (0.579-2.368)			
Length of article in pag-es	1.159 (1.045-1.285)	0.005	1.081 (0.925-1.263)	0.326
Study types		0.013		0.446
Research article	Reference		Reference	
Review	0.350 (0.154-0.798)		0.648 (0.212-1.982)	
Number of references	1.016 (1.003-1.029)	0.013	1.006 (0.988-1.025)	0.503
OA status		0.015		0.011
No	Reference		Reference	
Yes	0.423 (0.211-0.846)		0.391 (0.189-0.810)	

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Case Report

Life Conflux

Forearm necrotizing fasciitis caused by freshwater fish in an immunocompromised patient with rheumatoid arthritis for 20 years: a case report and review of the literature

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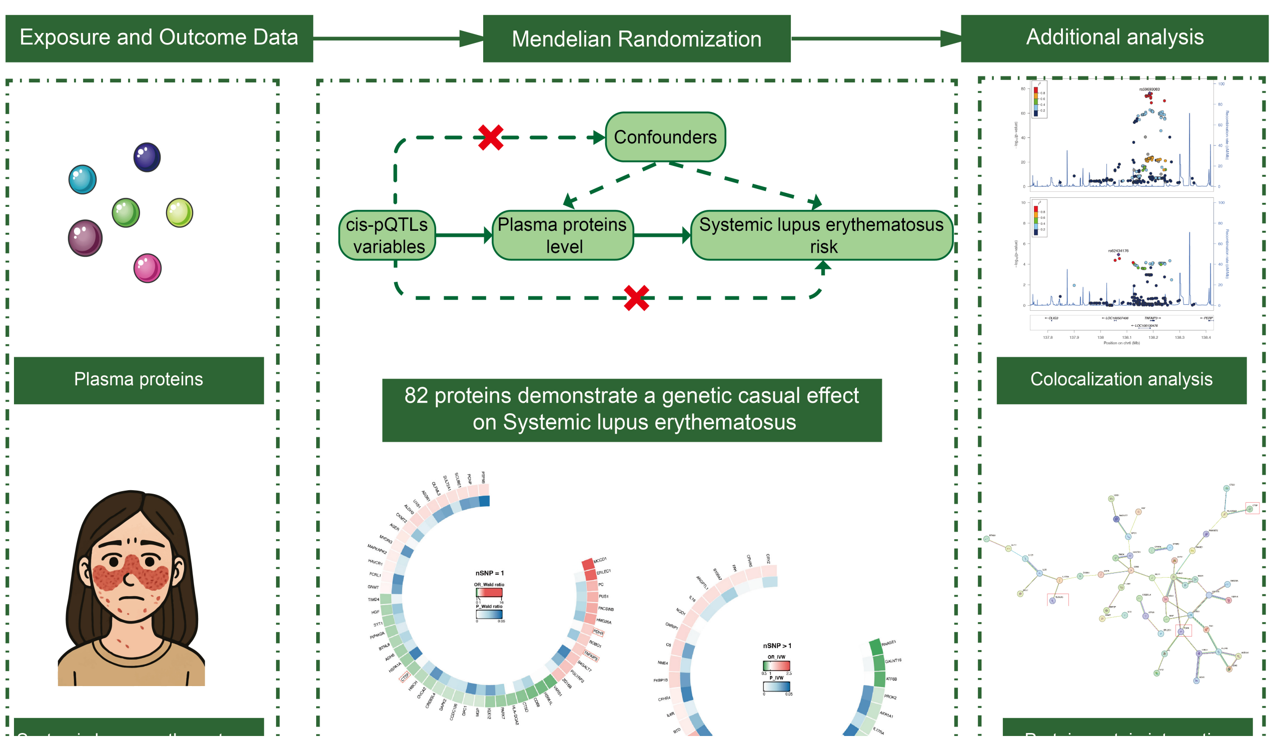
Graphical Abstract



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Forearm necrotizing fasciitis caused by freshwater fish in an immunocompromised patient with rheumatoid arthritis for 20 years: a case report and review of the literature

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Abstract

Necrotizing fasciitis (NF) is a life-threatening soft tissue infection typically associated with marine pathogens such as Vibrio vulnificus in cases involving fish-related injuries. This case report describes a rare instance of NF caused by Aeromonas sobria following a freshwater fish (Chinese carp) injury in a 58-year-old immunocompromised woman with a 20-year history of rheumatoid arthritis on long-term immunosuppressive therapy. The patient presented with rapid-onset swelling, pain, and septic shock, requiring intensive care, vasopressor support, and four surgical debridements with negative pressure wound therapy. Wound cultures confirmed Aeromonas sobria, contrasting with the more common marine-acquired Vibrio infections. Despite delayed diagnosis, aggressive multimodal management—including broad-spectrum antibiotics (piperacillin-tazobactam followed by meropenem) and repeated surgical interventions—resulted in survival after 41 days of hospitalization. This case highlights the importance of considering freshwater pathogens in NF, particularly in immunocompromised patients, and underscores the critical role of early surgical debridement and empiric antimicrobial coverage for atypical organisms. Clinicians should maintain a high suspicion for NF in fish-related injuries, even without marine exposure, to prevent fatal outcomes.

Keywords: Necrotizing fasciitis, Aeromonas sobria, Immunocompromised host, Septic shock.

Introduction

Necrotizing fasciitis is one of the fatal skin and soft tissue infections. In the cases caused by fish wounds, most of them are caused by marine fish wounds, and Vibrio vulnificus has been detected in almost all cases [1-4]. Even when combined with a clear history of seafood exposure and a positive vibrio vulnificus culture, and most physicians give early warning and treat it with caution, the prognosis of necrotizing fasciitis is not optimistic [5]. In the absence of typical contact history, some patients are likely to be missed, resulting in delayed treatment. This paper reports a case of necrotizing fasciitis in our center, which was injured by freshwater fish and cultured as mild aeromonas, and summarizes relevant literature to provide reference experience for clinical treatment of similar diseases.

Case Presentation

The 58-year-old female patient had a history of rheumatoid arthritis for 20 years. She had been taking tripterygium wirelli for immunosuppressive therapy for a long time. She had a history of right knee fracture surgery and binocular cataract surgery. Nine days earlier, a freshwater fish (Chinese carp) had stabbed the area between the thumb and forefinger of the left hand, and the following day the patient experienced swelling and pain in the affected area. The patient removed the scab on her own and disinfected the wound with iodine povidone, but her condition did not improve and the swelling worsened, increasing to the whole palm and wrist, so she was admitted to the emergency department. On the day of the patient's visit, she presented with severe symptoms of unconsciousness and a drop in blood pressure (62/42 mmHg), with further swelling of the stabbed area. Emergency physicians immediately supplemented blood volume, applied vasoactive drugs and transferred the patient to the intensive care unit. Critical care physicians applied piperacillin-tazobactam for anti-infective treatment and cultured mild Aeromonas sp. in the wound secretions, and continued aggressive rehydration with high doses of vasoactive medications (norepinephrine 160ug/min to

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maintain blood pressure around 120/70mmHg) to maintain the patient's vital signs. The patient underwent a first emergency surgical debridement on the first day of hospitalization, during which the surgeon incised the affected area for thorough debridement and placed a Vacuum Sealing Drainage (VSD) (Figure1A-B). After the operation, the patient was returned to the intensive care unit, where mechanical ventilation was continued, followed by an anti-infective regimen of meropenem (1gQ8H), vasoactive drugs, gammaglobulin, allogeneic plasma, and albumin to maintain the patient's vital signs. On the 20th day of hospitalization, the patient's vital signs stabilized, and he was extubated and resumed spontaneous respiration. The patient underwent the second, third, and fourth surgeries on the 8th, 19th, and 26th days after hospitalization, respectively (Figure1C-F). Finally, the patient was discharged from the hospital with a total of 41 days of hospitalization, including 27 days in the intensive care unit.

Discussion

Regarding the treatment of necrotizing fasciitis, there are no other than three problems: early diagnosis, antibacterial treatment and surgical intervention, but in practice all three issues are not easily resolved in clinical practice.

Diagnosis

Necrotizing fasciitis is a life-threatening soft tissue infection characterized by rapidly progressive destruction of the muscle fascia and surrounding soft tissues. Depending on the infecting bacteria, necrotizing fasciitis is usually classified into four types [6]: Type I: infections involving multiple microorganisms; Type II: infections caused by a single pathogen, mainly hemolytic streptococci; Type III: infections involving Clostridium, Vibrio, and Gram-negative bacteria; and Type IV: infections involving fungi. The initial symptoms of necrotizing fasciitis are often non-specific, presenting only non-characteristic symptoms such as pain, redness, swelling, and erythema, making clinical diagnosis a challenge. However, when severe manifestations of necrotizing fasciitis occur, such as localized twisting, skin necrosis, or even infectious shock, it is often indicative of a progression of the infection. Laboratory Risk Indicator for Necrotizing Fasciitis (LRINEC) is a clinical tool first described by Wong [7] et al. It is thought to be able to detect cases of necrotizing fasciitis in early clinical stages, but some scholars are skeptical about its practical application: the LRINEC score used to diagnose necrotizing fasciitis had a low Receiver Operating Characteristic Curve (ROC) in a study by Rav [8] et al. It is not recommended to be used alone and needs to be combined with history, physical examination and imaging; Chang [9] concluded that the accuracy of the LRINEC score is unreliable in necrotizing fasciitis involving Vibrio traumaticus.

Although the clinical symptoms of necrotizing fasciitis are similar to those of cellulitis and other soft-tissue infections in the initial stage, both of which manifest as erythema, localized fever, skin hardness and edema, necrotizing fasciitis often manifests itself as severe pain that is not proportional to the severity of the lesion [10]. And usually within two days, patients not only have more intense pain, but most of them develop systemic symptoms such as fever, confusion, diarrhea, nausea and vomiting, and after four days, patients may develop infectious shock or multi-organ failure, which includes hypotension, metabolic acidosis, coagulation disorders, and malnutrition [11]. In all cases with clinical symptoms similar to cellulitis, there is a need to be vigilant and a high suspicion of necrotizing fasciitis, especially when accompanied by severe pain that progresses rapidly and is not consistent with the condition. Sarani et al.[12] suggested local biopsy histopathology in highly suspicious cases to confirm the diagnosis of necrotizing fasciitis in an earlier study.

Medical imaging is a powerful tool in the diagnosis of necrotizing fasciitis. Although radiographs are less sensitive, they can also be used routinely as soft tissue gas can be observed in most case images of necrotizing fasciitis [13] and can rule out potential foreign bodies or fractures. Ultrasonography similarly lacks resolution of deeper structures and is therefore of limited diagnostic significance in necrotizing fasciitis. However, the visualization of fascial irregularities on ultrasound images, or abnormal fluid accumulation in deep fascial planes can also help to differentiate necrotizing fasciitis from cellulitis [14]. Computed tomography (CT) examination is the main imaging modality for necrotizing fasciitis due to its high spatial resolution, and the visualization of gas in the deep fascial planes on CT images is a diagnostic hallmark of necrotizing fasciitis [15], but it does not exclude the diagnosis of necrotizing fasciitis in some of the cases in which there is no soft-tissue gas [16]. Magnetic resonance imaging has an extremely high contrast resolution for the diagnosis of soft tissue infections and can achieve a sensitivity of 93% for the diagnosis of necrotizing fasciitis [17], which can be used as the gold standard for the diagnosis of necrotizing fasciitis. Thickening of the deep fascia and subfascial effusion observed on magnetic resonance imaging can suggest the diagnosis of necrotizing fasciitis, which can be confirmed when the thickness of the deep fascia is greater than 3 mm and is accompanied by the involvement of multiple fascial compartments.

Antimicrobial Therapy

The importance of rational antibiotic application for the treatment of necrotizing fasciitis cannot be overstated, but the clinical characteristics of necrotizing fasciitis also have special requirements for antibiotic application. Firstly, due to tissue hypoxia and ischemia at the site of infection, the blood is unable to deliver enough antibiotics to the site of infection [18], which means that a single antibiotic is of limited therapeutic value, and at the same time, most studies have shown that necrotizing fasciitis occurs mostly by a mixture of multiple bacterial infections [8, 19, 20], which calls for early and adequate combined application of broad-spectrum antibiotics in the treatment of necrotizing fasciitis. Due to the rapid onset of necrotizing fasciitis, its diagnosis and use of antibiotics cannot be completely dependent on bacterial cultures of samples, in the fatal case reported by Merola [21] et al. a 49 year old female patient with necrotizing fasciitis died of infectious shock after the initial bacterial cultures of samples were negative and the autopsy report showed the presence of Streptococcus pyogenes in the pleural and pericardial fluids. Thus the principle of antibiotic use in necrotizing fasciitis cannot be overemphasized.

Regarding the study of susceptible bacteria, Nazerani [22] et al. concluded that group A hemolytic streptococci were the

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Figure 1. Wound Progression. (**A**) On emergency admission, the patient had significant swelling of the left hand and forearm. (**B**) Debridement was performed for the first time, and VSD suction was placed postoperatively. (**C**) After the first VSD, a large amount of necrotic tissue was still seen. (**D**) After the second VSD, the local infection was significantly improved, and the basal granulation growth was unsatisfactory. (**E**) After the third VSD, the granulation tissue grew satisfactorily and healthy skin was grafted to the granulation tissue. (**F**) The skin survived, and left hand function was preserved.

most frequently detected microorganisms, while Molewa [23] et al. found that Staphylococcus aureus and Escherichia coli were the most prevalent gram-positive and gram-negative organisms, respectively, while they recommended amoxicillin clavulanate and clindamycin for empirical treatment. In the study of Guliyeva [24], similar findings were obtained that infection in necrotizing fasciitis was dominated by mixed bacterial infections, which accounted for 70-90% of all cases, with anaerobic bacteria being dominated by non-typable Streptococcus and Enterobacteriaceae bacteria, and aerobic bacteria being dominated by Staphylococcus aureus. In the choice of antibiotics, carbapenems (imipenem, meropenem, or ertapenem) or piperacillin-tazobactam plus vancomycin or daptomycin (good coverage for methicillin-resistant staphylococcus aureus (MRSA)) plus clindamycin (for toxin-secreting strains of GAS and Staphylococcus aureus) are usually recommended in the empiric phase of treatment. After bacterial culture results are obtained and necrotizing fasciitis is more accurately typed, then ampicillin/ampicillin-sulbactam in combination with metronidazole or clindamycin is recommended for type I necrotizing fasciitis; for type II necrotizing fasciitis, a first- or second-generation cephalosporin is used for coverage of Streptococcus pyogenes and Staphylococcus aureus; for type III necrotizing fasciitis, clindamycin and penicillin; and for type IV patients, fungal therapy such as fluconazole is emphasized [24].

Surgical Intervention

As stated earlier, the use of antibiotics for necrotizing fasciitis cannot be overemphasized, but paradoxically, the use of antibiotics alone has a limited therapeutic effect on necrotizing fasciitis because bacteria can rapidly multiply and progress along the deep fascial layers, and due to the hypoxia and ischemia of the tissues, the antibiotics are not efficiently transported to the tissues [18], therefore, compared to the application of antibiotics, the surgical intervention becomes more indispensable.

The importance of surgical intervention has been emphasized in almost all studies involving necrotizing fascial strictures [8,10,12,20,21], with agreement that early debridement improves patient survival. In terms of surgical approach, negative pressure wound therapy has been recognized by an increasing number of researchers [25], and also, in this case, we applied this technique several times. Negative pressure technique was first proposed by Morykwas [26] et al. in 1997 and is believed to stabilize the wound environment, reduce wound edema, decrease bacterial load, improve tissue attention, and stimulate granulation tissue and angiogenesis. In comparison with conventional dressings, negative pressure vacuum therapy is thought to offer superiority in preventing hematoma formation and infection [27], as well as promoting healing of burn wounds [28]. Today this technique is widely used for orthopedic infections [29], diabetic ulcers [30], and patients with abscessed chests [31].However, we must emphasize that due to the potential risks associated with anaerobic bacteria, we must exercise caution when using negative pressure wound therapy on wounds where infection has not been fully controlled. We recommend that patients with stable vital signs undergo thorough debridement treatment before undergoing negative pressure wound therapy.

After successful radical debridement, the patient's vital signs often stabilize, and the next step of repair and reconstruction is also a major challenge for the surgeon. In this case, we have applied a fracture skin graft to repair the forearm defect, which was obtained from the healthy chest wall, which is practicable and convenient in the case of well-germinated defects, but in the case of complex defects, a simple skin graft would not be able to solve the problem. Lotus flaps with perforating vessels are a good alternative when there is a defect in the perineal area [32]. Artificial tissue substitutes have been favored by clinical surgeons in recent years, such as Mirzania [33] using umbilical amniotic tissue (umbilical amniotic tissue) for eyelid reconstruction after debridement for necrotizing fasciitis and Tobalem [34] applying NovoSorb biodegradable temporizing matrix) for extensive trunk reconstruction in children after necrotizing fasciitis debridement, both with good results.

This Case

Immunodeficiency is a common risk factor for necrotizing fasciitis, and other common risk factors include diabetes mellitus, obesity, alcoholism, chronic renal failure, and organ transplant patients [35]. The patient in this case was a female patient with a 20-year history of rheumatoid arthritis who was in a hypo-immune state due to long-term oral administration of tripterygium wilfordii (a Chinese medicine that regulates the immune system). The patient's lymphocyte count was consistently below 20%, which resulted in a higher systemic immune-inflammatory index (SII), and a higher SII index has been associated with lethal necrotizing fasciitis in previous studies [36]. This may explain the rapid progression of this patient's disease. No subcutaneous friction rub or radiographic evidence of gas accumulation was observed in this case. We made a diagnosis of necrotizing fasciitis based on surgical exploration. Initial emergency surgical exploration: A longitudinal incision was made along the dorsal aspect of the left forearm, revealing diffuse grayish-black deep fascia. Resistance disappeared during blunt dissection of the subcutaneous tissue and fascia layer (positive "finger test"), and thin, turbid fluid exuded from the muscle surface after fascial incision (positive "drip sign"). The necrotic fascia was resected to the edge of bleeding. During the second debridement surgery, we found that the residual necrotic fascia had clear boundaries (separated from the viable tissue), and there was no capillary bleeding at the wound site. A subsequent pus culture confirmed a single-bacterium infection by A. sobria. During the third surgery, new granulation tissue was observed covering the viable fascia in the original surgical area, and the remaining necrotic foci were thoroughly removed. During the fourth surgery, the skin graft bed exhibited granular granulation tissue with no exposure of deep tissue, and an autologous skin graft was performed on the wound. In previous cases of fish stab wounds, stab wounds from marine fish or exposure to contaminated seawater were predominant, whereas in the present case, the patient had no history of seafood exposure and was only partially stabbed by the fin of a freshwater fish, which in bacterial cultures showed the causative organism to be Aeromonas mildewii, which is also different from Vibrio traumaticus found in most fish stab wounds [4]. Aeromonas sobria, also belonging to the family of Vibrio (Vibrionaceae), is a commensal conditionally pathogenic bacterium in humans, fish, and animals [37], widely found in soil and water bodies and parasitized in aquatic animals, and is often reported to be associated with acute gastroenteritis and bacterial peritonitis in humans. However, this case suggests that Aeromonas mildans may cause fatal necrotizing fasciitis in immunocompromised conditions.In previous studies, some scholars have reported similar cases, although they are not entirely identical to ours. Hutchinson [38] reported a case of necrotizing fasciitis caused by lake water in 2021. A previously healthy 66-year-old woman sustained a deep laceration on the posterior aspect of her right lower leg, which was subsequently contaminated with lake water. After the wound was irrigated and sutured, the patient developed NF. Al Nour AH [39] reported a more severe case in 2024. They described a fatal retroperitoneal NF caused by Aeromonas caviae in a patient with a history of gastric cancer. Chang [40] reported a case of necrotizing fasciitis in a patient with neutropenia. Das [41] reported a case of necrotizing fasciitis in a recipient of allogeneic unrelated hematopoietic stem cell transplantation. Tsai [42] reported two cases of long-term diabetic patients who developed fatal necrotizing fasciitis due to infection with Aeromonas sobria. In reported cases [40-42] involving patients with immunodeficiency, the outcome of necrotizing fasciitis is often worse, and we believe that immunosuppression is central to its rapid progression.Therefore, we recommend that any fish sting injury in immunocompromised patients (regardless of freshwater/saltwater) should be considered a surgical emergency. For such patients, physicians should immediately administer intravenous antibiotics targeting Aeromonas hydrophila and Vibrio species (e.g., third-generation cephalosporins + ciprofloxacin) and assess the LRINEC score every 4 hours within 24 hours. If the patient's SII > 900 or C-Reactive Protein (CRP) > 150 mg/L, we recommend early debridement.

Due to the urgent need for life-saving measures, no tissue pathological evidence was obtained. In future similar cases, we recommend obtaining additional specimens during a second debridement procedure in the stable phase to further investigate the underlying mechanisms.

Conclusion

We highlight the need for vigilance regarding necrotizing fasciitis in patients with fish-related injuries, even without seafood exposure—particularly in immunocompromised individuals or those infected with Aeromonas hydrophila. Early recognition and aggressive surgical debridement are critical for successful treatment.

Abbreviations

C-Reactive Protein: CRP; Laboratory Risk Indicator for Necrotizing Fasciitis: LRINEC; Methicillin-Resistant Staphylococcus Aureus: MRSA; Systemic Immune-inflammatory Index: SII; Vacuum Sealing Drainage: VSD.

Author Contributions

Zhengnan Zhao, Peng Wang, Lei Ma and Jiabao Yang designed the whole project. Yang Wang, Jiaping Guo wrote the manuscript. Chenglong Yao, Zefan Sun and Xiu Wang contributed to the data collection. Jiabao Yang contributed to the methodology. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Mendelian Randomization Reveals Height as a Risk Factor and Potential Susceptibility Genes for Testicular Cancer

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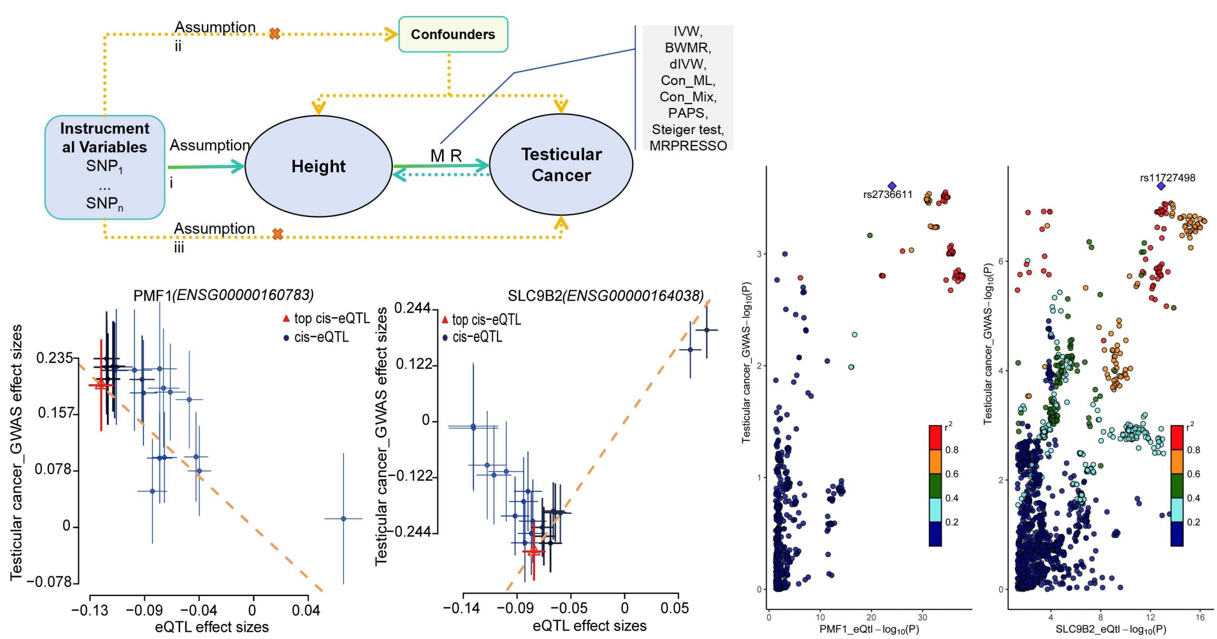
Graphical Abstract



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Mendelian Randomization Reveals Height as a Risk Factor and Potential Susceptibility Genes for Testicular Cancer

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Abstract

Background: Testicular cancer (TC) is the most common solid malignancy in young men, and the causal relationship between height and TC remains controversial to date.
Methods: We applied a two-sample Mendelian randomization (MR) framework using large-scale genome-wide association study summary statistics to estimate the causal effect of adult height on TC risk. This was supported by LD Score regression, stringent instrument selection, and an extensive sensitivity portfolio. Cohort-specific estimates were pooled via random-effects meta-analysis. Mechanistic inference included gene-set enrichment and an integrated Summary-data-based Mendelian Randomization (SMR)-colocalization pipeline to prioritize susceptibility genes.
Results: Height was causally associated with higher TC risk in two independent cohorts (OR 1.384, 95% CI 1.029-1.861; P<0.05) and in meta-analysis (pooled OR 1.354, 95% CI 1.112-1.644), with concordant directions across robustness estimators, no evidence of directional pleiotropy, and minimal heterogeneity. Bidirectional MR found no reverse effect of TC liability on height (P>0.05), and regional colocalization did not support shared causal variants between height and TC signals (PP.H4<0.50). Enrichment implicated a height-chondrogenesis-extracellular matrix-TGF-β/SMAD-endocrine axis. SMR and colocalization convergently nominated PMF1 (PP.H4=0.80) and SLC9B2 (PP.H4=0.95) as susceptibility genes with high posterior support across cohorts.
Conclusion: Genetically proxied height confers a modest but robust increase in TC risk, and PMF1 and SLC9B2 emerge as plausible mediators at colocalized regulatory loci.
Keywords: Height; Testicular cancer; Mendelian randomization; Bayesian colocalization; Susceptibility gene.

Introduction

Testicular cancer (TC) ranks as the most common solid tumor among males aged 15-44 years, comprising approximately 1-2% of all male malignancies worldwide [1]. In 2025, the United States is expected to report nearly 9,720 new cases and about 600 deaths, with a median age at diagnosis of 33 years. Incidence has risen steadily over recent decades, particularly in high-income regions: Western and Northern Europe exhibit the highest age-standardized incidence rates-8.7 and 7.2 per 100,000 men, respectively-while projections suggest a 24% increase in Europe by 2025 compared to 2005 levels [2,3]. Established risk factors encompass cryptorchidism, a positive family history, prior testicular pathology, and hormonal imbalances [4]. Epidemiological analyses indicate that each 5 cm increment in adult height correlates with a roughly 13% elevation in TC risk. Environmental and lifestyle exposures-such as cannabis use, agricultural chemicals, and occupational hazards-have also been implicated, though findings remain inconsistent [5,6].
Conventional observational studies, while foundational, often struggle with residual confounding, measurement error, and reverse causation. For example, analyses based on SEER registry data cannot disentangle whether height causally influences tumorigenesis or merely reflects early life nutritional and socioeconomic conditions. Consequently, the causal link between stature and TC remains unresolved [6].
Mendelian randomization (MR) offers a powerful alternative by employing germline variants associated with height as instrumental variables. This approach leverages the random assortment of alleles at conception to mitigate confounding

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and reverse causation, paralleling randomization in controlled trials, thus strengthening causal inference [7-9]. Moreover, summary-data-based MR (SMR) integrates genome-wide association study (GWAS) and expression quantitative trait loci (eQTL) data to colocalize genetic signals, pinpoint susceptibility genes, and nominate therapeutic targets [10-12].
Here, we apply state-of-the-art two-sample MR using extensive GWAS meta-analyses of adult height and TC, aiming to resolve the causal role of height in tumor risk. Concurrently, SMR analysis interrogates GWAS and eQTL summary statistics to identify and prioritize genes whose expression mediates testicular carcinogenesis.

Methods and Materials

Study Design

Genome-wide linkage disequilibrium (LD) score regression was used to characterize polygenicity and residual confounding and to estimate the genetic correlation between adult height and TC across cohorts. A two-sample MR framework based on GWAS summary statistics then quantified the causal effect of height on TC risk. The study adhered to STROBE-MR reporting standards and aligned with the three core instrumental-variable assumptions: (i) genetic instruments exhibit strong and robust associations with the exposure, (ii) instruments are independent of measured and unmeasured confounders, and (iii) instruments influence the outcome solely through the exposure (Figure 1) [13].
The analytical workflow comprised cohort-specific MR estimation, random-effects meta-analysis to pool causal effects across cohorts, and colocalization analyses to assess whether exposure and outcome signals share causal variants at implicated loci. Biological interpretation included gene-set and pathway enrichment analyses of mapped genes from associated variants. Target nomination leveraged SMR integrated with colocalization to prioritize putative therapeutic genes with convergent GWAS and eQTL evidence.

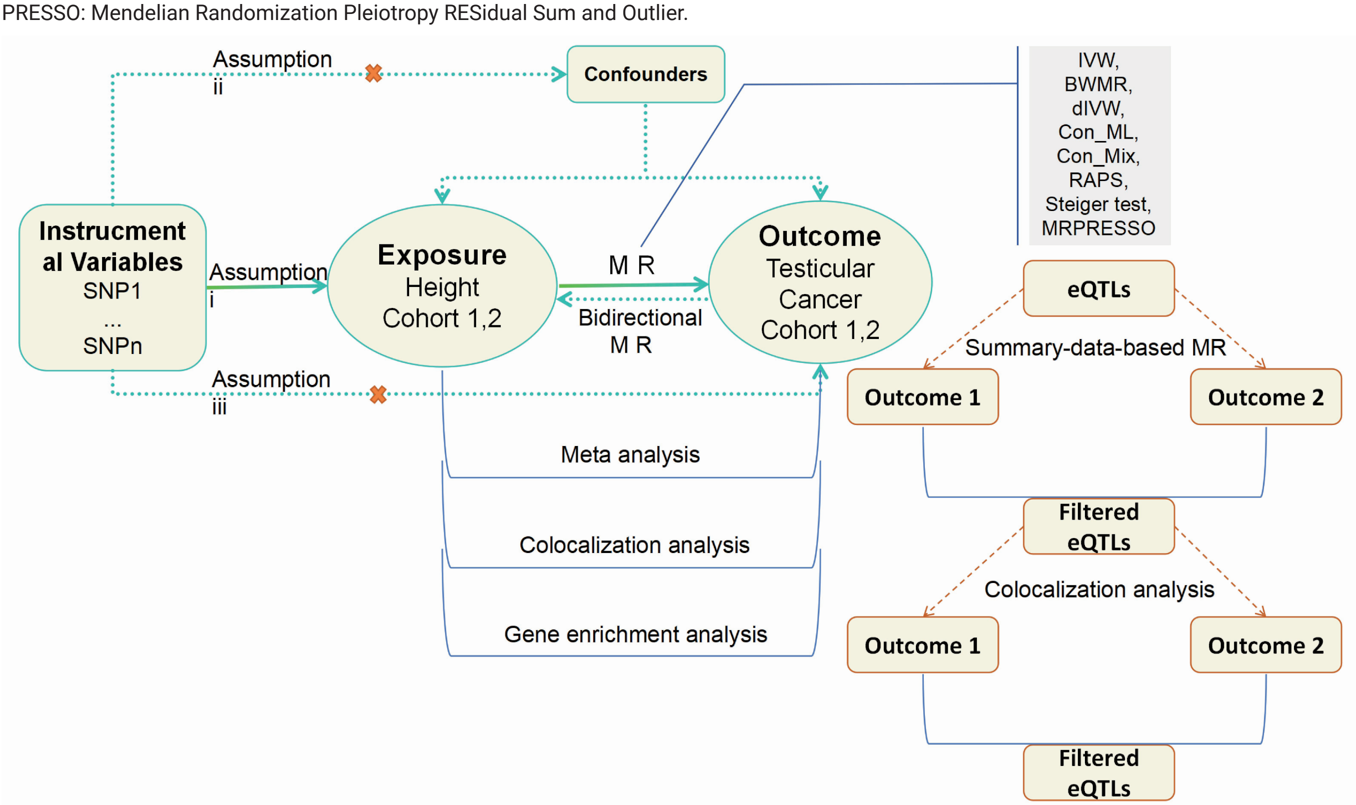
Data Sources

We leveraged data from two cohorts to ensure a broad and diverse genetic representation for our MR analysis. In Cohort 1, Height: Measurements were obtained from the UK Biobank (n = 336,474). TC: Diagnoses were derived from the latest FinnGen biobank release, specifically from the cancer registry using ICD-O-3 codes for neoplasms (total n = 144,160; cases = 536, controls = 143,624). In Cohort 2, Height: Measurements were obtained from the IEU GWAS database (n = 360,388). TC: Diagnoses were extracted from the UK Biobank, defined using PheCode 187.2 for malignant neoplasm of the testis (total n = 208,768; cases = 797, controls = 207,971). eQTLs data were sourced from the eQTLGen Consortium [14]. Table S1 provides a comprehensive overview of the datasets employed.

LD score regression

LD Score regression leverages GWAS summary statistics to estimate SNP-based heritability, partition that heritability across overlapping functional annotations, and quantify genetic correlations between phenotypes. For each variant, the LD score quantifies the extent of LD it captures and is defined as

Figure 1. An outline of the study's approach. Utilizing a bidirectional Mendelian randomization framework. SNP: single nucleotide polymorphism, IVW: Inverse-variance weighted method, dIVW: debiased inverse-variance weighted method, RAPS: Robust adjusted profile score Con_ML: constrained maximum likelihood and model averaging, Con_Mix: contamination mixture, BWMR: Bayesian weighted Mendelian randomization, MR-PRESSO: Mendelian Randomization Pleiotropy RESidual Sum and Outlier.



the sum of squared correlations with neighboring SNPs within a specified window. This study computed LD scores using the European-ancestry reference panel from the 1000 Genomes Project [15]. Univariate and cross-trait LD Score regression yielded intercepts indexing residual confounding, SNP-heritability estimates, genetic correlations, and related parameters used to assess the genetic contributions to the complex diseases and traits analyzed [16,17].

Selection of genetic instruments

Genetic instruments were restricted to variants reaching genome-wide significance ($p < 5.0\times10^{-8}$). Independence among instruments was enforced by LD clumping using $r^2 < 0.001$ within a 10 Mb window to minimize correlation among selected SNPs [18]. Instrument strength was quantified with the F-statistic, and only variants with $F > 10$ were retained to limit weak-instrument bias; during screening, the F-statistic was approximated as $F = (\beta/se)^2$ [19,20]. Directionality was evaluated with the Steiger test to remove variants that explained more variance in the outcome than in the exposure [21]. Harmonization procedures excluded palindromic SNPs with intermediate allele frequencies to avoid strand ambiguity and preserved alignment of effect alleles across datasets.

For SMR analyses, instruments were limited to cis-eQTLs located within ±1 Mb of the gene region. Eligible eQTLs showed GWAS with gene expression measured in whole blood ($p < 5.0\times10^{-8}$) and had a minor allele frequency greater than 1%. This selection ensured biologically proximate regulatory instruments with adequate statistical strength for downstream inference [22].

MR analysis and sensitivity analyses

The primary causal estimator was the IVW method [23], selected for its efficiency and conservative behavior under heterogeneity and implemented under a multiplicative random-effects framework where appropriate. Outlier detection and correction were performed with MR-PRESSO, including the global test and outlier removal [24].

Sensitivity analyses included complementary robustness estimators: BWMR to downweight pleiotropic instruments [25]; the Con_Mix model to accommodate mixtures of valid and invalid instruments [26]; MR-RAPS for robustness under weak instruments and idiosyncratic pleiotropy [27]; Con_ML to address correlated and uncorrelated pleiotropy [28]; and the dIVW estimator to reduce weak-instrument bias [29]. Single-variant effects were summarized with the Wald ratio. Additional procedures comprised leave-one-out analyses to assess leverage by individual instruments and repeated MR-PRESSO outlier checks after instrument refinement. Sample overlap was assessed with MRlap [30]; when p_difference > 0.05, any overlap is unlikely to materially affect statistical power. Heterogeneity was quantified using Cochran's Q [31]; a non-significant p-value (>0.05) indicated no substantial between-instrument heterogeneity, whereas a significant result prompted use of the IVW multiplicative random-effects specification. Horizontal pleiotropy was evaluated with the MR-Egger intercept [32]; a non-significant p-value (>0.05) was taken as no evidence for directional pleiotropy.

For SMR, instrument validity was assessed with the HEIDI test, with $p > 0.05$ indicating no significant heterogeneity in variant effects on the gene-trait relationship. The parameter heidi-mtd governed implementation (0 for the original algorithm; 1 as the default improved procedure using up to 20 SNPs for enhanced performance) [22,33]. Potential confounding was examined by querying external variant–phenotype resources to identify links between candidate instruments and established TC factors (HIV infection, family history, age, or cryptorchidism [4,5,34]). Variants flagged as associated with confounders were removed, and causal estimates were re-derived to confirm the stability of inference.

Colocalization analysis

The Bayesian colocalization framework quantified whether the same causal variant drives two association signals by calculating approximate Bayes factors and transforming them into posterior probabilities for five mutually exclusive hypotheses. The hypotheses comprised: H0, neither the exposure signal nor the GWAS trait harbors a causal variant within the locus; H1/H2, only the exposure or only the GWAS trait contains a causal variant; H3, both traits contain distinct causal variants; and H4, both traits share a single causal variant. Genomic windows were defined as ±50 kb around index variants for GWAS-GWAS comparisons and ±1 Mb around the gene for eQTL-GWAS analyses [35, 36]. Evidence for colocalization was classified as suggestive at Posterior probabilities for hypothesis H4 (PP.H4) ≥ 0.50 and strong at PP.H4 ≥ 0.75 [37, 38].

Statistics

Analyses were conducted in R (version 4.4.3) using the following packages: TwoSampleMR (v0.5.10), MendelianRandomization (v0.8.0), coloc (v5.1.1), meta (v6.2-1), SMR (v1.03), MRPRESSO (v1.0), MRlapPro (v0.0.3) [30], Bioconductor (v3.21), clusterProfiler (v4.16.0) [39], and gprofiler2 (v0.2.3) [40]. Statistical power was evaluated with an online calculator [41]. SNP-gene set enrichment was performed with g: Profiler [40]. Statistical significance was defined as a two-sided p-value < 0.05 unless otherwise specified.

Results

LD Score regression and Gene Set Enrichment Analysis Reveal Genetic Correlation for Height and TC

LD Score regression, which distinguishes polygenicity from confounding in GWAS, was applied to assess genetic overlap between adult height and TC. In cohort 1, the height phenotype yielded a Z-score of 18.75 ($p = 1.62\times10^{-78}$), whereas TC produced a Z-score of 1.46 (p=0.146). Cohort 2 showed analogous findings, with Z-scores of 20.42 ($p = 1.16\times10^{-92}$) for height and 3.44 ($p = 5.87\times10^{-4}$) for TC (Figure S1A). Lambda GC values for the TC phenotype remained below 1.05, indicating minimal population stratification or systematic bias in both datasets [42].

A genome-wide significance threshold ($p<5\times10^{-8}$) in cohort 1 identified 607 independent height-associated SNPs. Annotation of these variants mapped to 393 genes, which were most strongly enriched for the "Body height" term in DisGeNET (adjusted $p = 1.88\times10^{-8}$) (Figure S1B; Table S2). Similarly, 81 TC-associated SNPs corresponded to genes enriched in the "TC" category of the Jensen_DISEASES database (adjusted $p=4.89\times10^{-5}$) (Figure S1C; Table S2). These enrichment results corroborate that the selected SNPs capture phenotype-specific genetic signals.

Association of Genetically Predicted Height with TC Risk

Cohort 1 supported a causal association between height and TC. After LD pruning, minor allele frequency filtering, and harmonization, 578 height-associated variants were identified; eight variants linked to potential confounders were removed, leaving 570 instruments for analysis. The IVW estimator indicated higher TC risk with greater genetically predicted height (Odds ratio (OR) 1.384; 95% Confidence Interval (CI) 1.029-1.861; p = 0.031; Figure 2A).

Robustness assessments were consistent with the primary finding. MR-PRESSO returned a significant result (p = 0.032), and concordant estimates arose from Con_Mix, RAPS, dIVW, Con_ML, and BWMR methods (Figure 2A). Power to detect the observed effect was 0.618. Leave-one-out analyses showed no variant materially altered the association (Table S3). The MR-Egger intercept provided no evidence of directional pleiotropy (p = 0.535), and Cochran's Q suggested no substantial heterogeneity across instruments (p = 0.070) (Table S4). Cohort 2 provided independent validation. After excluding six pleiotropic variants, the IVW estimate remained significant (OR 1.328; 95% CI 1.024-1.723; p = 0.032), with supporting results across sensitivity analyses (Figure 2A; Table S4).

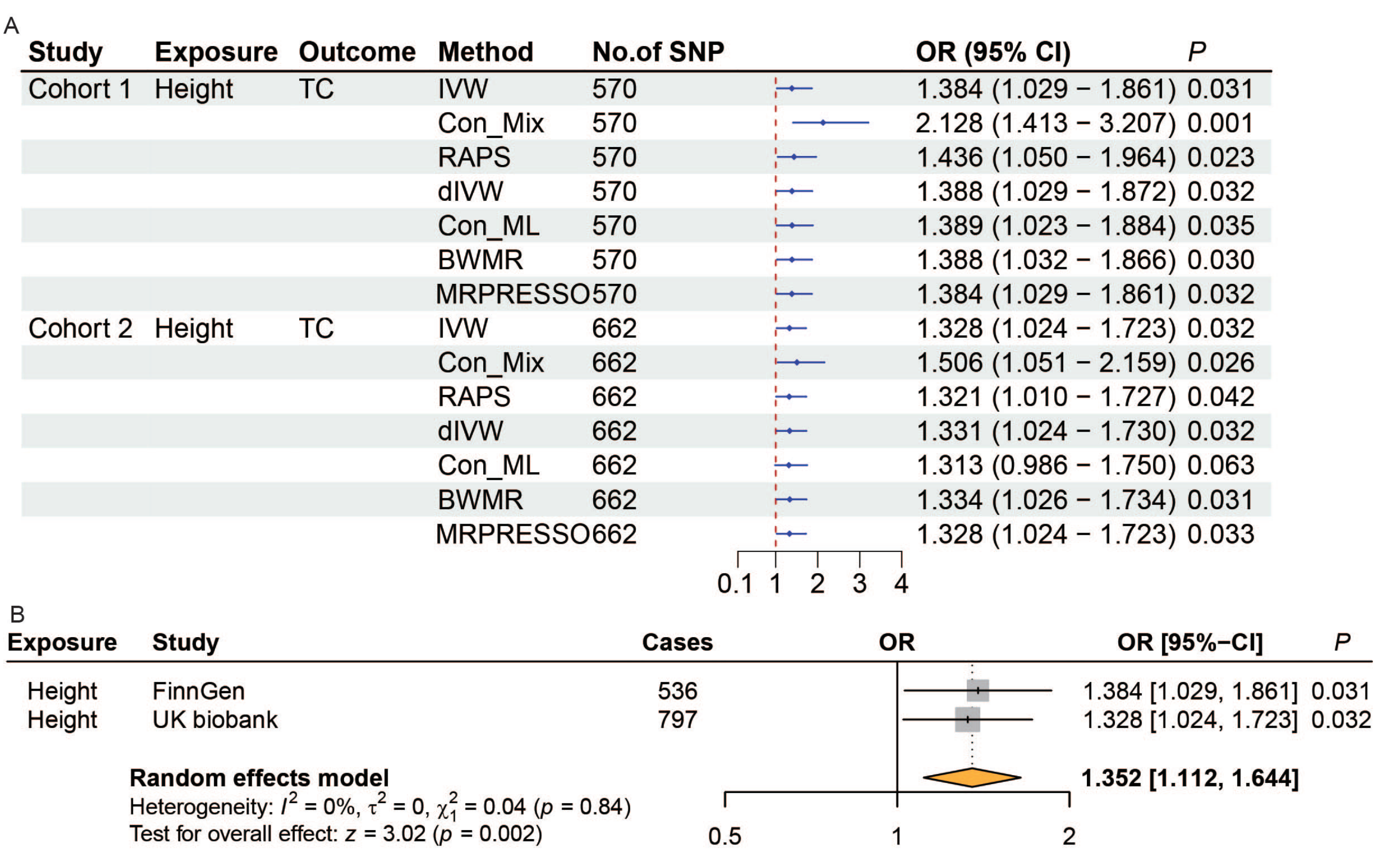
A random-effects meta-analysis pooled cohort-specific estimates. The combined effect size demonstrated a robust association between genetically predicted height and TC risk (pooled OR 1.35; 95% CI 1.12-1.64; p = 0.002; Figure 2B). A non-significant heterogeneity test (p = 0.84) indicated minimal between-cohort variability, reinforcing the consistency and reliability of the causal estimate.

Absence of reverse causality between height and TC based on bidirectional MR and colocalization analyses

Bidirectional MR assessed whether genetic liability to TC influences adult height under the same instrument selection criteria used for the forward direction. Reverse-direction instruments comprised two SNPs in Cohort 1 and six SNPs in Cohort 2, and IVW estimates were null (p = 0.759 and p = 0.834, respectively; Table S5), indicating no evidence that TC genetic predisposition causally affects height. Colocalization analysis evaluated whether height and TC association signals share a causal variant within ±50 kb windows around index loci [35]. PP.H4 were 0.04 in Cohort 1 and 0.02 in Cohort 2, values that do not support colocalization of the two phenotypes in the queried regions. Concordant null results from reverse-direction MR and low colocalization probabilities indicate a primarily unidirectional relationship, whereby genetic determinants of adult height modulate TC risk rather than the converse.

SNP-gene Enrichment Reveals Potential Mechanisms Linking Height to TC Risk

Figure 2. Causal effect of genetically proxied height on TC risk. (A) Forest plots of two-sample MR estimates from two independent cohorts. IVW and MR-PRESSO are prespecified primary estimators; RAPS, dIVW, Con_ML, ConMix, and BWMR serve as sensitivity analyses. Effect sizes are OR for TC per 1 SD increase in genetically predicted height with 95% CI; OR > 1 indicates higher risk. **(B)** Random-effects meta-analysis pooling cohort-specific IVW estimates. The pooled effect is reported as OR with 95% CI and two-sided P value. Between-cohort heterogeneity was evaluated using Cochran's Q; P_heterogeneity > 0.05 indicates no material heterogeneity.

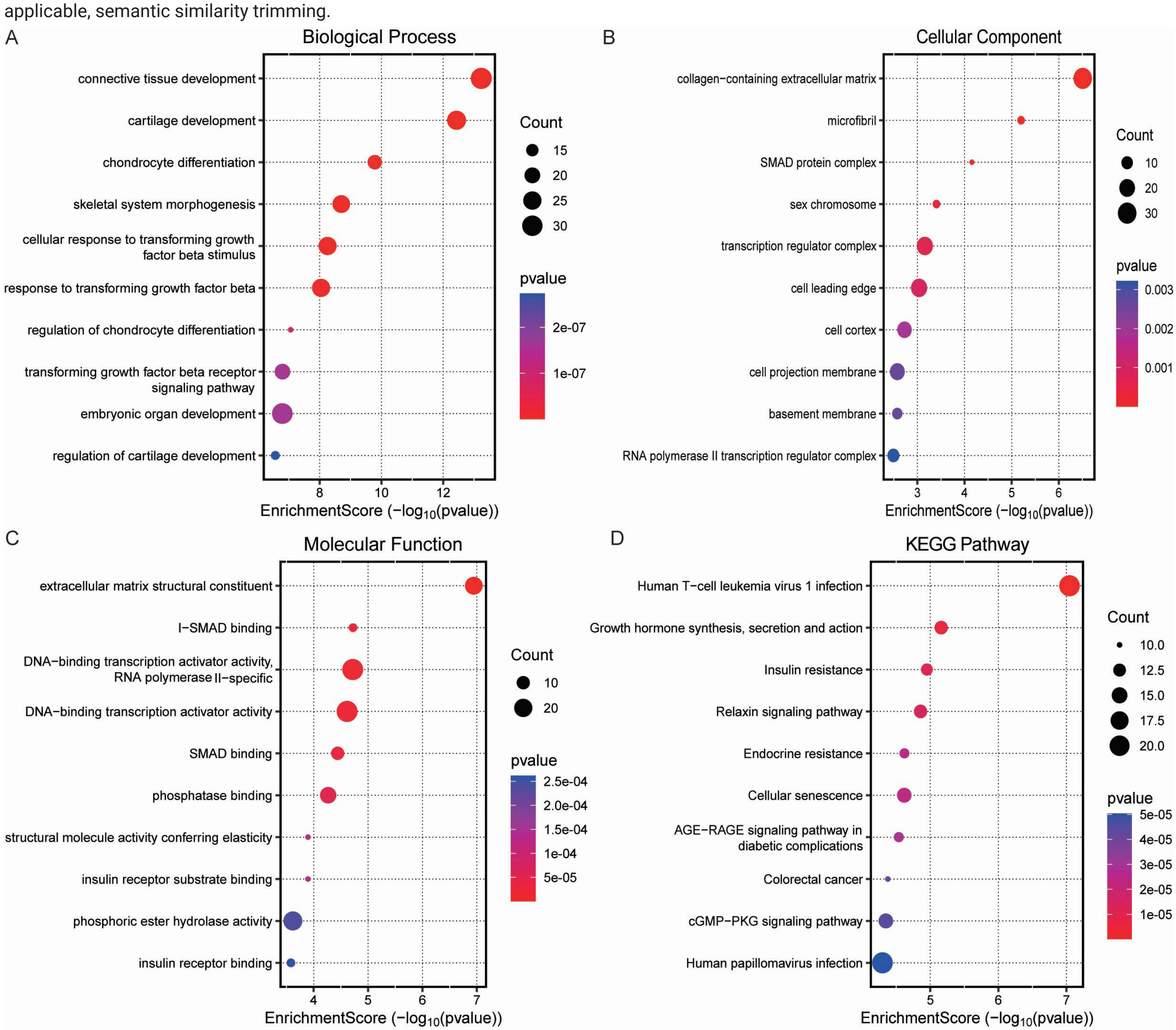


To delineate putative mechanistic links between the height phenotype and TC, the analysis compiled 1,232 SNPs from both cohorts. It removed 111 duplicates, followed by functional annotation with g: Profiler [40], which mapped 506 unique genes. Gene Ontology (GO) enrichment indicated predominant biological process terms including connective tissue development, cartilage development, chondrocyte differentiation, skeletal system morphogenesis, and cellular response to transforming growth factor beta stimulus (Figure 3A). Cellular component terms were enriched for collagen-containing extracellular matrix (ECM), microfibril, SMAD protein complex, sex chromosome, and transcription regulator complex (Figure 3B). Molecular function terms were enriched for extracellular matrix structural constituent, I-SMAD binding, RNA polymerase II-specific DNA-binding transcription activator activity, and SMAD binding (Figure 3C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlighted Human T-cell leukemia virus 1 infection, Growth hormone synthesis, secretion and action, Insulin resistance, Relaxin signaling pathway, and Endocrine resistance (Figure 3D, Table S6). These enrichment profiles cohere with a height-chondrogenesis-extracellular matrix-endocrine axis and provide biologically plausible support for the inferred causal relationship between height and TC.

PMF1 and SLC9B2 as potential susceptibility genes in TC from SMR and colocalization analysis

SMR analysis prioritized TC-associated genes using eQTL instruments. In Cohort 1, application of p_SMR < 0.05 and p_HEIDI > 0.05 identified 721 genes (Figure 4A). In Cohort 2, the same criteria yielded 711 genes (Figure 4B). The intersection of the two gene sets resulted in 44 candidates (Table S7,

Figure 3. Functional enrichment of height-TC-associated genes. The enrichment analysis using clusterProfiler identified significant terms in (**A**) GO Biological Process, (**B**) GO Cellular Component, (**C**) GO Molecular Function, and (**D**) KEGG pathways. Dots encode term size (Count) and adjusted significance, x-axis shows EnrichmentScore=−log10(p). Terms shown are the top 10 per panel after filtering at p.adjust<0.05 and, where applicable, semantic similarity trimming.



S8). Colocalization analysis was then performed for these 44 genes in both cohorts. Five genes in Cohort 1 (CES4A, PMF1, LRRC37A15P, MAP2K1, and SLC9B2) and six genes in Cohort 2 (PRR13, PMF1, CLPTM1L, RP11-10L12.2, BDH2, and SLC9B2) showed PP.H4 values above the suggestive threshold of 0.5 (Figure 4C). Only PMF1 and SLC9B2 replicated across cohorts, with PP.H4 of 0.65 (Cohort 1) and 0.80 (Cohort 2) for PMF1, and 0.95 (Cohort 1) and 0.92 (Cohort 2) for SLC9B2, surpassing the high colocalization threshold of 0.75. The top SNP for SLC9B2 was rs11727498, and the top SNP for PMF1 was rs2736611 (Figure 4D, E).

These results indicate high-confidence colocalization between the GWAS signal for TC and the regulatory signal for PMF1 and SLC9B2 at their respective loci, consistent with a shared causal variant. The pattern supports PMF1 and SLC9B2 expression as plausible mediators of the observed TC association within these regions.

Discussion

This study supports a modest but consistent causal effect of genetically predicted height on TC risk and nominates PMF1 and SLC9B2 as credible susceptibility genes by integrating MR, SMR, and colocalization evidence. The findings align with developmental and endocrine biology of growth, while highlighting mechanistic hypotheses that merit functional validation.

A European-ancestry two-sample MR analysis indicated a statistically significant, positive causal association between genetically proxied height and TC risk (OR 1.38 per exposure unit, typically one SD of height; $p<0.05$), thereby rejecting the null hypothesis at the 5% significance level. This direction and magnitude are consistent with conventional observational evidence; for example, a meta-analysis of American and Dutch cohorts reported a 13% increase in TC risk per 5 cm of height [43] and a large Korean cohort observed a 9% increase in overall cancer hazards per 5 cm, with one of the strongest site-specific associations for TC [44]. The present estimates offer a more credible causal interpretation because MR is less susceptible to residual confounding and reverse causation than standard observational designs. Under the core instrumental-variable assumptions, the IVW estimate remained robust after MR-PRESSO and a suite of sensitivity analyses, including methods with stronger resistance to pleiotropy. A random-effects meta-analysis further strengthened the reliability of the association. This multi-method strategy enhances confidence in the findings and helps explain inconsistencies in the literature. For instance, one MR analysis based on 5,518 TC cases and 19,055 controls reported no association [6], a discrepancy plausibly attributable to differences in data quality, population heterogeneity, and methodological choices. It is important to note that the reported association reflects a relative risk increase; given the low baseline incidence of TC, the absolute risk elevation remains modest. Although height is not a modifiable target, it provides etiologic insight and can contribute to risk stratification.

Mechanistic considerations linking height to TC risk remain incompletely resolved, yet several coherent hypotheses emerge from our data and prior biology. Taller individuals likely have a greater number of susceptible cells, which increases opportunities for malignant transformation [45]. Endocrine correlates of stature, notably growth hormone and insulin-like growth factor 1 (IGF1), exert mitogenic and anti-apoptotic effects through PI3K–AKT and MAPK cascades, plausibly elevating baseline oncogenic signaling [46]. Height-associated variants may also reside in LD with loci that influence cancer susceptibility, providing an alternative genetic conduit for risk [47,48].

Pathway enrichment in this study converges on a height-chondrogenesis-ECM-TGF-β/SMAD-endocrine axis that plausibly shapes the testicular germ-cell niche across fetal and pubertal windows. Within this axis, the insulin/IGF system is essential for late-fetal and neonatal Sertoli-cell proliferation and ultimately determines adult testicular capacity [49,50]; perturbation of insulin/IGF signaling diminishes testis size and sperm output, underscoring a developmental bottleneck with downstream implications for carcinogenesis [51]. In parallel, ECM components-including collagen-rich matrices and microfibrils-and SMAD complexes point to fibrillin-1-mediated control of latent TGF-β bioavailability. This mechanism fine-tunes local proliferative and survival cues [52,53]. Given that TC often originates as in-utero germ cell neoplasia in situ and progresses under pubertal hormonal surges with niche remodeling, the convergence of endocrine tone and ECM-TGF-β coupling provides a biologically credible route by which genetic liability to height increases the conditional probability of malignant progression [54]. While these observations offer a unifying framework, targeted functional and longitudinal studies are needed to define the specific mediators and developmental windows that causally bridge height biology to TC risk.

In the SMR framework, a significant p_SMR indicates an instrumented association between the phenotype and gene expression; when accompanied by a non-significant HEIDI test and high PP.H4 from colocalization, the evidence more convincingly excludes horizontal pleiotropy and distinct nearby causal variants, strengthening coherence along the proposed causal chain. Population genetics and functional inference evidence support PMF1 (rs2736611) and SLC9B2 (rs11727498) as credible TC susceptibility genes, consistent with the present SMR and colocalization signals. PMF1 at 1q22 has been repeatedly noted in testicular germ cell tumor (TGCT) genome-wide analyses and reviews, within risk categories enriched for microtubule and chromosome assembly-domains in which PMF1 is highlighted alongside TEX14 and related cytoskeletal regulators [55]. Recent large-scale meta-analytic syntheses further list PMF1 among genes with moderate-to-high likelihood of TGCT involvement, aligning with pan-cancer resources that report PMF1 expression, while underscoring the need for TC-specific, compartment-resolved validation [56].

SLC9B2 (NHA2), a mitochondrial Na+/H+ exchanger that governs pH and apoptotic tone, sits within the broader NHE family implicated in tumor proliferation and prognosis [57]; locus-level fine-mapping places TGCT credible variants upstream of SLC9B2, supporting overlap between disease association and regulatory terrain [58]. An early negative report in testis homogenates predates cell–type–resolved profiling and does not exclude testis- or tumor-compartment-specific regulation at this locus [59]. Together, convergent GWAS synthesis (PMF1), credible-set overlap (SLC9B2), and ion homeostasis biology (NHA2) provide a coherent rationale for mechanistic follow-up, with priorities including single-cell testis datasets and perturbation studies in seminoma and non-seminoma models.

Several limitations warrant consideration when interpreting

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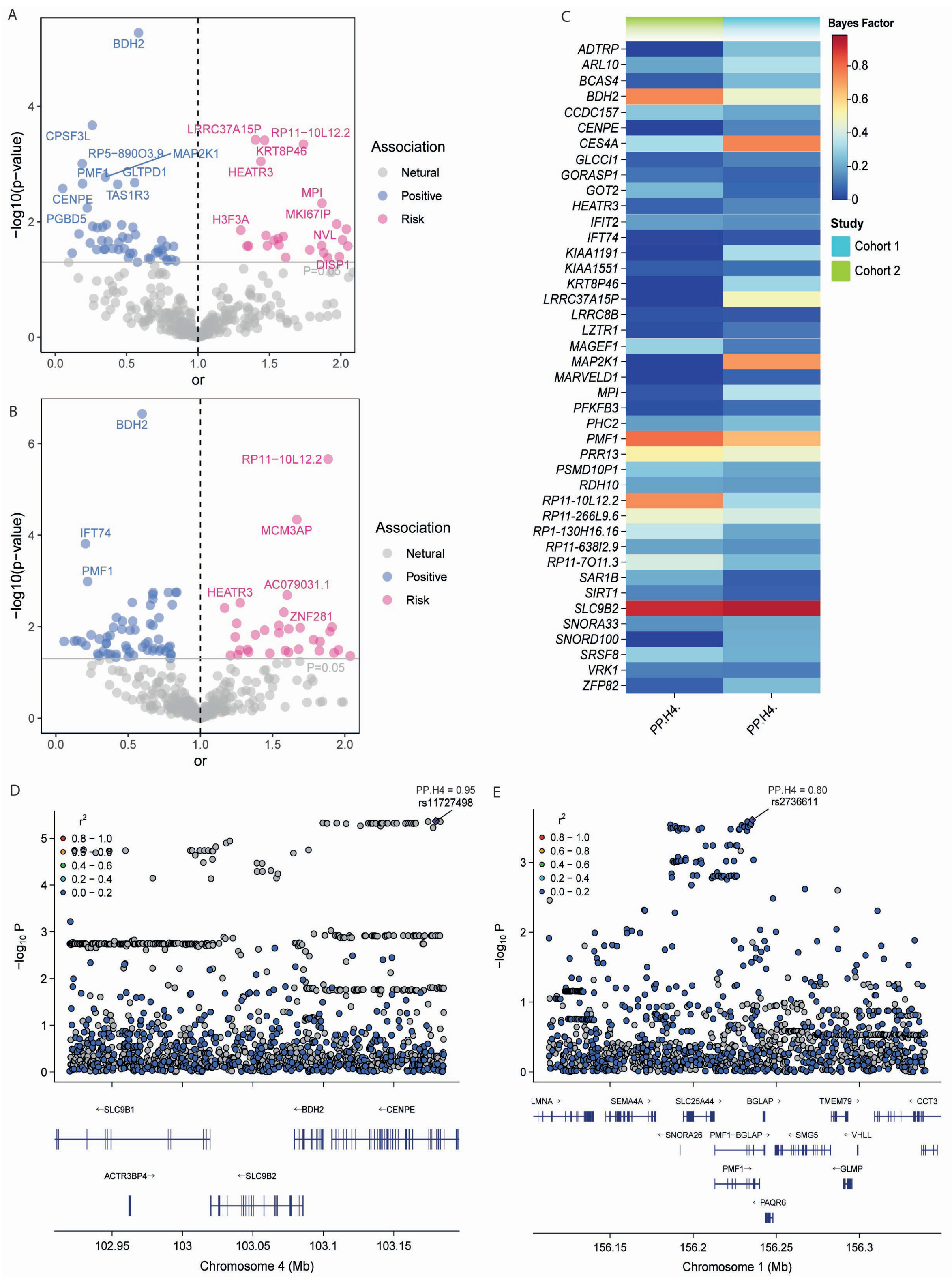
Figure 4. Prioritization of putative susceptibility genes for TC. (A–B) Volcano plots summarizing SMR associations between cis-eQTL-regulated gene expression and TC risk in Cohort 1 (A) and Cohort 2 (B). The x-axis denotes SMR effect size (OR) and the y-axis -log10(P_SMR). **(C)** Colocalization results at candidate loci across both cohorts, indicating the posterior probability of a shared causal variant (PP.H4) according to the thresholds specified in Methods. FAM22D and MKI67IP showed reportable SMR associations after quality control but yielded no evaluable colocalization results; accordingly, they are not displayed in panel C. **(D-E)** Regional SNP-level scatter/LD plots showing the correspondence between eQTL effects and TC GWAS effects for SLC9B2 in Cohort 1 (D) and PMF1 in Cohort 2 (E). Points are colored by LD (r^2) with the lead variant; neighboring genes are displayed to provide genomic context.



these findings. The MR framework estimates causal effects between genetically proxied height and TC risk, yet confirmation of causality benefits from triangulation across study designs, including longitudinal cohorts, quasi-experiments where feasible, and expanded multi-ancestry GWAS resources to strengthen external validity. The analytic sample derives predominantly from European populations, which constrains generalizability; replication in non-European ancestries is essential to address allele frequency and LD differences and assess effect size transportability. Pathway enrichment is hypothesis-generating rather than confirmatory and has not been validated by targeted functional or longitudinal studies. Testis- and cell type-resolved eQTL/pQTL datasets, single-cell transcriptomics, and perturbation assays will be required to establish whether the highlighted endocrine-ECM-TGF-β/SMAD programs operate in the relevant germ cell and Sertoli cell compartments in vivo. The primary analyses assumed a linear exposure-response between height liability and risk; potential non-linear or threshold effects, developmental timing, and gene-environment interactions should be explored in future work to refine risk modeling. The susceptibility genes and loci identified through SMR and colocalization nominate plausible targets but remain provisional. Model choices in SMR/colocalization (priors, windows, single- vs multi-causal architectures) can influence posterior evidence. Fine-mapping with credible sets, cross-ancestry replication, molecular validation-CRISPR perturbation, allelic reporter assays, and orthogonal proteogenomic readouts are necessary to establish causality and evaluate the translational potential of these candidates.

Conclusion

This work implicates height as a causal risk factor for TC. It highlights PMF1 and SLC9B2 as convergent susceptibility candidates, providing testable targets for tissue-specific functional validation and future risk stratification frameworks.

Abbreviations

Bayesian Weighted Mendelian Randomization: BWMR; Contamination Mixture method: Con_mix; Constrained Maximum Likelihood and Model Averaging: Con_ML; Confidence Interval: CI; Debiased Inverse-Variance Weighted: dIVW; Expression quantitative trait loci: eQTL; Extracellular matrix: ECM; Gene Ontology: GO; Genome-wide association studies: GWAS; Inverse-Variance Weighted: IVW; Instrumental variables: IV; Kyoto Encyclopedia of Genes and Genomes: KEGG; Linkage disequilibrium: LD; Mendelian Randomization: MR; Mendelian Randomization Pleiotropy RESidual Sum and Outlier: MR-PRESSO; Odds ratio: OR; Robust adjusted profile score: RAPS; Standard Error: SE; Single nucleotide polymorphisms: SNP; Summary-data-based Mendelian Randomization: SMR; Testicular germ cell tumor: TGCT; Testicular cancer: TC.

Author Contributions

Yuangao Xu conceived the study; Yuanbo Xu, Jieyu Xiong and Yikun Wu acquired and curated the data and performed all analyses; Yuanlin Wang, Hua Shi and Shuxiong Xu provided essential resources and supervised the experimental campaign; Yuanbo Xu, Jieyu Xiong and Yuangao Xu drafted the initial manuscript, Yuangao Xu critically revised and finalized the manuscript. All authors contributed to the interpretation of the data, iteratively refined the manuscript with substantive feedback, and have read and approved the final version for publication.

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Ethics Approval and Consent to Participate

Not applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data availability

Not Applicable.

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Causal Relationships between 1400 Serum Metabolite Traits, 179 Plasma Lipids, and Hemorrhoids: A Mendelian Randomization Study and Meta-analysis

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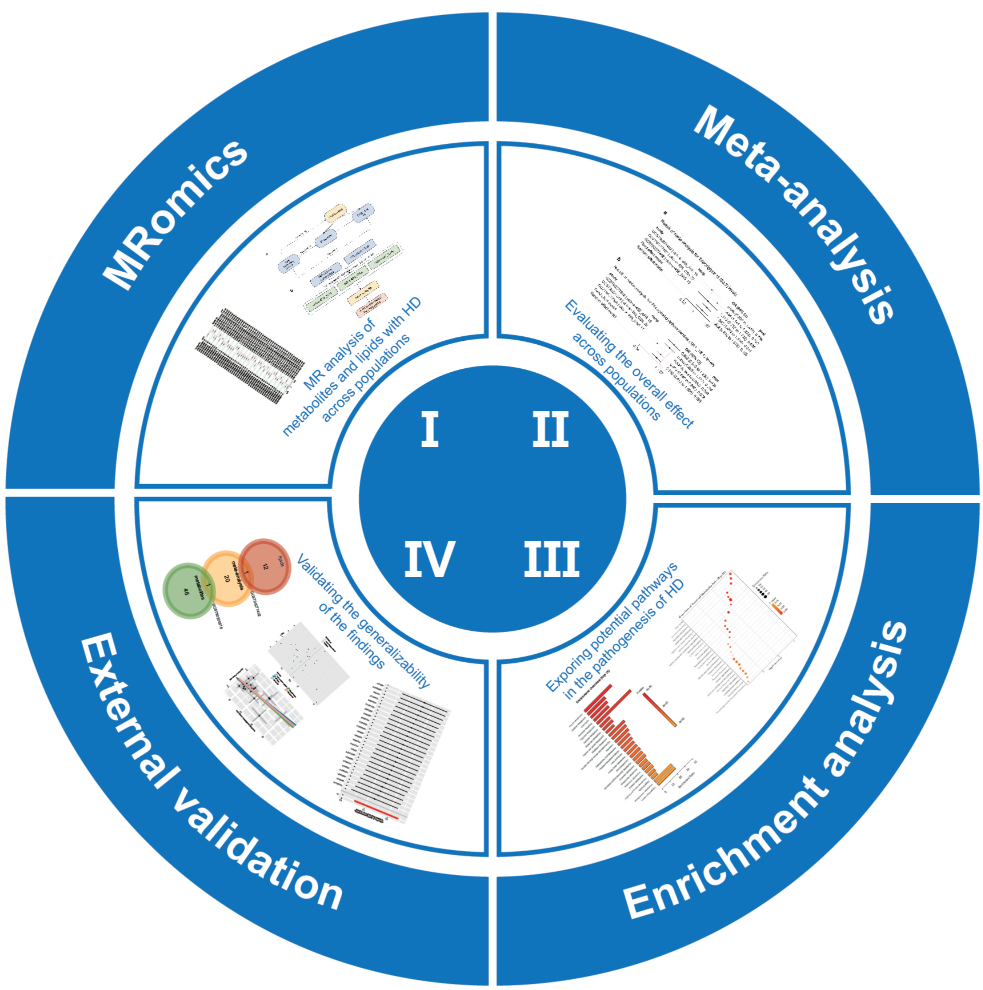
Graphical Abstract



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Causal Relationships between 1400 Serum Metabolite Traits, 179 Plasma Lipids, and Hemorrhoids: A Mendelian Randomization Study and Meta-analysis

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Abstract

Background: Hemorrhoids is a common anorectal disorder that significantly impacts patients' quality of life over the long term and imposes a substantial economic burden. However, the potential link between HD and serum metabolites and lipids has been scarcely studied, and its pathogenesis remains unclear.

Methods: The causal relationships between serum metabolite traits, plasma lipids, and HD were evaluated using Mendelian Randomization. Inverse Variance Weighted was the most important analysis approach. Sensitivity analysis was used to assess the robustness of the results. Enrichment analysis was applied to the metabolites obtained from the results of MR analysis and meta-analysis.

Results: The risk factors included Citramalate levels (OR = 1.487, 95%CI = 1.172-1.888, P = 0.001), P-cresol sulfate levels (OR = 1.476, 95%CI = 1.111-1.961, P = 0.007), and Triacylglycerol (53:3) levels (OR = 1.262, 95%CI = 1.049-1.518, P = 0.014). The protective factors included Imidazole lactate levels (OR = 0.865, 95%CI = 0.756-0.989, P = 0.034), 1-(1-enyl-stearoyl)-GPE (p-18:0) levels (OR = 0.722, 95%CI = 0.552-0.944, P = 0.017), and Phosphatidylethanolamine (18:1_18:1) levels (OR = 0.803, 95%CI = 0.666-0.969, P = 0.022). Enrichment analysis showed that risk factors were enriched in Alanine Metabolism (Holm P = 0.0039), Cysteine Metabolism (Holm P = 0.0146), Urea Cycle (Holm P = 0.0181), and Ammonia Recycling (Holm P = 0.0245). Protective factors were enriched in the Urea Cycle (Holm P = 0.00754), Ammonia Recycling (Holm P = 0.0102), Aspartate Metabolism (Holm P = 0.0147), Glutamate Metabolism (Holm P = 0.038), Arginine and Proline Metabolism (Holm P = 0.0479), and Malate-Aspartate Shuttle (Holm P = 0.0495).

Conclusion: Previous studies often focused on the causal relationships between a few potential risk factors and HD in a single population. Our study is the first to address this gap by integrating a broad range of 1400 metabolites and 179 lipids with HD across diverse populations, and identifying specific metabolic pathways involved in HD development.

Keywords: Serum Metabolites; Plasma Lipids; Hemorrhoids; Mendelian Randomization; Meta-analysis**.**

Introduction

Hemorrhoids (HD) is one of the most common types of anorectal disorders. Epidemiological studies indicate that over 50% of individuals experience at least one episode of HD before the age of 50 [1]. Furthermore, studies have suggested that HD may be associated with an increased risk of colorectal cancer [2]. The pathogenesis of HD is complex and involves multiple factors, including dietary habits, lifestyle, local inflammation, and abnormalities in local anatomical structures [3, 4]. HD is prevalent worldwide and has a long-term and significant negative impact on patients' quality of daily life. However, the scientific community has yet to fully understand the specific pathophysiological processes of HD and its association with risk factors.

Numerous studies have indicated significant correlations between serum metabolites and HD triggers, such as constipation, diarrhea, and varicose veins. For example, C4 and fibroblast growth factor 19 (FGF19) in serum are considered biomarkers for bile acid diarrhoea [5], highlighting the importance of serum metabolites in the pathogenesis of diarrhea, which itself may trigger HD. Notably, many studies have discovered

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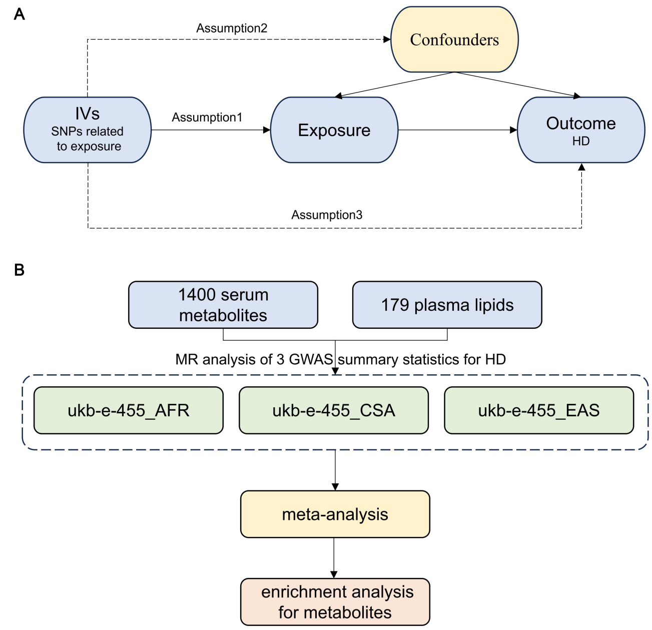
complex interactions between serum metabolites and the gut microbiota [6-8]. Evidence from Mendelian Randomization studies supports a causal correlation between the gut microbiota and the occurrence of HD [9]. Although existing evidence suggests a potential link between serum metabolites and HD, the direct causal relationship between the two requires further research to be clarified.
Lipids are important components of cell membranes and are involved in many physiological functions, including energy storage, signal transduction, and immune responses [10-12]. Studies indicate that plasma lipids may be associated with various inflammatory diseases [13], and HD, as an inflammatory anorectal disease, may be related to vascular inflammation and tissue damage. For instance, oxidized low-density lipoprotein (ox-LDL) can activate endothelial cells and smooth muscle cells, promoting the release of inflammatory factors [14, 15], which may exacerbate the local inflammatory state in HD patients. Additionally, dyslipidemia is related to an imbalance in the gut microbiota [16], which has been proven to be associated with various intestinal diseases, including HD [9, 17]. Therefore, regulating plasma lipid levels and improving dyslipidemia may have positive significance in the prevention and treatment of HD. However, further in-depth research is needed to clarify the specific mechanisms between dyslipidemia and HD.
Mendelian Randomization (MR), as an innovative method for causal inference, has been widely applied in epidemiological and genetic research in recent years. MR utilizes single nucleotide polymorphisms (SNPs) as naturally occurring instrumental variables (IVs). By analyzing the association between genetic variants and diseases, MR infers the potential causal relationship between exposure factors and outcomes. Since genetic variations are determined before an individual's birth and are not directly influenced by environmental factors, MR analysis can simulate the conditions of randomized controlled trials, effectively reducing confounding factors and reverse causation, thereby providing more reliable causal inference evidence.
Previous MR studies have identified obesity and plasma low-density lipoprotein (LDL) levels as risk factors for HD [18, 19], but these studies often focused on only a few potential risk factors. For the first time, this study conducted a comprehensive scan of the causal relationship between HD and metabolites and lipids in blood, using 1,400 serum metabolites and 179 plasma lipids as exposure factors. Moreover, previous MR studies often focused on a single population, raising concerns about the generalizability of their conclusions to other populations. To address this issue, we obtained GWAS data from individuals of African American or Afro-Caribbean, South Asian, and East Asian descent, and performed a meta-analysis of MR results across these populations to obtain robust conclusions. We also conducted external validation using GWAS data on HD from individuals of European ancestry.

Materials and methods

Study design

The three assumptions underlying the causal interpretation of MR analysis and the design of this study are displayed in Figure 1. The IVs used in the analysis need to satisfy three assumptions: (1) IVs must be strongly associated with the exposure variable; (2) IVs must follow the principle of random

Figure 1: Flowchart of study design. (**A**) Three assumptions of MR analysis. (**B**) Study workflow.



assignment and not be influenced by confounding factors; (3) IVs should not be directly related to the outcome but should influence the outcome only through the potential causal relationship between the exposure and the outcome [20].

GWAS data for serum metabolites and HD

The data used in this study were all obtained from publicly available GWAS datasets. GWAS summary statistics for 1,400 serum metabolite traits were acquired from the genome-wide association study by Chen et al. (SNPs = 1,540,000,000 approximately). The study cohort included 8, 299 individuals of European ancestry [21]. GWAS summary statistics for plasma lipids were obtained from the genome-wide association analysis by Ottensmann et al. [22] (SNPs = 11,318,730). The study sample comprised 7,147 Finnish individuals. HD data were retrieved from the IEU Open GWAS [23], sourced from the UK Biobank. The three HD datasets used in this study have the following GWAS IDs: ukb-e-455_AFR (SNPs = 15,526,533), ukb-e-455_CSA (SNPs = 981,032), and ukb-e-455_EAS (SNPs = 8,191,759). The ukb-e-455_AFR dataset includes samples from 6,458 African American or Afro-Caribbean individuals. The ukb-e-455_CSA dataset includes samples from 8,640 South Asian individuals. The ukb-e-455_EAS dataset includes samples from 2,658 East Asian individuals. Table S1 presents the baseline characteristics of the GWAS summary statistics used in preliminary analysis. In external validation, we selected ebi-a-GCST90086078 as the GWAS dataset for HD. The ebi-a-GCST90086078 data were derived from 56,637 individuals of European ancestry.

Selection of instrumental variables

The selected IVs must meet three criteria: First, SNPs associated with serum metabolite traits and plasma metabolites must have a genome-wide significance threshold of 1×10^{-5}. Second, to ensure the independence of selected SNPs, the linkage disequilibrium (LD) threshold is set to $r^2 \leq 0.001$ with a physical distance greater than 10,000 kb to eliminate LD. Third, the strength of the IVs is evaluated using the F-statistic, and SNPs with an F-statistic less than 10 are excluded.

Mendelian randomization analysis

MR analysis was performed using the "TwoSampleMR" package (version 0.6.4) in R (version 4.3.1). Statistical significance was defined as a $P < 0.05$. The causal relationship between exposure and HD was estimated using three methods: Inverse Variance Weighted (IVW), MR-Egger, and Weighted Median. IVW was the primary analysis method, as it employs weighted linear regression to assess the association between IVs and the outcome. When the genetic variants satisfy the three assumptions of instrumental variables and are not affected by pleiotropy, IVW provides a consistent estimate of the causal effect between exposure and outcome.MR-Egger accounts for potential heterogeneity in IVs and can detect and correct for bias, thus being used to evaluate horizontal pleiotropy [24]. When the results of these three methods were inconsistent, the IVW results were used as the primary evaluation, while the "forest plot" packages were employed for graphical representations. We followed the STROBE-MR (Strengthening the reporting of observational studies in epidemiology using mendelian randomization) guidelines for MR results reporting [25].

Sensitivity analysis

Sensitivity analyses included heterogeneity testing, horizontal pleiotropy testing, and leave-one-out testing. Heterogeneity was assessed using MR-Egger and IVW methods, quantified by Cochran's Q statistic. A $P > 0.05$ indicated that heterogeneity was not statistically significant. Horizontal pleiotropy was evaluated using the MR-Egger regression intercept and MR-PRESSO. For the MR-Egger regression intercept, a $P > 0.05$ suggested that horizontal pleiotropy could be ignored [26]. For MR-PRESSO, a $P > 0.05$ after the Global Test indicated that horizontal pleiotropy could be ignored. The stability of the results was assessed using the leave-one-out method; if the remaining results showed no significant changes after removing a single SNP, the results were considered stable.MR-PRESSO was performed using the MR-PRESSO package (version 1.0) in R (version 4.3.1), while the "forest plot" packages were employed for graphical representations. Funnel plots were used to assess whether there were biases in the results.

Enrichment analysis

Enrichment analysis for metabolites was conducted using MetaboAnalyst 6.0 [27]. Metabolites involved in serum metabolite traits with causal relationships to HD were classified into risk factor groups and protective factor groups according to the following rules: First, for serum metabolite levels, metabolites were classified as either risk factors or protective factors based on the sign of the beta value. For serum metabolite ratios, if the beta value was positive, the numerator component was classified as a risk factor, and the denominator component was classified as a protective factor; if the beta value was negative, the classification was reversed. Enrichment analysis was conducted separately for the two groups. The data were sourced from The Small Molecule Pathway Database (SMPDB). A significance threshold of $P < 0.05$ was set for this study.

Results

Causal effects of serum metabolite traits on HD

Results of African American or Afro-Caribbean population

After rigorous and comprehensive sensitivity analysis and excluding all results that failed the sensitivity analysis, 23 serum metabolite traits were identified as having a reliable causal relationship with HD, with 13 serum metabolite levels and ratios being risk factors for HD and 10 being protective factors (Figure S1-4). Among the risk factors, the top three with the most significant causal relationship with HD were: Cortolone glucuronide (1) levels (OR = 1.544, 95%CI = 1.124-2.123, P = 0.007), 3-methyl-2-oxobutyrate levels (OR = 2.079, 95%CI = 1.189-3.635, P = 0.010), and Glycocholate levels (OR = 1.405, 95%CI = 1.063-1.857, P = 0.017). Among the protective factors, the top three with the most significant causal relationship with HD were: 1-(1-enyl-palmitoyl)-2-oleoyl-GPE (p-16:0/18:1) levels (OR = 0.566, 95%CI = 0.413-0.776, P = 0.0004), X-11299 levels (OR = 0.439, 95%CI = 0.257-0.747, P = 0.002), and Aspartate to phosphate ratio (OR = 0.422, 95%CI = 0.226-0.791, P = 0.007). All MR results and sensitivity analysis outcomes are listed in Table S2.

Results of South Asian population

After conducting rigorous and comprehensive sensitivity analysis and excluding results that did not pass the sensitivity test, 32 results were considered reliable. 17 serum metabolite traits were identified as risk factors for HD (Figure S1, 5-7), with the three most significant causal effects being: Gamma-glutamyltyrosine levels (OR = 1.748, 95%CI = 1.258-2.427, P = 0.001), Adenosine 5'-monophosphate (AMP) to phosphate ratio (OR = 1.910, 95%CI = 1.267-2.879, P = 0.002), and Guaiacol sulfate levels (OR = 1.851, 95%CI = 1.245-2.750, P = 0.002). 15 serum metabolite traits were identified as protective factors for HD, with the top three significant causal effects being: 1-(1-enyl-stearoyl)-GPE (p-18:0) levels (OR = 0.623, 95%CI = 0.447-0.867, P = 0.005), Sphingomyelin (d18:2/24:1, d18:1/24:2) levels (OR = 0.570, 95%CI = 0.385-0.845, P = 0.005), and Glycerol to mannitol to sorbitol ratio (OR = 0.704, 95%CI = 0.541-0.914, P = 0.009). All MR results and their sensitivity analysis results are shown in Table S3.

Results of East Asian population

34 serum metabolite traits with causal relationship with HD was identified by MR analysis and sensitivity analysis, including 14 risk factors and 20 protective factors (Figure S1, 8-10). The top three risk factors with the most significant causal relationship with HD are: X-25271 levels (OR = 4.134, 95%CI = 1.798-9.507, P = 0.001), 5-hydroxyindole sulfate levels (OR = 2.610, 95%CI = 1.469-4.638, P = 0.001), and Spermidine to pyruvate ratio (OR = 2.405, 95%CI = 1.347-4.294, P = 0.003). The top three protective factors with the most significant causal relationship with HD are: Xanthurenate levels (OR = 0.409, 95%CI = 0.220-0.759, P = 0.005), Homostachydrine levels (OR = 0.318, 95%CI = 0.140-0.723, P = 0.006), and X-21796 levels (OR = 0.402, 95%CI = 0.203-0.799, P = 0.009). All MR results that passed the sensitivity analysis and their sensitivity analysis results are shown in Table S4.

Causal effects of plasma lipids on HD

Results of African American or Afro-Caribbean population

After MR analysis and comprehensive sensitivity analysis, 4 lipids were identified to have a causal relationship with HD, 2 of which are risk factors and 2 are protective factors for HD (Figure S11-14). Phosphatidylcholine (18:2_20:4) levels (OR = 1.588, 95%CI = 1.097-2.299, P = 0.014)and Phosphatidylcholine (O-16:1_16:0) levels (OR = 1.676, 95%CI = 1.109-2.533, P = 0.014) were identified as increasing the risk of HD. Phosphatidylcholine (O-16:0_20:3) levels (OR = 0.462, 95%CI = 0.293-0.727, P = 0.001) and Phosphatidylethanolamine (O-18:1_18:2) levels (OR = 0.569, 95%CI = 0.384-0.842, P = 0.005) were identified as protective factors. Details of the results of MR analysis and sensitivity analysis were shown in Table S5.

Results of South Asian population

After a rigorous and comprehensive sensitivity analysis, 10 plasma lipids were considered to have a reliable causal relationship with HD (Figure S11-14). Among them, 8 plasma lipids were identified as risk factors for HD: Triacylglycerol (53:4) levels (OR = 1.247, 95%CI = 1.021-1.523, P = 0.030), Triacylglycerol (52:3) levels (OR = 1.332, 95%CI = 1.062-1.672, P = 0.013), Triacylglycerol (56:7) levels (OR = 1.340, 95%CI = 1.038-1.730, P = 0.024), Triacylglycerol (56:4) levels (OR = 1.373, 95%CI = 1.019-1.850, P = 0.037), Triacylglycerol (56:8) levels (OR = 1.389, 95%CI = 1.095-1.761, P = 0.007), Triacylglycerol (53:2) levels (OR = 1.399, 95%CI = 1.045-1.874, P = 0.024), Triacylglycerol (53:3) levels (OR = 1.444, 95%CI = 1.141-1.828, P = 0.002), Ceramide (d42:1) levels (OR = 1.462, 95%CI = 1.102-1.939, P = 0.008). Additionally, 2 plasma lipids were identified as protective factors against HD: Sphingomyelin (d36:2) levels (OR = 0.730, 95%CI = 0.540-0.986, P = 0.040), Sterol ester (27:1/18:1) levels (OR = 0.772, 95%CI = 0.609-0.979, P = 0.033). All MR results and their sensitivity analysis results are shown in Table S6.

Results of East Asian population

After MR analysis and sensitivity analysis, 7 plasma lipids were considered to have a reliable causal relationship with HD, and all were identified as protective factors for HD (Figure S11-14): Sterol ester (27:1/20:3) levels (OR = 0.541, 95%CI = 0.331-0.885, P = 0.014), Ceramide (d42:1) levels (OR = 0.610, 95%CI = 0.387-0.960, P = 0.033), Phosphatidylcholine (16:0_16:1) levels (OR = 0.468, 95%CI = 0.243-0.901, P = 0.023), Phosphatidylcholine (18:0_18:3) levels (OR = 0.400, 95%CI = 0.164-0.977, P = 0.044), Phosphatidylcholine (O-16:0_16:0) levels (OR = 0.352, 95%CI = 0.160-0.770, P = 0.009), Phosphatidylethanolamine (18:1_18:1) levels (OR = 0.540, 95%CI = 0.343-0.850, P = 0.008), Triacylglycerol (54:7) levels (OR = 0.600, 95%CI = 0.365-0.987, P = 0.044). Details of the results of MR analysis and sensitivity analysis were shown in Table S7.

Meta-analysis of the results of MR

A meta-analysis will be conducted on the MR analysis results of serum metabolite traits and plasma lipids whose results of MR analysis were positive in at least one population, to evaluate the overall effect of serum metabolite traits and plasma lipids on HD across samples from different populations.

Meta-analysis of the causal relationships between serum metabolite traits and HD

Figure 2 shows the meta-analysis results of the effects of serum metabolites on HD. The heterogeneity test results indicate that a fixed-effect model should be chosen (P values are all greater than 0.05). The factors that increase the risk of HD are: Citramalate levels (OR = 1.487, 95%CI = 1.172-1.888, P = 0.001), P-cresol sulfate levels (OR = 1.476, 95%CI = 1.111-1.961, P = 0.007), Guaiacol sulfate levels (OR = 1.481, 95%CI = 1.113-1.971, P = 0.007), Gamma-glutamyltyrosine levels (OR = 1.472, 95%CI = 1.127-1.923, P = 0.005), Glycocholate levels (OR = 1.202, 95%CI = 1.018-1.420, P = 0.030), X-12839 levels (OR = 1.205, 95%CI = 1.007-1.442, P = 0.042), X-21258 levels (OR = 1.287, 95%CI = 1.007-1.645, P = 0.044), Adenosine 5'-monophosphate (AMP)to phosphate ratio (OR = 1.665, 95%CI = 1.219-2.275, P = 0.001), Pyruvate to 3-methyl-2-oxobutyrate ratio (OR = 1.341, 95%CI = 1.094-1.643, P = 0.005) (Figure 2A). The factors that reduce the risk of HD are: Imidazole lactate levels (OR = 0.865, 95%CI = 0.756-0.989, P = 0.034), 1- (1-enyl-stearoyl)-GPE (p-18:0) levels (OR = 0.722, 95%CI = 0.552-0.944, P = 0.017), Lanthionine levels (OR = 0.767, 95%CI = 0.597-0.983, P = 0.037), Sphingomyelin (d18:2/24:1, d18:1/24:2) levels (OR = 0.735, 95%CI = 0.548-0.985, P = 0.040), Dihomo-linolenoylcarnitine (C20:3n3 or 6) levels (OR = 0.827, 95%CI = 0.705-0.971, P = 0.020), X-17325 levels (OR = 0.803, 95%CI = 0.656-0.984, P = 0.034), X-17351 levels (OR = 0.786, 95%CI = 0.622-0.992, P = 0.043), X-24306 levels (OR = 0.765, 95%CI = 0.609-0.960, P = 0.021), Alpha-ketoglutarate to kynurenine ratio (OR = 0.817, 95%CI = 0.686-0.973, P = 0.024), Aspartate to phosphate ratio (OR = 0.665, 95%CI = 0.460-0.962, P = 0.030), Salicylate to oxalate (ethanedioate) ratio (OR = 0.705, 95%CI = 0.537-0.925, P = 0.012) (Figure 2B).

Meta-analysis of the causal relationships between plasma lipids and HD

Figure 3 shows the meta-analysis results of the effects of plasma lipids on HD. The heterogeneity test results indicate that a fixed-effect model should be chosen (P values are all greater than 0.05). Triacylglycerol (53:3) levels have been identified as a risk factor for HD (OR = 1.262, 95%CI = 1.049-1.518, P = 0.014) (Figure 3A). Phosphatidylethanolamine (18:1_18:1) levels are a protective factor against HD (OR = 0.803, 95%CI = 0.666-0.969, P = 0.022) (Figure 3B).

Enrichment analysis of serum metabolites

According to the rules proposed in the Methods section, the serum metabolite characteristics with positive meta-analysis results were classified into risk factor groups and protective factor groups. Serum metabolites that could not be retrieved in HMDB, PubChem, and KEGG were excluded (refer to Table S8 for metabolite information in different databases).Metabolite enrichment analysis showed that the enriched metabolic pathways for the risk factor group were: Alanine Metabolism (Holm P = 0.0039), Cysteine Metabolism (Holm P = 0.0146), Urea Cycle (Holm P = 0.0181), Ammonia Recycling (Holm P = 0.0245) (Figure 4A, Table S9).The enriched metabolic pathways for the protective factor group included: Urea Cycle (Holm P = 0.00754), Ammonia Recycling (Holm P = 0.0102), Aspartate Metabolism (Holm P = 0.0147), Glutamate Metabolism (Holm P = 0.038), Arginine and Proline Metabolism (Holm P = 0.0479), Malate-Aspartate Shuttle (Holm P = 0.0495) (Figure 4B, Table S10).

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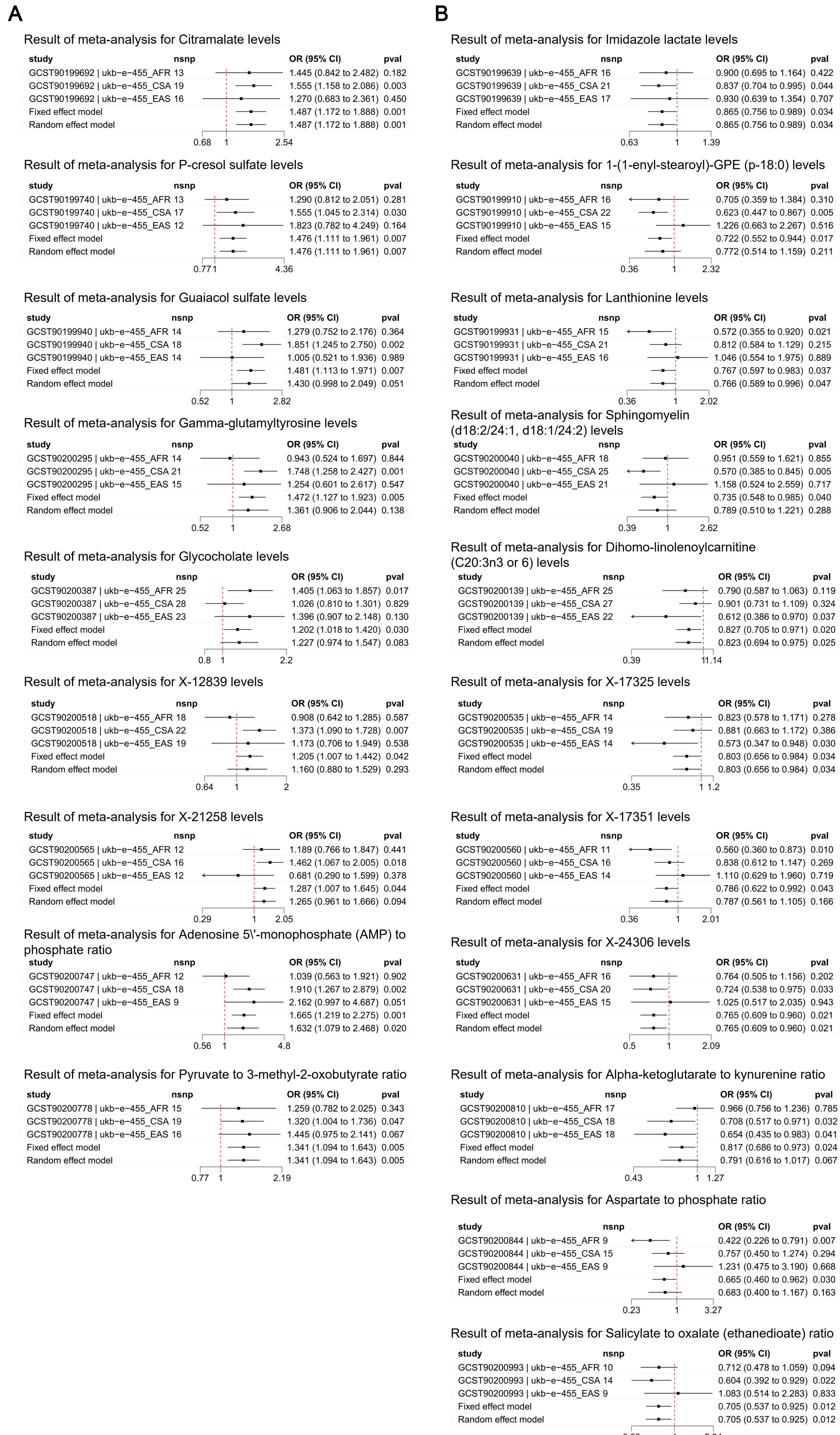
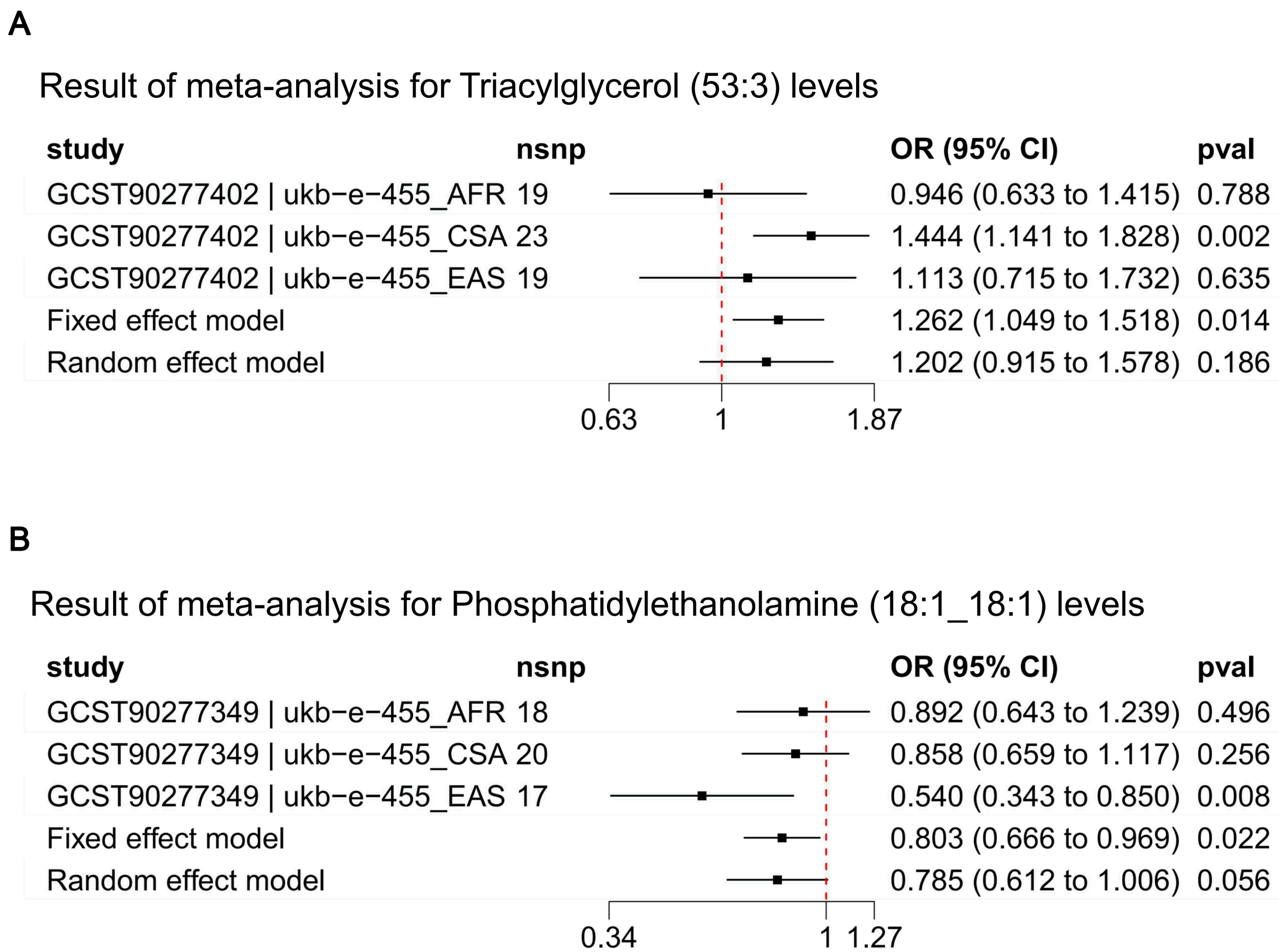


Figure 2: Meta-analysis results of serum metabolite traits. (**A**) The meta-analysis identified 9 serum metabolite traits as risk factors for HD. (**B**) The meta-analysis identified 11 serum metabolite traits as protective factors for HD.

Figure 3: Meta-analysis results of plasma lipids. (**A**) The meta-analysis identified Triacylglycerol (53:3) levels as risk factors for HD. (**B**) The meta-analysis identified Phosphatidylethanolamine (18:1_18:1) levels as protective factors for HD.



Discussion

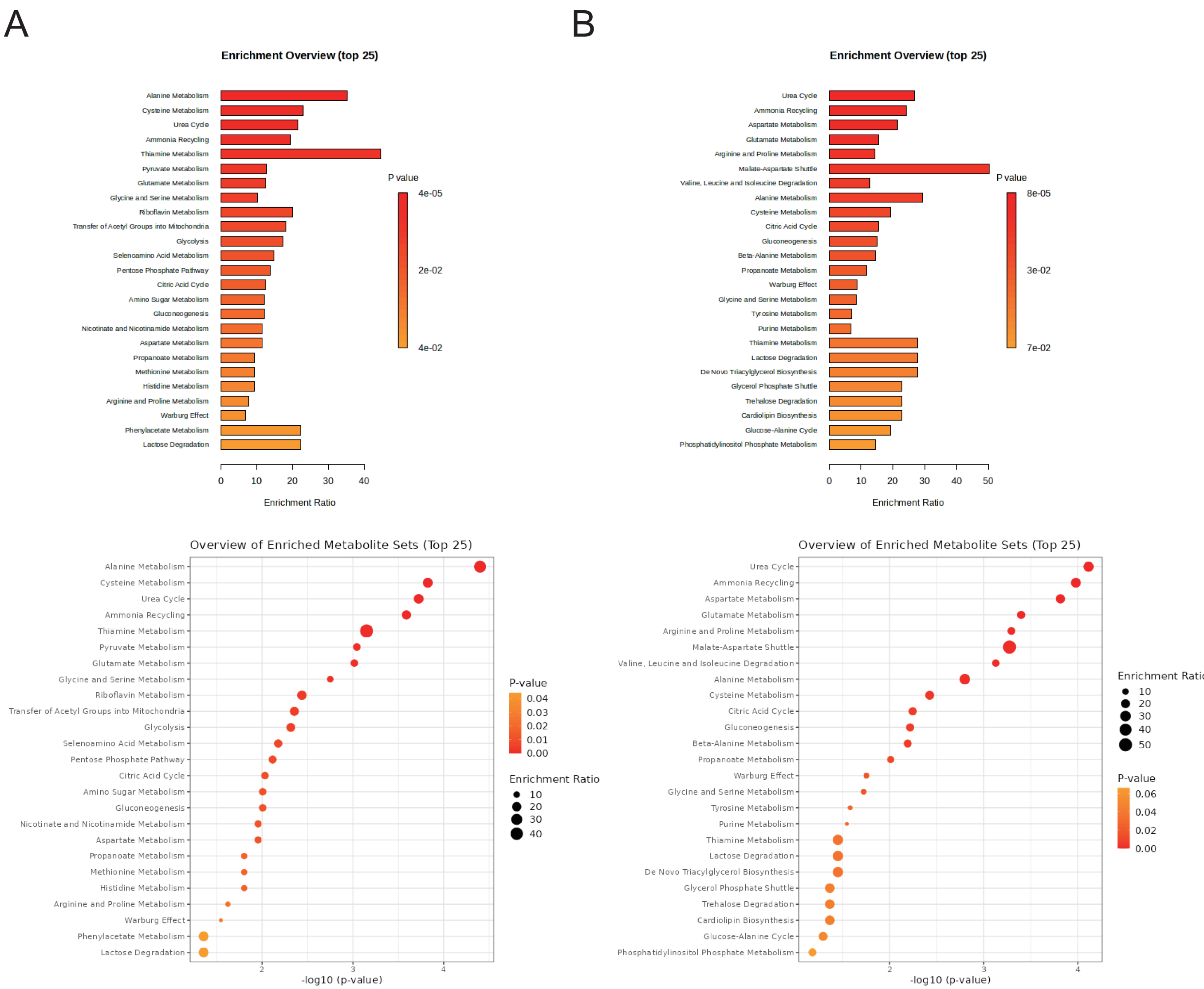
In the development of HD, the dilation and congestion of hemorrhoidal veins are the main pathological features. Inflammation and damage of local vascular endothelium play a crucial role in the occurrence of HD [3]. However, it remains unclear whether local inflammation in hemorrhoid patients causes changes in blood composition or if abnormal blood components trigger local inflammation, leading to the development of HD. In this study, we combined metabolomics, lipidomics, and genomics for the first time to explore the causal relationships between serum metabolites, plasma lipids, and HD using MR analysis, and employed meta-analysis to investigate the overall effects of MR results across different population samples.

It was observed that many lipids or lipoids and their metabolites are included among the protective factors for HD. Lipoids such as ceramides can maintain the integrity of the intestinal barrier by enhancing tight junctions in intestinal epithelial cells [28], thereby preventing harmful substances and bacteria from entering the bloodstream [29]. Additionally, fatty acids, especially unsaturated fatty acids, play an important role in maintaining vascular health. Polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) can reduce the risk of HD by inhibiting platelet aggregation and decreasing inflammation and damage in the vascular endothelium [30].

Metabolite enrichment analysis results indicate that Alanine metabolism and Cysteine metabolism may play key roles in the pathogenesis of HD. Abnormal activation of Alanine metabolism may influence the tumor necrosis factor-alpha (TNF-α) signaling pathway, thereby modulating the inflammatory response and lipid metabolism processes [31, 32]. Additionally, enhanced Cysteine metabolism is closely associated with the inflammatory response in venous tissue [33]. This inflammatory response may lead to endothelial damage in the venous wall. Furthermore, enhanced Cysteine metabolism is associated with increased production of matrix metalloproteinases (MMPs) [34-36]. The upregulation of MMPs expression promotes collagen degradation and extracellular matrix (ECM) remodeling, leading to significant changes in the structure and function of the venous wall. These changes may ultimately result in the development of varicose veins [37, 38]. When these pathological changes occur in the veins of the anorectal region, they may trigger the formation of HD.

Metabolite enrichment analysis revealed a series of metabolic

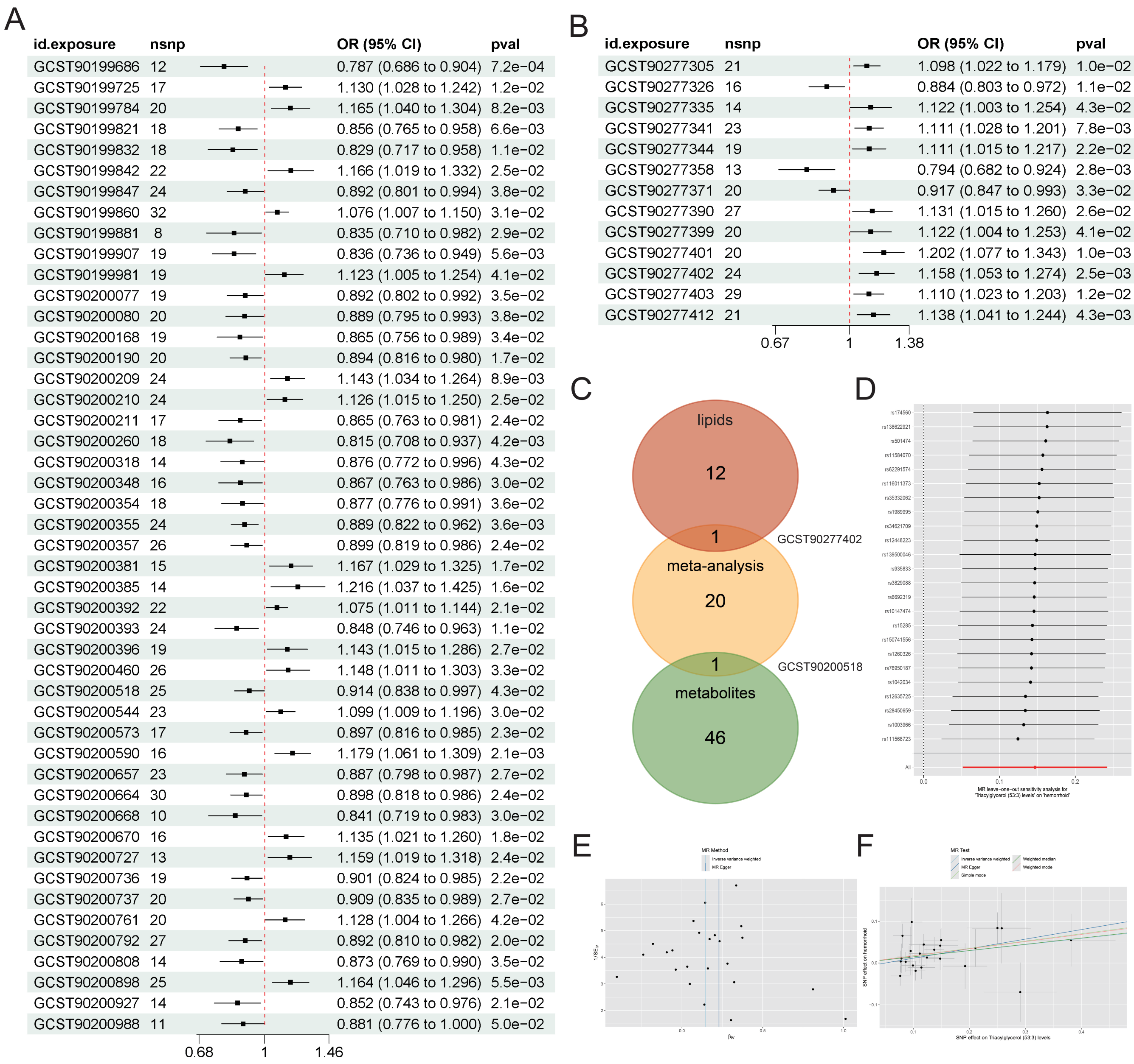
Figure 4: Serum metabolites enrichment analysis results. (**A**) Enrichment analysis results of serum metabolites in the risk factor group. (**B**) Enrichment analysis results of serum metabolites in the protective factor group.



pathways with potential protective effects. Specifically, Aspartate metabolism, Glutamate metabolism, Arginine and Proline metabolism, and the Malate-Aspartate shuttle play key roles in regulating the pathophysiological processes related to HD. Aspartate metabolism reduces oxidative stress by affecting intracellular metabolic processes such as the TCA cycle and pyruvate metabolism, thereby improving endothelial function [39-41]. Regulation of Glutamate metabolism may have anti-thrombotic effects by modulating the expression of nitric oxide synthase 3 (NOS3) and the metabolism of Arginine, Glutamate, and Glutamine [42-45]. The metabolic product of Arginine, nitric oxide (NO), has been shown to suppress inflammation, reduce inflammatory responses, and minimize tissue damage [46]. Proline metabolism reduces inflammation-induced tissue damage by activating immune cells such as T cells and natural killer (NK) cells [47]. The Malate-Aspartate shuttle plays a crucial role in maintaining intracellular NADH balance by transporting NADH to the mitochondria, reducing cytoplasmic NADH concentration, and thus lowering oxidative stress production. Excess accumulation of NADH in the cytoplasm may provide substrates for NAD (P) H oxidase, thereby promoting oxidative stress and inflammatory responses [48, 49]. The Malate-Aspartate shuttle may alleviate inflammation in the anorectal region by inhibiting this pathway, thereby preventing the occurrence of HD. In summary, the regulation of these metabolic pathways may offer new insights into the prevention and treatment of HD.

Interestingly, urea cycle and ammonia recycling were observed to exhibit both promoting and inhibiting effects in the development of HD. Urea cycle and ammonia recycling are key pathways in the metabolism, utilization, and excretion of ammonia, and are crucial for maintaining ammonia homeostasis in the internal environment [50-53]. On the one hand, high ammonia concentrations have been shown to promote inflammatory responses [54, 55], which may enhance the release of inflammatory mediators and, in turn, contribute to the development of HD. On the other hand, moderate ammonia levels have been found to mitigate the damage of inflammatory responses to

Figure 5: Results of external validation. (**A**) Forest plot shows the causal associations between HD and serum metabolites in the European ancestry population. (**B**) Forest plot shows the causal associations between HD and plasma lipids in the European ancestry population. (**C**) Venn diagram illustrates the overlap between the MR analysis in the European ancestry population and the previous meta-analysis results. (**D**) Leave-one-out analysis shows the influence of each individual SNP on the overall causal effect between GCST90277402 and HD, used to assess horizontal pleiotropy in the MR analysis. (**E**) Funnel plot indicates no apparent bias among SNPs in the MR analysis between GCST90277402 and HD. (**F**) Scatter plot exhibits the beta estimates of the MR results between GCST90277402 and HD using different analytical methods.



pancreatic β-cells through the regulation of glutamine metabolism [56, 57], suggesting that ammonia may have anti-inflammatory effects at certain concentrations. Therefore, urea cycle and ammonia recycling play a critical role in maintaining ammonia balance in the internal environment, and subtle changes in ammonia levels can influence the development of HD by modulating inflammatory responses. This finding not only reveals the complex roles of the urea cycle and ammonia recycling in the pathophysiology of HD but also provides new perspectives for further research, suggesting that regulating ammonia metabolism pathways may offer potential strategies for the prevention and treatment of HD.

Triacylglycerol (53:3) levels were found to be positively associated with HD risk, while Phosphatidylethanolamine (18:1_18:1) levels were negatively correlated with HD risk. Research suggests that Triacylglycerol (53:3) may increase the risk of thrombosis [58], as well as induce inflammatory responses such as leukocyte adhesion and exudation, which in turn may promote the development of varicose veins [59, 60]. When these pathological changes are localized to the anorectal region, they may lead to the formation of HD. Phosphatidylethanolamine (18:1_18:1) is an important phospholipid compound with various physiological functions in the human body, but research on this specific phospholipid remains limited. These findings provide new insights into the role of plasma lipids in the pathogenesis of HD and may offer a scientific basis for developing new therapeutic strategies.

This study reports for the first time the causal relationship between serum metabolites, plasma lipids, and the occurrence of HD. It identifies risk factors for HD and provides new insights into the mechanisms underlying HD. Through MR analysis, we identified a series of metabolites and lipids associated with the risk of HD occurrence, and explored their overall causal relationships with HD in different population samples through meta-analysis. These findings provide a new theoretical foundation for the clinical diagnosis and treatment strategies of HD.

We must acknowledge that, while the results showed consistency across diverse populations, the genetic and environmental backgrounds of these specific populations may influence the generalizability of the findings. Therefore, we conducted external validation in a European ancestry population. We selected ebi-a-GCST90086078 as the GWAS dataset for HD and conducted MR analysis using 1,400 serum metabolites and 179 plasma lipids as outcomes. The ebi-a-GCST90086078 data were derived from 56,637 individuals of European ancestry. SNPs were selected with a genome-wide significance threshold of 1×10^{-5}. Based on the MR analysis results, in the European ancestry population, the causal relationships between HD and metabolites and lipids are illustrated in Figure 5. By intersecting the MR analysis results with those of the previous meta-analysis, we observed that Triacylglycerol (53:3) levels (GCST90277402) showed a significant positive association with HD in the results of European ancestry population, in line with the meta-analysis results of African American or Afro-Caribbean, South Asian, and East Asian populations. This indicated the generalizability of this finding. GCST90200518 also demonstrated a causal relationship with HD in both the external validation and meta-analysis; however, the direction of the effect was opposite. For detailed results, please refer to the Raw Data. Nevertheless, we observed that the results in the European ancestry population differ markedly from those in the other three populations, reminding us that the population limitations still exist.

Besides, since we only used summary statistics for estimation, we did not evaluate the causal association in different groups, such as different gender or age groups. Another major limitation of this study is the lack of consideration of potential confounding factors, including BMI, smoking status, diet, environmental exposures and so on. These confounders may also contribute to the development of HD and thereby compromise the accuracy and reliability of the analytical results.

However, despite these preliminary results providing valuable clues for the pathophysiological research of HD, there is still a lack of in-depth understanding regarding the precise connection between these metabolites and lipids and HD on a multi-omics level. Therefore, future research needs to adopt multi-omics approaches, such as proteomics, metabolomics, and genomics, to comprehensively explore the interactions and potential molecular mechanisms between these biomarkers and HD. Through this interdisciplinary research approach, we aim to further uncover the complex pathological processes of HD and provide a scientific basis for developing more effective prevention and treatment strategies.

Conclusion

In-depth MR analysis and meta-analysis were conducted in this study to reveal the potential causal relationship between serum metabolites, plasma lipids, and the occurrence of HD. Specific metabolites and lipid markers were found significantly associated with the risk of HD and identified metabolite-enriched pathways, providing new insights into its pathophysiology. Our findings offer new insights into the clinical diagnosis and treatment of HD and provide a reference for future multi-omics studies to uncover deeper molecular mechanisms, laying the foundation for the development of effective prevention and treatment strategies.

Abbreviations

Extracellular matrix: ECM; Eicosapentaenoic acid: EPA; Genome-wide association study: GWAS; Hemorrhoids: HD; Instrumental variable: IV; Inverse Variance Weighted: IV; Low-density lipoprotein: LDL; Matrix metalloproteinase: MMP; Mendelian randomization: MR; Natural killer: NK; Nitric oxide: NO; Nitric oxide synthase 3: NOS3; Polyunsaturated fatty acid: PUFA; Single nucleotide polymorphism: SNP; Strengthening the reporting of observational studies in epidemiology using mendelian randomization: STROBE-MR; Tumor necrosis factor-alpha: TNF-α.

Author Contributions

Lu Hu and Danyang Wang made equal contributions to this manuscript. Lu Hu: Writing - original draft, Investigation, Data curation, Project administration. Danyang Wang: Formal analysis, Methodology, Supervision, Writing - review & editing. Chang You: Writing - review & editing. Ziyan Zhao: Writing - review & editing. Wanlin Zheng: Writing - review & editing. Luyao Wang: Writing - review & editing. Yutong Li: Visualization. Liangzhe Dai: Visualization. Hongkai Yu: Writing - original draft, Investigation, Data curation, Project administration. All authors read and approved the final manuscript.

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Ethicals Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data availability

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

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The early stage of the COVID-19 Pandemic's Psychological Footprint: A Study on the Surge of Mental Health Concerns in China as Reflected by Baidu Index

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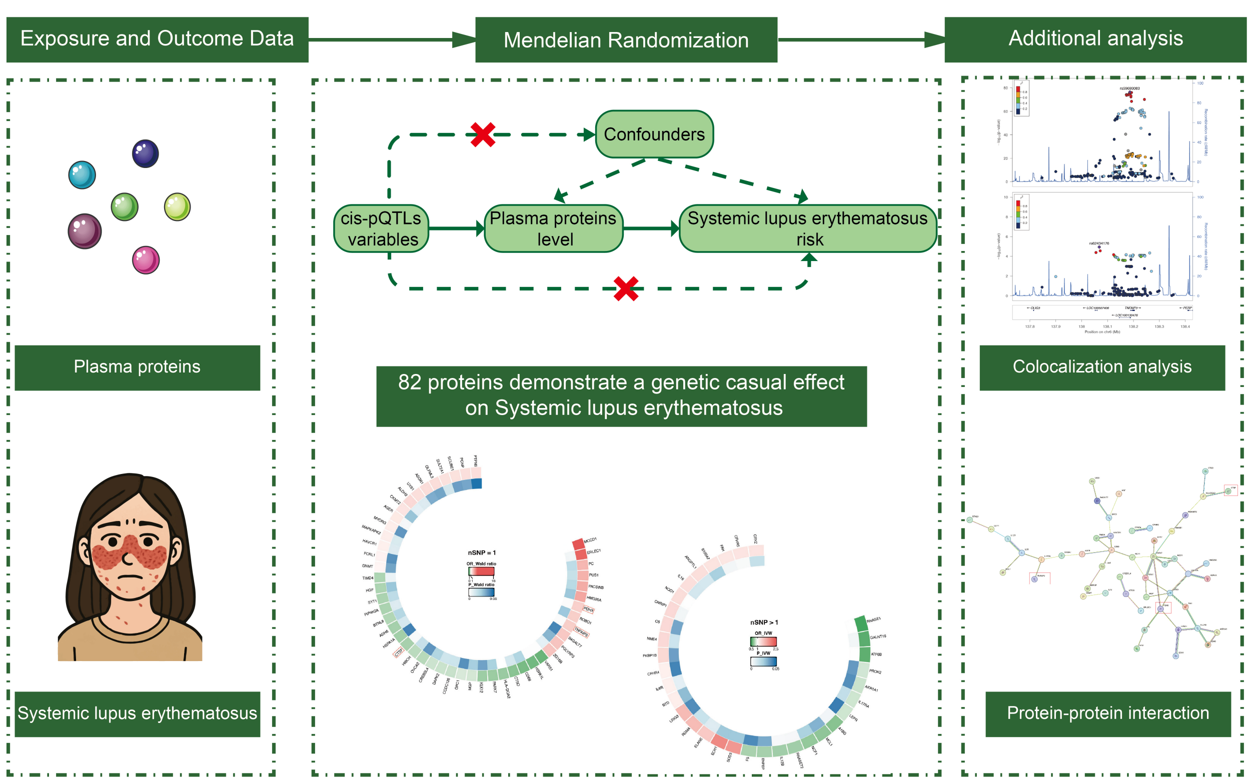
Graphical Abstract



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The early stage of the COVID-19 Pandemic's Psychological Footprint: A Study on the Surge of Mental Health Concerns in China as Reflected by Baidu Index

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Abstract

Background: The outbreak of COVID-19 has posed an enormous threat to the health of people worldwide, both physically and mentally. We aimed to investigate the impact of COVID-19 on Chinese people's mental health via the Baidu Index, especially during the early period of the outbreak.
Methods: We collected people's search data regarding mental illness and COVID-19 from the Baidu index. Spearman's correlation analysis was applied to explore the correlations among mental illness search index values, COVID-19 search index values, and the number of confirmed cases in China. We implemented a dynamic series analysis to show the changing trend of Baidu index search values. Gender, age, and regional distribution of search values were also observed. Internet searches for mental illness increased significantly after the quarantine measures were implemented.
Results: The number of COVID-19 cases were positively correlated to the overall search index values for mental illnesses (r_s=0.766, p=1.041×10^{-12}), and negatively correlated to search index values for COVID-19 (r_s=-0.236, p=0.023). The searches for COVID-19 was positively correlated to the daily growth of cases (r_s=0.861, p=2.310×10^{-18}). No lag pattern exists between Internet searches for mental illness and the number of confirmed cases. Male and people over 50 years old searched less than other groups. Besides, the highest search behaviors appeared in southeastern China. Public search behaviors indicate that since the outbreak of COVID-19, the psychological problems of the Chinese people have been extremely prominent.
Conclusion: Baidu Index offers a valuable tool for guiding effective intervention and prevention efforts aimed at mitigating psychological stress. Its ability to reflect public search behaviors allows for the timely provision of mental health support during the COVID-19 pandemic and could serve as a model for responding to the mental health challenges posed by future large-scale infectious disease outbreaks.

Keywords: COVID-19; Mental illness; Psychological stress; Baidu index.

Introduction

The emergence of the coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has unveiled a global health crisis that has not only posed an immediate threat to physical well-being but also cast a long shadow over mental health [1]. The pandemic has resulted in a surge of infections and fatalities, with the numbers continuing to rise and reshape our understanding of global health [2]. The psychological impact of COVID-19 is equally pervasive, ranging from heightened anxiety and depression to post-traumatic stress disorder, with the early stages of the outbreak being particularly distressing due to the novel nature and severity of the virus [1]. Despite the growing recognition of the pandemic's psychological impact, few studies have focused on the early weeks of the outbreak, a period critical for understanding how the public's mental health was affected and for identifying the most vulnerable populations in need of immediate intervention. While several studies have been published on the acute effects and features of COVID-19, few have focused on the psychological effects on the public [3, 4]. Since the timely treatment of psychological stress would enhance public resilience and strength [5], understanding the epidemiological characteristics of psychological problems caused by COVID-19 is of great importance.
To interrupt the transmission of the epidemic, the Chinese government has put in place strong quarantine measures, which also influence the routinely psychological counseling procedure. Thus, it is difficult to get data on public mental disorders during the epidemic from the hospital. Since the epidemiologi-

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cal data on mental illness is missing, the best way to deal with these challenges keeps unknown [6]. Today, social media and medical forums have been an essential medium for public access to information [7]. Almost eighty percent of Internet users have used the Internet as the source of health-related information. It is becoming increasingly clear that using an Internet search engine to gather and conduct the data of users' search behavior can improve the surveillance of epidemics and public interest in health topics [7, 8]. In China, people are more likely to retrieve the diagnosis and treatment information through the Internet compared with the past [9]. Baidu, as a search tool with the largest consumers in China, public search terms through this engine could represent their interest in the topics concerned, especially in places with a high Internet penetration [10]. Several examinations had used Baidu Index to explore people's search behavior in the health-related field, including HIV/AIDS [10], dengue fever [11], and H7N9 virus [12]. Besides, a positive correlation was observed between these search behaviors and epidemics.

Our study leverages Baidu Index data to scrutinize the psychological responses among the Chinese population during the nascent stages of the COVID-19 outbreak, from January 10, 2020, to March 9, 2020. The choice of this early period is deliberate and significant for several reasons. First, the initial shock of the pandemic led to a surge in public anxiety and a scramble for information, which is often reflected in online search behaviors. Second, by analyzing these search trends, we can detect the early signs of psychological distress and the specific mental health concerns that were most prevalent. This approach allows us to provide insights into the psychological impact of COVID-19 that can inform targeted interventions to mitigate long-term mental health consequences. Our study contributes to the existing literature by highlighting the importance of early psychological surveillance during pandemics. It underscores the need for a proactive approach to mental health support, particularly during the initial stages of a health crisis when public anxiety is at its peak [13]. Based on the hypothesis that Internet query data can reflect the epidemics of psychological stress during the outbreak (such as the types of mental illness with higher concern, the gender, age, and regional distribution of the vulnerable population), data from Baidu search engines could help the nation to face the challenges of public mental illness with more timely and effective ways. By identifying the early psychological repercussions of COVID-19 through Baidu Index data, our study aims to inform public health strategies that address not only the physical health threats but also the mental health challenges posed by such pandemics.

Methods

Baidu Index

As the major Internet search engine in China, Baidu's market share has exceeded eighty percent [14]. Moreover, 92.1% of search engine users conducted their searches by Baidu [15]. By analyzing and calculating the weighted sum of the search times of the entering queries, Baidu users can view the characteristics of their searching behaviors [15]. In the current study, we collected the daily number of cases confirmed with COVID-19 data from the National Health Commission of People's Republic of China [16]. Since the National Health Commission has reported the number of new people daily since January 10,2020, we retrieved daily search metrics data between 2020/1/10 to 2020/3/9 from the Baidu index.

Keywords of Screening

As a Chinese search engine, search queries are all entered as Chinese characters and can be expressed with various related keywords among populations. Thus, the point for Internet surveillance is how to recognize these keywords. To analyze the psychological impact of COVID-19 during the outbreak, we selected the Chinese search terms, which were highly correlated to "COVID-19" and "SARS-CoV-2" in the study based on the function of the keyword analysis provided by Baidu Index. First, we entered the official Chinese names of "COVID-19" and "SARS-CoV-2" as keywords. Then, the keyword analysis function generated a series of related keywords automatically, ranging from high to low by the search volumes related to our topics. Different terms can be combined with the sign (+), which means "OR" to represent the multiple terms. According to prior research that analysis Spanish students' use of the Internet as the source of mental health information, the keywords that reflect public psychological stress can be defined as follows: "Depression", "Bipolar disorder", "Anxiety", "Obsessive", "Panic", "Suicide", "Schizophrenia", "Stress", "Dementia", "Personality disorders", "Addictions", "Post-natal depression" and "Eating disorder" [17]. Since the Baidu index did not provide adequate search data for "Eating disorder," we could not include this term in our study. Besides, we did not include the keyword of "Post-natal depression" because of its low correlation with our study. Eventually, seven COVID-19-related and 44 mental health-related keywords were selected for our analysis (Table S1). To analyze the overall characteristics of public psychological stress, we added up together each keyword of mental illness to get an aggregate daily data to perform our research. Furthermore, gender, age, and regional distributions of users who conducted mental illness searches were examined in our study.

Statistical analysis

Using software SPSS 23.0, we performed the correlation analysis to examine the correlations among the daily number of confirmed cases in China, the daily Baidu Index values for COVID-19, and the daily Baidu Index values for psychological stress. We also performed a lag Spearman correlation to analyze the lag pattern of the relationship between the number of COVID-19 cases and internet searches for mental disorders. $P < 0.05$ was set for statistical significance between variables (two-sided test). Besides, we used software GraphPad Prism 8.2 and ArcGIS 10.6 to create figures.

Dynamic series analysis

We applied a dynamic series analysis to describe the dynamic change in Baidu index search values for the entered queries. Since public awareness to COVID-19 was not high until Wuhan (China) had suspended the transportation (23 January 2020) [6], we conducted the dynamic series analysis between 24 January 2020 to 9 March 2020 to better reflect the mental impact of COVID-19 on Chinese people. We provided the daily development rate and increase rate of the search values as the statistical indicators. The ratio of fixed base and link-relative was also adopted to investigate the dynamic change of our object. Comparing the search index values in a given time with the baseline, we get the daily development of a fixed base rate, which can indicate the general development direction and speed of the search index values in a given time. While the daily development rate of link-relative was defined to describe the day by day growth rate of the search index values by making a comparison between a day's search values and the previous day's search index values. Moreover, we also calculated an average development rate (the geometric mean of the link-relative development) to compare the development speed of people's search behaviors for our observing topics. Besides, by decreasing the development by 100%, we get the increase rate.

Results

Correlation analysis among search index values of mental illness, search index values of COVID-19, and number of cases in China

Table 1 shows the correlation among the overall search index values of mental illness, the search index values of COVID-19, and the daily number of cases confirmed with COVID-19 in China. Table 1 also presents the correlations between the daily number of confirmed cases in China and the search index values of each topic regarding mental illness. We observed a strong positive correlation between the number of cases and the relative Baidu index values for "Anxiety" (r_s=0.879, p=2.42×10^{-20}), "Addiction" (r_s=0.555, p=4.23×10^{-6}), "Suicide" (r_s=0.849, p=1.132×10^{-17}), "Schizophrenia" (r_s=0.774, p=4.218×10^{-13}), "Panic" (r_s=0.946, p=4.984×10^{-30}), "Obsessive" (r_s=0.839, p=5.811×10^{-17}), "Stress" (r_s=0.895, p=5.856×10^{-22}), "Dementia" (r_s=0.772, p=4.896×10^{-13}), "Personality disorders" (r_s=0.790, p=5.865×10^{-14}), and "Sleep" (r_s=0.778, p=2.499×10^{-13}) ("Depression" and "Bipolar disorder" presented with slightly positive correlations, r_s=0.261, p=0.044, and r_s=0.394, p=0.002, respectively).

As for the correlations between the search index values of each topic regarding mental illness and the index values of COVID-19. We observed negative correlations between relative search index of COVID-19 and search index for nine of twelve mental illnesses: "Depression" (r_s=-0.862, p=1.219×10^{-28}), "Anxiety" (r_s=-0.207, p=0.046), "Addiction" (r_s=-0.596, p=2.844×10^{-10}), "Suicide" (r_s=-0.272, p=0.008), "Schizophrenia" (r_s=-0.495, p=4.458×10^{-7}), "Obsessive" (r_s=-0.262, p=0.011), "Stress" (r_s=-0.352, p=0.001) ("Dementia", "Personality disorders" and "Sleep" did not show such correlations, r_s=-0.014, p=0.894, r_s=0.042, p=0.691, and r_s=0.078, p=0.458, respectively, "Panic" presented with positively correlation: r_s=0.538, p=2.742×10^{-8}). The correlations between the number of confirmed cases in China, the daily growth of cases in China, and search values of COVID-19 were shown in Figure1A-C.

In our analysis, we observed a robust positive correlation be-

Table 1. Correlation among search index values of mental illness, number of cases, and search index values of COVID-19

Keywords/Overall	Search index values of mental health and number of cases		Search index values of mental health and values of COVID-19	
	r_s	*p* value	r_s	*p* value
Overall	0.766	1.041×10^{-12}	-0.236	0.023
Depression	0.261	0.044	-0.862	1.219×10^{-28}
Anxiety	0.879	2.42×10^{-20}	-0.207	0.046
Addiction	0.555	4.234×10^{-6}	-0.596	2.844×10^{-10}
Suicide	0.849	1.132×10^{-17}	-0.272	0.008
Schizophrenia	0.774	4.218×10^{-13}	-0.495	4.458×10^{-7}
Panic	0.946	4.984×10^{-30}	0.538	2.742×10^{-8}
Obsessive	0.839	5.811×10^{-17}	-0.262	0.011
Stress	0.895	5.856×10^{-22}	-0.352	0.001
Dementia	0.772	4.896×10^{-13}	-0.014	0.894
Personality disorders	0.790	5.865×10^{-14}	0.042	0.691
Sleep	0.778	2.499×10^{-13}	0.078	0.458
Bipolar disorder	0.394	0.002	-0.642	4.232×10^{-12}

tween the frequency of COVID-19-related searches and the daily increase in confirmed cases in China (Figure 1a, r_s=0.861, p=2.310×10^{-18}). This suggests that public interest in COVID-19 was closely aligned with the early surge in case numbers. However, we did not detect a significant correlation between the volume of COVID-19 searches and the cumulative number of confirmed cases (Figure 1b, r_s=-0.006, p=0.966). Despite the continuous rise in confirmed cases throughout our study period, the frequency of COVID-19-related searches exhibited a decline, indicating a waning public interest and sensitivity towards the pandemic. This trend may reflect a decrease in public concern and attention as the outbreak progressed during the early outbreak of COVID-19.

We observed an intriguing negative correlation between search indices for mental illness and COVID-19, with a Spearman's rho of -0.236 (Figure 1c, p=0.023). This inverse relationship suggests that as the immediate threat of the pandemic receded from public focus, attention shifted towards the enduring psychological impacts of the crisis. Significantly, we identified a sustained rise in searches related to mental health, concomitant with increasing cumulative COVID-19 cases during the same timeframe (Figure 1d, r_s=0.766, p=1.041×10^{-12}). This pattern of search behavior indicates that while the direct attention on COVID-19 waned, there was an escalating awareness and concern regarding mental health issues-potentially a secondary repercussion of the pandemic's influence on daily life and overall well-being. This evolution highlights the critical need to address not only the direct health impacts of COVID-19 but also its indirect psychological ramifications for the broader population.

Lag correlation between the number of confirmed cases and search index values of mental illness

Figure 2 and Table S2 presents the lag correlation between the number of confirmed cases and search index values of mental illness. Generally, we observed a declined trend of correlation coefficient with the increasing lag of time. We found the highest correlation with the Baidu searches for “Depression” (r_s=0.261), “Bipolar disorder” (r_s=0.394), “Anxiety” (r_s=0.879), “Obsessive” (r_s=0.839), “Suicide” (r_s=0.849), “Schizophrenia” (r_s=0.774), “Stress” (r_s=0.895), and “Dementia” (r_s=0.772) 0 days earlier for the number of COVID-19 cases (Table 1). As for other search topics of mental illness, the results of time lag correlation showed that the highest correlation between Baidu searches for “Panic”, “Personality disorders”, “Addictions” and the number of reported COVID-19 cases 2 days, 2 days, and 11 days earlier with the correlation coefficient as 0.949, 0.802, and 0.772, respectively (Table S2) (p<0.05 for all).

Search trends in Web-based data of mental illness

The top five searched topics were "Depression" (with the ratio of 33.15%), "Addiction" (12.91%), "Anxiety" (12.23%), "Suicide" (8.87%), and "Schizophrenia" (7.28%) (Figure S1). Figure 3 shows the search trend of the 12 search topics regarding psychological stress in China during the outbreak of COVID-19. The overall search trends of index values for “Depression”,

Figure 1. Analysis of Search Trends During the COVID-19 Pandemic in China. **A.** Correlation between COVID-19-related search frequency and daily increase in confirmed cases in China; **B.** Correlation between COVID-19 search volume and cumulative confirmed cases in China; **C.** Correlation between search indices for mental illness and COVID-19; **D.** Correlation between search indices for mental illness and the cases of cumulative COVID-19 in China.

"Addictions", "Anxiety", "Suicide", "Schizophrenia", and other seven search topics declined in the earlier period of observing time combined with a little of fluctuation. Then the trend increased around 24 January 2020 and kept at a higher level. The dynamic series of the overall and top five search index values for topics regarding mental illness is presented in Table 2, Table S3. A steady increase trend was observed for all the topics contained with the average increase rate of 52.11% for "Depression", 154.33% for "Addictions", 112.66% for "Anxiety", 66.96% for "Suicide", and 42.77% for "Schizophrenia" (Figure S2).

Gender distribution of public search behaviors

For all the search topics on mental illness in our study, women searched more than men, especially for "Depression" (nearly female: 72%, male: 28%) and "Addictions" (nearly female: 79%, male: 21%) (Figure 3).

Figure 2. Lag correlations between the number of confirmed cases and each/overall search topics of mental illnesses.

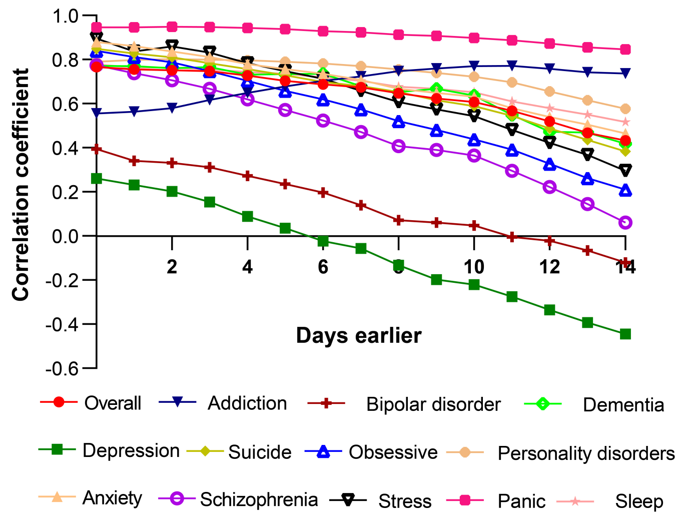
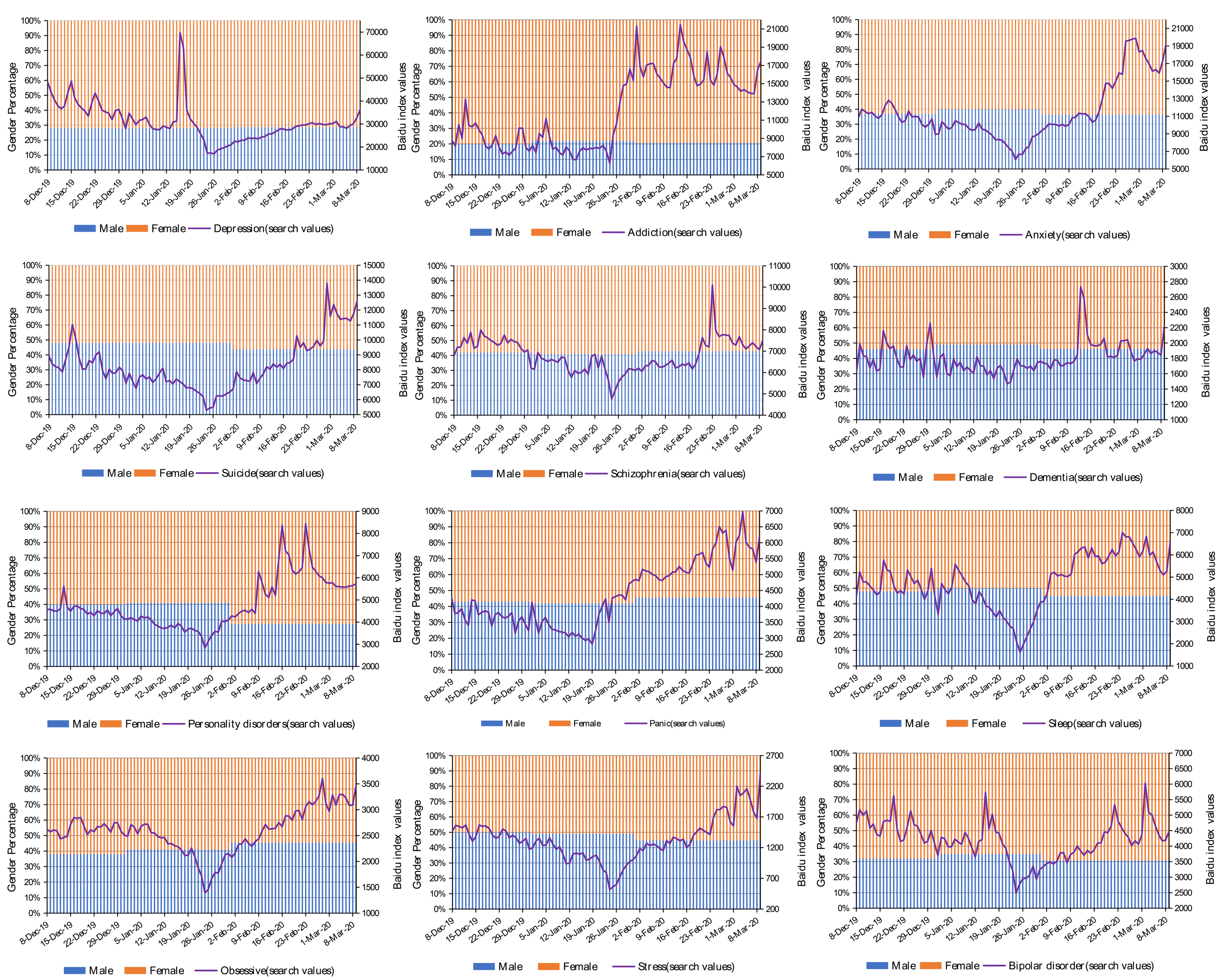


Figure 3. Gender distribution and time series of search index values for the 12 search queries on mental illness from 8 December 2019 to 9 March 2020.



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Age distribution of public search behaviors

Figure 4 presents the age distribution for mental illness-related searches between 8 December 2019 to 9 March 2020, in China. Generally, people aged between 20-49 presented higher search behaviors for each topic regarding mental illness, while the group with people aged over 50 years old had the lowest search behaviors.

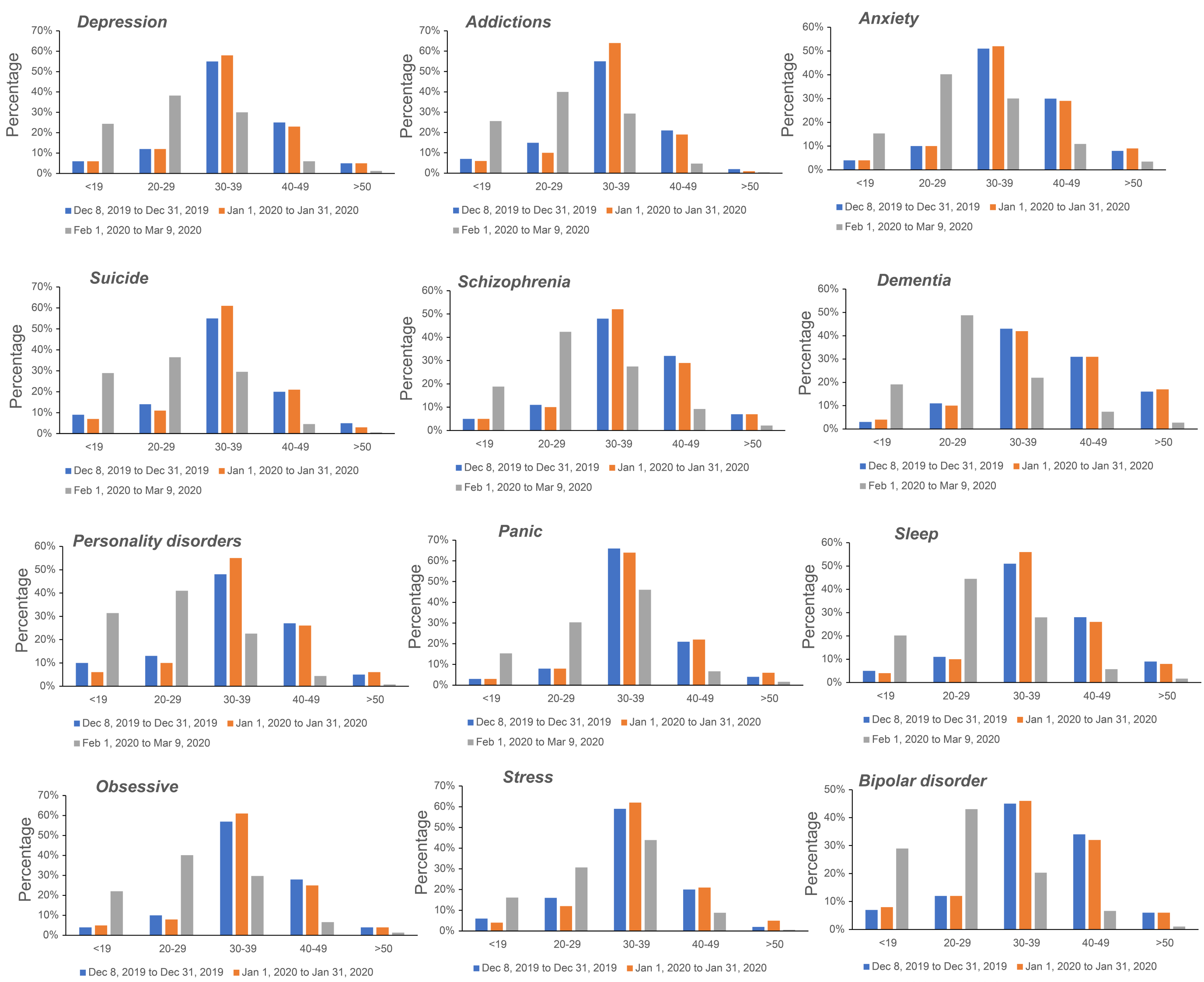
Regional distribution of public search behaviors

Figure 5 performs the regional distribution of the online searches for mental illness and the cases of COVID-19 in 34 provinces and autonomous regions in China. We observed that populations in Guangdong Province searched most while people in Macao and Taiwan searched least compared with the population in other places. For the top five keywords related to mental illness, the population in southeastern China (Guangdong Province, Shandong Province, Jiangsu Province, Hebei Province, Zhejiang Province, Henan Province) and Sichuan Province had the highest search values for mental illness. While search values in northwestern China (Tibet, Ningxia Hui autonomous region, and Qinghai Province) as well as in Hongkong and Taiwan were the lowest. Besides, followed by central, northeast, south, north, and west China ranked second to sixth regarding search index for mental health. Similar results were also found in the regional distribution of confirmed cases. The public in southeastern China showed the highest search behaviors (Figure 5).

Discussion

Our study contributes to the existing literature by highlighting the importance of early psychological surveillance during pandemics. It underscores the need for a proactive approach to mental health support, particularly during the initial stages of a health crisis when public anxiety is at its peak. Based on the hypothesis that Internet query data can reflect the epidemics of psychological stress during the outbreak, data from Baidu search engines could help the nation to face the challenges of public mental illness with more timely and effective ways. By

Figure 4. Age distribution of the searches for 12 topics regarding mental illness from 8 December 2019 to 9 March 2020.



identifying the early psychological repercussions of COVID-19 through Baidu Index data, our study aims to inform public health strategies that address not only the physical health threats but also the mental health challenges posed by such pandemics, providing valuable insights for future infectious disease outbreaks as well.

Our results suggest that public Baidu index search values for mental illness were significantly positively correlated to the number of confirmed cases in China and slightly negatively correlated to the Baidu index search values for COVID-19. Internet searches for topics of mental illness increased significantly after the quarantine measures were implemented. For most Internet searches of mental disorders, there is no lag pattern of the relationship between the number of COVID-19 cases and internet search for most kinds of mental illnesses. We also observed that women searched more about mental health-related information via Baidu than men, and people over 50 years old had the least search behaviors. Besides, people in southeastern China searched for information related to mental illness via Baidu more than in other places.

The results suggest that when real-time information is missing (e.g., routinely face-to-face psychological counseling process cannot proceed), search engine data can serve as a useful method for investigating the epidemiological characteristics of psychological stress. Each topic related to mental illness presented positive correlations for the Baidu index with the number of confirmed cases in China, which shows that with the confirmed cases soaring, public interest to mental problems is increasing as well. At the same time, the Baidu index for COVID-19 was positively correlated to the daily growth of COVID-19. An explanation could be that with the robust surveillance and quarantine measures taken by the Chinese government and the joint efforts of all walks of life, the COVID-19 epidemic in China have been gradually controlled. Therefore, with the daily increase of cases decreasing, the public's concern about this novel virus declined as well. Instead, the searches

Table 2. Dynamic series analysis of the overall search index values for the topic regarding mental illness in China

Date	Search index values	Absolute increment		Development rate(%)		Increment rate(%)	
		Cumulative	Day on day	Fixed base ratio	Link relative ratio	Fixed base ratio	Link relative ratio
24-Jan	54202	_	_	100.0	100.0	_	_
25-Jan	59693	5491	5491	110.1	110.1	10.1	10.1
26-Jan	61899	7697	2206	114.2	103.7	14.2	3.7
27-Jan	68379	14177	6480	126.2	110.5	26.2	10.5
28-Jan	71648	17446	3269	132.2	104.8	32.2	4.8
29-Jan	74948	20746	3300	138.3	104.6	38.3	4.6
30-Jan	78297	24095	3349	144.5	104.5	44.5	4.5
31-Jan	78901	24699	604	145.6	100.8	45.6	0.8
1-Feb	87395	33193	8494	161.2	110.8	61.2	10.8
2-Feb	84621	30419	-2774	156.1	96.8	56.1	-3.2
3-Feb	85957	31755	1336	158.6	101.6	58.6	1.6
4-Feb	87246	33044	1289	161.0	101.5	61.0	1.5
5-Feb	88745	34543	1499	163.7	101.7	63.7	1.7
6-Feb	88274	34072	-471	162.9	99.5	62.9	-0.5
7-Feb	87706	33504	-568	161.8	99.4	61.8	-0.6
8-Feb	85408	31206	-2298	157.6	97.4	57.6	-2.6
9-Feb	88530	34328	3122	163.3	103.7	63.3	3.7

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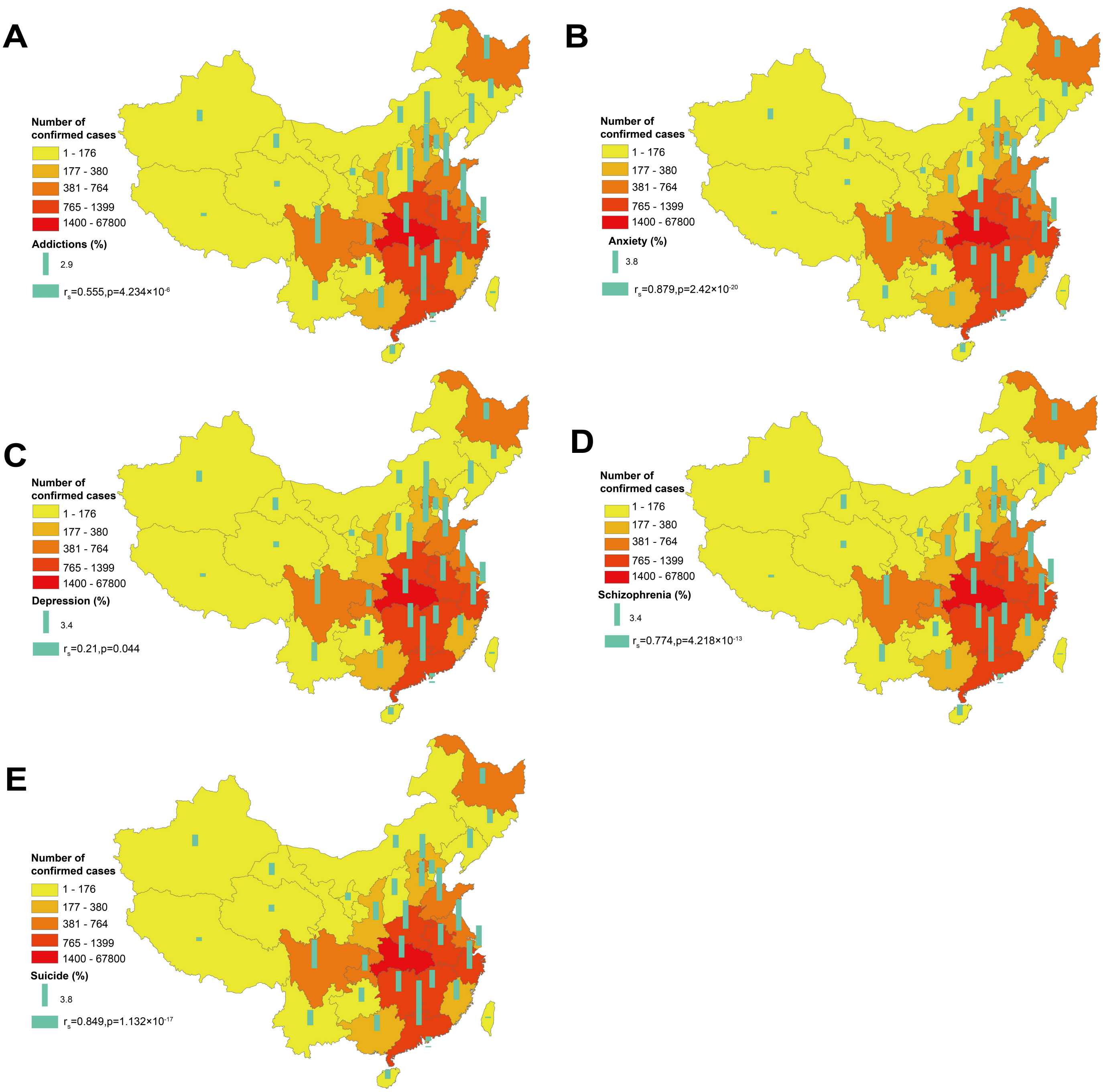
10-Feb	90371	36169	1841	166.7	102.1	66.7	2.1
11-Feb	92003	37801	1632	169.7	101.8	69.7	1.8
12-Feb	94788	40586	2785	174.9	103.0	74.9	3.0
13-Feb	97757	43555	2969	180.4	103.1	80.4	3.1
14-Feb	101543	47341	3786	187.3	103.9	87.3	3.9
15-Feb	101580	47378	37	187.4	100.0	87.4	0.0
16-Feb	100170	45968	-1410	184.8	98.6	84.8	-1.4
17-Feb	98902	44700	-1268	182.5	98.7	82.5	-1.3
18-Feb	98363	44161	-539	181.5	99.5	81.5	-0.5
19-Feb	100615	46413	2252	185.6	102.3	85.6	2.3
20-Feb	105560	51358	4945	194.8	104.9	94.8	4.9
21-Feb	105632	51430	72	194.9	100.1	94.9	0.1
22-Feb	108496	54294	2864	200.2	102.7	100.2	2.7
23-Feb	110842	56640	2346	204.5	102.2	104.5	2.2
24-Feb	110075	55873	-767	203.1	99.3	103.1	-0.7
25-Feb	109541	55339	-534	202.1	99.5	102.1	-0.5
26-Feb	117519	63317	7978	216.8	107.3	116.8	7.3
27-Feb	114798	60596	-2721	211.8	97.7	111.8	-2.3
28-Feb	113430	59228	-1368	209.3	98.8	109.3	-1.2
29-Feb	114608	60406	1178	211.4	101.0	111.4	1.0
1-Mar	110069	55867	-4539	203.1	96.0	103.1	-4.0
2-Mar	116200	61998	6131	214.4	105.6	114.4	5.6
3-Mar	109532	55330	-6668	202.1	94.3	102.1	-5.7
4-Mar	109584	55382	52	202.2	100.0	102.2	0.0
5-Mar	106313	52111	-3271	196.1	97.0	96.1	-3.0
6-Mar	106640	52438	327	196.7	100.3	96.7	0.3
7-Mar	105794	51592	-846	195.2	99.2	95.2	-0.8
8-Mar	112299	58097	6505	207.2	106.1	107.2	6.1
9-Mar	123929	69727	11630	228.6	110.4	128.6	10.4

for COVID-19 was negatively correlated to searches for most of the mental health-related disorders, which suggests the public's interest in mental problems is increasing even though the concern to COVID-19 is decreasing. Especially for "Depression", the Baidu index for this topic showed the strongest negative correlation with the Baidu index for COVID-19. In addition, since the gradual control of the epidemic, public interest to COVID declined, and people's fearless declined as well. Besides, for most search topics of mental illness, there was no lag time between the number of cases and the Baidu searches data of mental health-related disorders, which imply that the impact of such heal-emergencies on the public's mental health is immediate. Thus, when reporting the epidemic situation of COVID-19, the government must take into account the impact of these data on public mental health so as to make appropriate guidance and intervention to protect the public's mental health.

Adhered to previous studies, when suffering from health emergencies, fear and anxiety appeared quickly and did not last for too long, while depression lasts for a long time [18, 19]. According to several prior studies about the mental-related symptoms after the outbreak of SARS in 2003, 17.3% of the workers

Figure 5. Graph map and correlation for the top five most commonly searched topics regarding mental health and the number of confirmed cases in China (A-E, the height of the straight bar in various regions represents the search values of various mental disorders, the higher the bar, the higher the values).



served in the health-care system showed mental symptoms after the epidemic of SASR was controlled, and there were still 15.4% of them presented with mental symptoms one year after [20]. Thus, the mental disorders caused by heal-emergencies on the public tend to be a long-term and continuous process. As a result, although people's interest in COVID-19 declined, the searches for mental-illness information on the Internet increased instead. Such research may also be an explanation for results that search trends for topics regarding mental illness kept at a higher level compared with the earlier observing time [20, 21]. To control the further spread of COVID-19, Wuhan had suspended public transportation since 23 January 2020, indefinitely, and pronounced that citizens should not leave Wuhan without special reasons [22].

On the one hand, such quarantine measures could result in several outcomes, including the absence of interpersonal communication and the usual psychological counseling process [23]. On the other hand, with quarantine, people were required to stay at home. Consequently, they had more time to surf the Internet. Because of the increase of people's online activities, they were more vulnerable to the impact of the news about the COVID-19 epidemic on the Internet, which would also lead to an increase in psychological pressure. This may explain why the increasing turning point started around 24 January 2020, with the average increase rate of overall search values for mental illness as 78.95% since then.

Interestingly, public search behaviors for addictions presented with the fastest increase speed. Moreover, the lag pattern of the relationship between Baidu searches for "addiction", and the number of confirmed cases indicated that such addictive behaviors did not appear immediately. An explanation could be that when facing with the negative emotions caused by COVID-19 and those compulsory isolation measures, people tend to adopt some habitual ways to deal with it, which could result in the increase of various addictive behaviors, including smoking, drinking, and gambling, and it would take several days before people realize that they were addicted.

In terms of gender differences, some reasons may contribute to the phenomenon that women searched more than men. On the one hand, the utilizes of smartphones in women is higher among men, and when facing the crush of psychological stress and mental disorders, they were more likely to look for mental-related information as a form of help[24, 25]. On the other hand, even though under high mental pressure, men were not willing to seek help because of the idea of "collective face-saving," which was linked to the characteristics of masculinity, confidence, and robustness in East Asian culture [24]. Thus, a man would be embarrassed if his soft side was presented. Thus, even though the female is more prone to suffering from psychological stress, additional attention should be paid to the male group to help them to face such challenges.

There are more than 177 million order adults beyond 60 years in China consisted of the largest aging population in the world, and a large number of them are suffering from disabilities, mental health problems, and loneliness [26]. The remarkable transmission speed and high death rate of COVID-19 would aggravate the possibilities of getting mental problems, harsh their existing symptoms, and even damage to their cognitive function. Since the elders have limited access to Internet-based information for help, the mental health-protection initiatives should care more about this age group to provide them with high-quality and timely psychological services. We also found that the distribution of online search behaviors was different across regions. The overall regional distribution of online searches for mental illness was consistent with the regional distribution of confirmed cases in China, with the highest search behavior that appeared in southeastern China. Besides, the spatial heterogeneity, demographical, economical, and educational medical disparities may also contribute to the regional differences of online search behaviors for mental illness.

Interestingly, similar results were also found in a study of public online search behaviors of cancer in China by Xu et al [27]. They observed that people in places with dense population and developed economic (e.g., cities in eastern China) had more access to the Internet and higher awareness of health-related information Compared with people in cities with sparsely population and developing economies, they were more likely to look for health-related information on the Internet. Thus, local authorities need to put in place robust measures to ensure that people nationwide have enough access to Web-based information, especially for places with an underdeveloped economy.

Given the difficulty of retrieving public mental-related data because of the quarantine and isolation strategies during the outbreak of COVID-19, the characteristics of public mental problems and psychiatric morbidity remained unavailable. Establishing timely and efficient mental illness- control methods has become an essential part of the overall victory against the epidemic of COVID-19. As an Internet search toll with the largest consumers in China, gathering data of public real-time search behaviors from Baidu engines can be the source of information that supports mental illness prevention and control. People's attention to topics related to mental illness, as well as the population characteristics, can help the authorities to take prevention and intervention measures in a more targeted way, so as to lower the loss of public mental health caused by COVID-19. Besides, the epidemic of COVID-19 in China has been initially controlled due to the series of strong measures (e.g., quarantine, isolation, and social distancing) taken by the government). Thus, other countries where the epidemic is beginning to spread are encouraged to put in place similar strategies to control the transmission of the epidemic. Therefore, the characteristics of public psychological stress and mental disorders in those countries may also be similar to the characteristics in China. Our results can also help the authorities in those countries to make timely and effective measures to prevent and control the mental catastrophe.

As the first of exploring the psychological characteristics of the public during the outbreak of COVID-19 in China using the Baidu Index, some limitations need to bear in mind. For instance, we only applied the Baidu engine to retrieve public search behavior data, so the data that people who were suffering from mental disorders and did not seek help from Baidu is missing. Besides, only people with access to the Internet can perform those searches. So, it is insufficient to extrapolate our conclusion to the whole population. Finally, this study focused on the early phase of the COVID-19 pandemic (January–March 2020), and thus does not capture potential long-term psychological adaptations or clinically diagnosed mental health outcomes. Future research should extend the observation period to evaluate whether public sentiment stabilized or required sustained intervention.

Conclusions

Our study has demonstrated that the early stages of the COVID-19 pandemic were associated with a significant increase in online searches related to mental health among the Chinese population. This correlation underscores the immediate impact of the pandemic on mental well-being and the need for targeted interventions. Looking ahead, our findings suggest that monitoring online search trends could serve as an effective tool for detecting psychological distress during future pandemics, aiding in the development of timely and responsive public health strategies to address mental health needs. This approach could provide valuable insights into the psychological implications of major infectious disease outbreaks and inform preparedness plans to mitigate their mental health impacts. While our findings highlight the acute psychological distress during the initial outbreak, further research is needed to determine whether these effects were transient or required sustained intervention. Monitoring search trends can serve as an early warning system, but policy responses should be calibrated to avoid unnecessary panic.

Abbreviations

Severe Acute Respiratory Syndrome Coronavirus 2: SARS-CoV-2;

Author Contributions

Nana Meng: Conceptualization, Methodology, Data curation, Writing - Original Draft, Writing - Review & Editing. Yuan Chen: Data collection, Formal analysis, Validation, Writing - Review & Editing. Danna Zhao: Supervision, Writing - Review & Editing. Dingtao Hu: Supervision, Project administration, Writing - Review & Editing. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

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Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

Not Applicable.

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