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Homocysteine and risk of interstitial lung disease: A mendelian randomization approach to causal inference

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Abstract. *Background and aim:* Homocysteine (Hcy) has been implicated in inflammatory, oxidative stress (OS), and endoplasmic reticulum (ER) stress mechanisms, which are hypothesized to contribute to the pathogenesis of interstitial lung disease (ILD). Given the paucity of evidence regarding Hcy's role in ILD, a two-sample Mendelian randomization study was performed to investigate the causal association between Hcy and ILD. *Methods:* We sourced data for total plasma Hcy from genome-wide association studies (GWAS) involving 44,147 European individuals. Data for ILD, idiopathic pulmonary fibrosis (IPF), IPF-related respiratory insufficiency, and systemic autoimmune disease-associated IPF were derived from the FinnGen consortium. To evaluate the causal association of reduced total plasma Hcy with ILD and related conditions, a range of Mendelian randomization (MR) analytical techniques were utilized to analyze the data. The results are reported as odds ratios (ORs) with corresponding 95% confidence intervals (CIs). We conducted sensitivity analyses through leave-one-out procedures and Radial MR plots. *Results:* Our IVW estimates suggested that total plasma Hcy had a potential causal association with IPF (OR=0.649, 95%CI: 0.495-0.851), indicating that along with total plasma Hcy depressed 1 μmol/L, odds of IPF decreased 0.351. Although it seemed that decreased total plasma Hcy level is associated with lower odds of IPF-related respiratory insufficiency (OR=0.672, 95%CI: 0.489-0.924), due to the existence of horizontal pleiotropy, this causal association was not robust. In addition, MR leave-one-out and Radial MR sensitivity analyses showed there is no outlier among the selected IVs that could affect the potential causal relationship between Hcy and IPF. *Conclusions:* The levels of total plasma Hcy may bear a significant association with the risk of developing IPF, a specific form of ILD. However, additional well-controlled prospective studies are indispensable to definitively establish a causal relationship between Hcy and the pathogenesis of ILD.

Key words: homocysteine, interstitial lung disease, idiopathic pulmonary fibrosis, Mendelian randomization, causal association

Introduction

Interstitial lung disease (ILD) represents a constellation of intricate and heterogeneous disorders of the lung parenchyma, whose central pathogenic mechanism hinges on aberrant repair processes following alveolar epithelial cell injury, leading to inflammation and fibrosis (1). This process is especially pronounced in conditions such as systemic sclerosis, wherein immune-mediated injury takes center stage, driving the upregulation of cytokines such as IL-1β, TNF-α, and TGF-β. These inflammatory mediators not only escalate the inflammatory cascade but also stimulate fibroblast activation, culminating in excessive collagen deposition and scarring of the lung tissue (2). Characterized predominantly by progressive dyspnea and impairment of physical capabilities, ILDs significantly compromise patients' quality of

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life, with idiopathic pulmonary fibrosis (IPF) being the most prevalent and severe subtype. Compared to other ILD categories, IPF carries a grimmer prognosis (3, 4). As our understanding of the pathophysiology of ILDs deepens, encompassing the challenges of gas exchange disruption and decline in lung function, novel therapeutic strategies are emerging, heralding new hope for afflicted individuals (5). The onset and progression of interstitial lung disease (ILD) are multifaceted, encompassing a plethora of pathogenic mechanisms, prominently featuring oxidative stress (OS) and endoplasmic reticulum (ER) stress (1, 6). Homocysteine (Hcy), an intermediary metabolite of the indispensable amino acid methionine, has garnered attention for its potential to instigate inflammation, oxidative stress, and ER stress, thereby contributing to a milieu conducive to ILD pathology (7, 8). Preclinical models have substantiated this notion, demonstrating that Hcy can exacerbate ER stress, culminating in the induction of apoptosis in lung epithelial cells (9). Empirical evidence gleaned from recent case-control studies has posited a compelling association between elevated Hcy levels and an augmented risk of ILD in dermatomyositis and systemic sclerosis patients (10, 11). This correlation hints at a mechanistic role of Hcy in the pathogenesis of ILD. Nonetheless, the extant literature on Hcy's involvement in ILD remains scant, and traditional epidemiological investigations are inherently vulnerable to confounding biases and reverse causality, casting uncertainty over the purported causal link between Hcy and ILD. Mendelian randomization (MR), utilizing single nucleotide polymorphisms (SNPs) as unbiased instrumental variables, has established itself as a robust technique for investigating potential causal links between environmental exposures and disease outcomes (12, 13). Recent studies have further elucidated the gut-lung axis, particularly the causal relationships between inflammatory bowel disease (IBD) and interstitial lung disease (ILD). Zhang et al. and Luo et al. conducted Mendelian randomization studies, finding significant causal associations between IBD and ILD, suggesting that genetic predispositions to IBD may also influence the risk of developing ILD (14, 15). Furthermore, Zhao et al. investigated the causal link between serum metabolites and ILD, indicating that specific serum metabolites might be involved in the onset of ILD (16). Despite its growing application, the causal link between Hcy levels and the risk of ILD has not been explored using this approach—until now. In this research, we utilize Mendelian randomization (MR) to explore the previously unstudied causal relationship between Hcy and ILD. By adopting this innovative approach, we aim to contribute to the preventive strategies and potential therapeutic targets for ILD, thereby advancing our understanding of this disease's pathophysiology.

Methods

Data sources

As depicted in Figure 1, this is a two-sample Mendelian randomization analysis, where total plasma Hcy concentration data were derived from genome-wide association studies (GWASs) involving 44,147 individuals of European descent, with units expressed in micromoles per liter (μmol/L) (17). The data on ILD and associated conditions, including IPF,

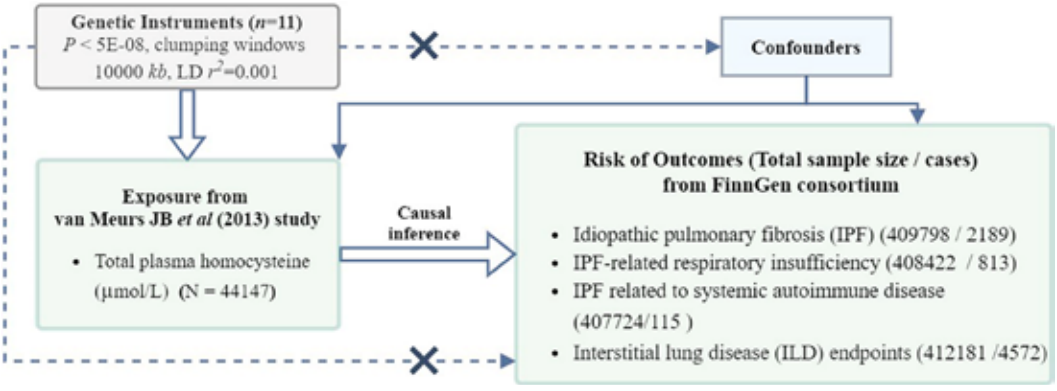

Figure 1. The directed acyclic graph of Hcy and ILD

Table 1. The information of lipid metabolism biomarkers and Outcomes

Phenotype	Total sample size (cases)	GWAS ID	PMID
Exposure			
Total plasma Hcy	44147	GCST002087	23824729
Outcomes			
ILD	412181 (4572)	R10_ILD_ENDPOINTS	-
IPF-related respiratory insufficiency	408422 (813)	R10_ILD_INSUFFICIENCY_ILD	-
IPF-related systemic autoimmune disease	407724 (115)	R10_ILD_SYST_AUTO_ILD	-
IPF	409798 (2189)	R10_IPF	-

Abbreviations: Hcy: homocysteine, ILD: interstitial lung disease, IPF: idiopathic pulmonary fibrosis.

IPF-related respiratory insufficiency and systemic autoimmune disease-related IPF, were extracted from the FinnGen consortium of its recent release the data of version 10 (https://www.finngen.fi/en/access_results). More details on the data source were presented in the Table 1. Each participant (or their legal guardian for those under 18 years old) provided informed consent for their data to be used in the GWAS studies, which had been approved by the relevant ethics committees. Given that the databases were publicly available, our institutional review board determined that additional ethical approval was not required.

Single Nucleotide Polymorphisms (SNPs) selection

SNPs significantly associated with Hcy were selected as potential IVs, with a selecting threshold of $P<5.0\times10^{-8}$. We removed SNPs with linkage disequilibrium (LD), with a threshold of $r^2=0.001$ and a clumping distance of 10,000 kb. We excluded palindromic SNPs to ensure that each allele is associated with a unique effect on the exposure and outcome, as required by the MR principle. We used MR-Egger regression to detect potential horizontal pleiotropy, which would violate the second assumption of MR. A significant intercept in the MR-Egger regression indicates the presence of pleiotropy.

The assumptions of MR analysis

The MR analysis is based on three key assumptions to minimize bias. Firstly, the IVs should be independent of confounders related to both the exposure and outcome. Secondly, the IVs should be significantly associated with the exposure (18, 19):

$$R^2 = \sum_{i=1}^{k} \frac{2\beta_i^2 MAF_{SNPi}(1-MAF_{SNPi})}{SD^2}$$

$$F = \frac{R^2(n-1-k)}{(1-R^2)k}$$

MAF represents the minor allele frequency, β denotes the effect size of the SNP on the exposure, SD is the standard deviation, k is the number of instrumental variables, and n is the sample size related to the exposure. An F-statistic less than 10 (20) indicates a weak relationship between the instrumental variables and the exposure. Additionally, the instrumental variables should affect the outcome solely through their impact on the exposure, meaning there should be no direct effect (horizontal pleiotropy) of the instrumental variables on the outcome.

Statistical analysis

The calculation of the causal effect values between a decrease in total plasma Hcy concentration and ILD and related diseases employed the inverse variance weighted (IVW) test. This is the principal method for obtaining unbiased estimates in the absence of horizontal pleiotropy. The magnitude of the effect was quantified using odds ratios (ORs) along with their 95% confidence intervals (CIs). A p-value less than 0.05 indicated the statistical significance of the evidence for a potential causal effect. We also used the weighted-median, weighted mode, MR-Egger, Radial MR, MR-Pleiotropy RESidual Sum and Outlier (MR-PRESSO), MR-Robust Adjusted Profile Score (RAPS), and Constrained Maximum

Likelihood and Model Averaging (cML-MA) methods to evaluate the causal relationship between Hcy and ILD. Specifically, the weighted-median method provides a robust and consistent estimate of the effect, even if up to 50% of the genetic variants are invalid instruments (20). The MR-Egger method uses a weighted linear regression; however, its estimates typically exhibit low precision and can be influenced by outlying genetic variants (21). The weighted mode, also known as the mode-based estimate (MBE), is designed to derive a single causal effect estimate from multiple genetic instruments (22). The radial plot helps identify a single outlying variant that could account for substantial discrepancies between the IVW and MR-Egger regression estimates (23). The MR-PRESSO analysis detects and aims to mitigate horizontal pleiotropy by excluding significant outliers (24). The MR-RAPS method provides an opportunity to investigate issues of weak instrument bias and improves efficiency by incorporating many weak instrumental variables. The cML-MA approach is robust against violations of both assumption 2 (the IV is not associated with the outcome conditional on the exposure) and assumption 3 (the IV is not associated with unmeasured confounders), while effectively controlling type I errors with high power (25). Heterogeneity was assessed using Cochran's Q test, and IVs with a p-value less than 0.05 were considered heterogeneous. Furthermore, a sensitivity analysis was conducted using the MR leave-one-out test. All statistical analyses were performed using R version 4.2.0 (Institute for Statistics and Mathematics, Vienna, Austria). The "TwoSampleMR" R package was utilized for the Mendelian Randomization (MR) analysis of the causal relationship between Hcy and ILD.

Results

Instrumental variables selection and test

Table 2 outlines the process of IV screening. Initially, 18 SNPs were extracted as potential IVs associated with total plasma Hcy levels. After excluding SNPs in LD and palindromic SNPs, 11 SNPs remained eligible, with an F-value of 96.16. Subsequently, horizontal pleiotropy and heterogeneity tests were conducted on the selected IVs (Table 3). The MR-Egger test indicated horizontal pleiotropy between the selected IVs and IPF-related respiratory insufficiency (P = 0.039), suggesting that the exposure may influence the outcome through other pathways. Additionally, both the MR-Egger regression (P = 0.035) and the IVW Q-test (P = 0.045) detected heterogeneity between the selected IVs and interstitial lung disease (ILD), requiring the application of the IVW random effects model for the causal association analysis between total plasma Hcy and ILD.

Causal association of total plasma Hcy with ILD and related diseases

According to the IVW estimates of the random effect model, total plasma Hcy had a potential causal association with IPF (OR = 0.649, 95% CI: 0.495–0.851), This suggests that a 1 μmol/L decrease in total plasma Hcy corresponds to a 0.351-fold reduction in the risk of developing IPF. Although it seemed that a lower total plasma Hcy level is associated with a lower risk of IPF-related respiratory insufficiency (OR = 0.672, 95% CI: 0.489–0.924), due to the presence of horizontal pleiotropy, this pathway of causal association was not robust (Table 4). Figure 2 shows a forest plot from the exploratory analysis of the causal relationship between Hcy and ILD, using various MR methods. Consistent with the IVW results, the cML-MA (OR=0.646, 95% CI: 0.490-0.852), MR-RAPS (OR=0.644, 95% CI: 0.489-0.848), and weighted median (OR=0.683, 95% CI: 0.487-0.958) methods also suggested a potential causal association between total plasma Hcy and ILD. Although the MR-Egger test suggested a potential causal link between total plasma Hcy and systemic autoimmune disease-related IPF (OR=0.037, 95% CI: 0.003-0.457), the MR-Egger method typically has low precision and can be affected by outlying genetic

Table 2. The IVs screening procedure of homocysteine levels

Exposure	Selected SNP (*P*<5E-08)	Omitted LD SNP	Drop palindromic SNP	*F*-value	R^2(%)
Total plasma homocysteine	18	16	11	96.16	4.1

Abbreviations: SNP: single nucleotide polymorphism, LD: linkage disequilibrium.

Table 3. The pleiotropic and heterogeneity test of IVs of homocysteine levels

Outcomes	SNPs	Horizontal pleiotropy		Heterogeneity			
		Egger Intercept	*P*	MR Egger Q	*P*	IVW Q	*P*
IPF	11	-0.01	0.750	16.11	0.065	16.30	0.091
IPF-related respiratory insufficiency	11	0.23	**0.039**	9.83	0.364	16.22	0.094
IPF-related systemic autoimmune disease	11	0.01	0.869	5.12	0.824	5.15	0.881
ILD	11	-0.01	0.584	17.99	**0.035**	18.63	**0.045**

Abbreviations: SNP: single nucleotide polymorphism, MR: Mendelian randomization, IVW: inverse variance weighted test, ILD: interstitial lung disease, IPF: idiopathic pulmonary fibrosis.

Table 4. Causal association of Hcy with ILD

Variables	Method	SNPs	OR (95% CI)	*P*
IPF	IVW (Fixed effect)	11	0.649 (0.495-0.851)	**0.002**
	IVW (Random effect)	11	0.649 (0.459-0.918)	**0.015**
IPF-related systemic autoimmune disease	IVW (Fixed effect)	11	0.559 (0.175-1.790)	0.328
	IVW (Random effect)	11	0.559 (0.127-2.460)	0.442
IPF-related respiratory insufficiency	IVW (Fixed effect)	11	0.672 (0.432-1.047)	0.079
	IVW (Random effect)	11	0.672 (0.489-0.924)	**0.014**
ILD	IVW (Fixed effect)	11	0.830 (0.688-1.002)	0.053
	IVW (Random effect)	11	0.830 (0.642-1.074)	0.156

Abbreviations: Hcy: homocysteine, ILD: interstitial lung disease, IPF: idiopathic pulmonary fibrosis, SNP: single nucleotide polymorphism, OR: odds ratio, CI: confidence interval, IVW: inverse variance weighted

variants. No significant relationship was observed between total plasma Hcy and ILD. Furthermore, the association between Hcy and IPF explored via different MR methods showed a similar trend (Figure 3A), and the effect of most selected SNPs on IPF was concentrated to the left of "0" (Figure 3B), indicating that the odds of IPF decreased with decreasing total plasma Hcy concentrations.

Sensitivity analyses

Additionally, sensitivity analyses using the MR leave-one-out and Radial MR methods were performed to evaluate the robustness of the potential causal relationship between total plasma Hcy and IPF. As depicted in Figure 4, no outliers were identified among the 11 selected IVs. Additionally, the Radial plot (Figure 5) revealed no single variant that significantly contributed to the discrepancies between IVW and MR-Egger regression estimates. These findings further supported the robustness of the potential causal relationship between total plasma Hcy and IPF.

Discussion

The results in this study further substantiated the robustness of the potential causal relationship between total plasma Hcy and IPF. Based on large-scale summary statistics from GWAS, we found a potential causal link between decreased total plasma Hcy levels and a reduced likelihood of IPF in the European population. However, the role of Hcy in ILD is still unclear and requires further investigation. There is growing evidence that higher plasma Hcy levels are linked to an increased risk of central and peripheral vascular disorders, including carotid, coronary, and peripheral arterial diseases (26). Although the role of Hcy in ILD remains unclear, previous studies have reported the associations between

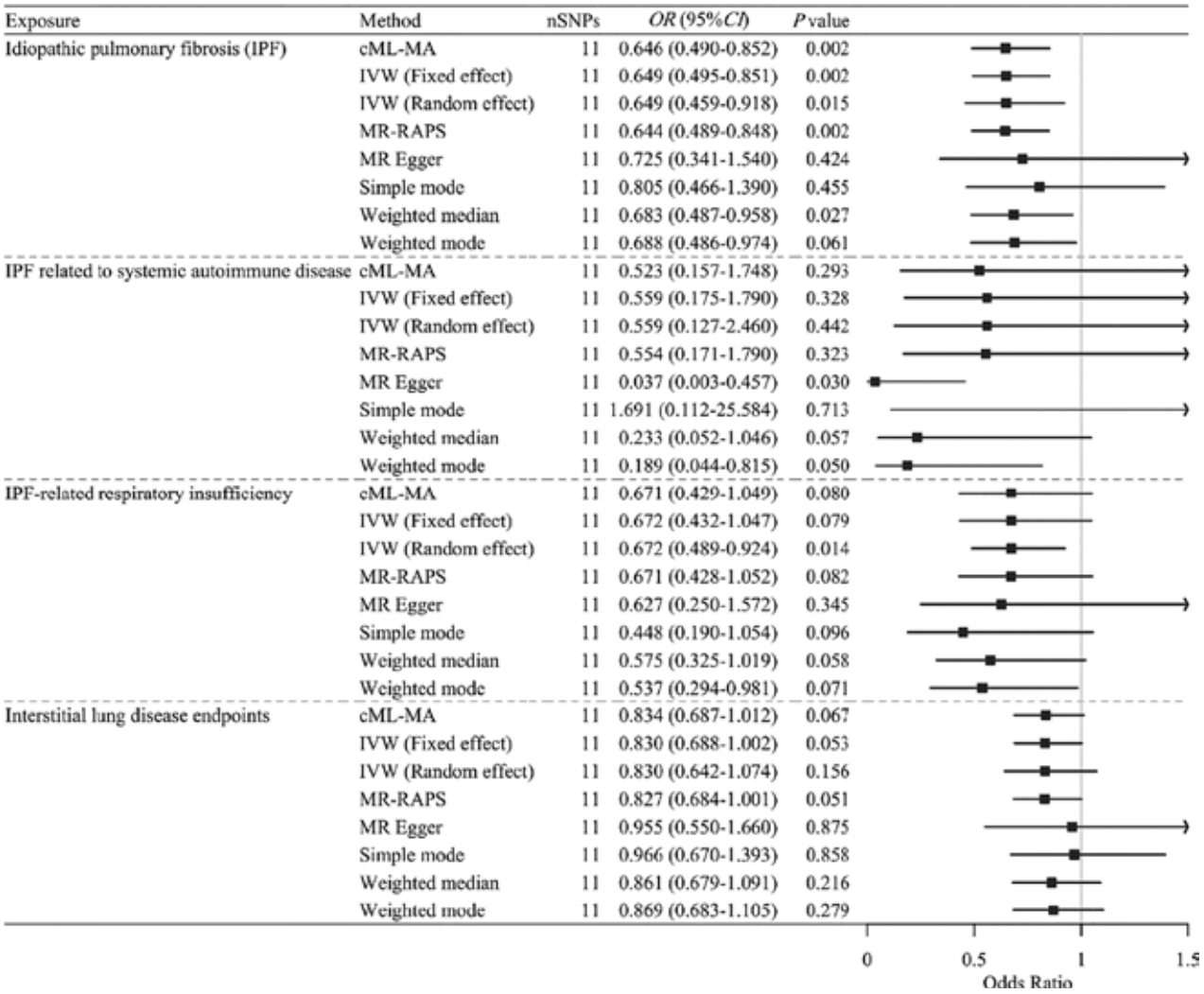

Figure 2. Potential causal associations of plasma total Hcy level with IPF, IPF-related systemic autoimmune disease, IPF-related respiratory insufficiency, and ILD.

Hcy levels and different pulmonary diseases, which may provide a basis for inferring a causal association between Hcy and ILD. Sekiguchi et al. (11) noted that plasma Hcy levels were significantly higher in dermatomyositis patients compared to healthy controls. Furthermore, they found that the prevalence of ILD was substantially higher among dermatomyositis patients with elevated plasma Hcy levels. A meta-analysis conducted by Zinellu et al. (27) showed that serum homocysteine concentrations were significantly higher in patients with chronic obstructive pulmonary disease (COPD). However, a recent MR study showed no significant association between plasma Hcy and COPD (28). Similarly, this study, also based on the MR method, observed that a 1 μmol/L decrease in total plasma Hcy was associated with a 0.649-fold decrease in the odds of IPF. Comparing to previous studies, the present research, which utilized genetic variation to control for unmeasured confounders and reverse causality, provides a more robust investigation of the relationship between Hcy and IPF. Notably, multiple factors can

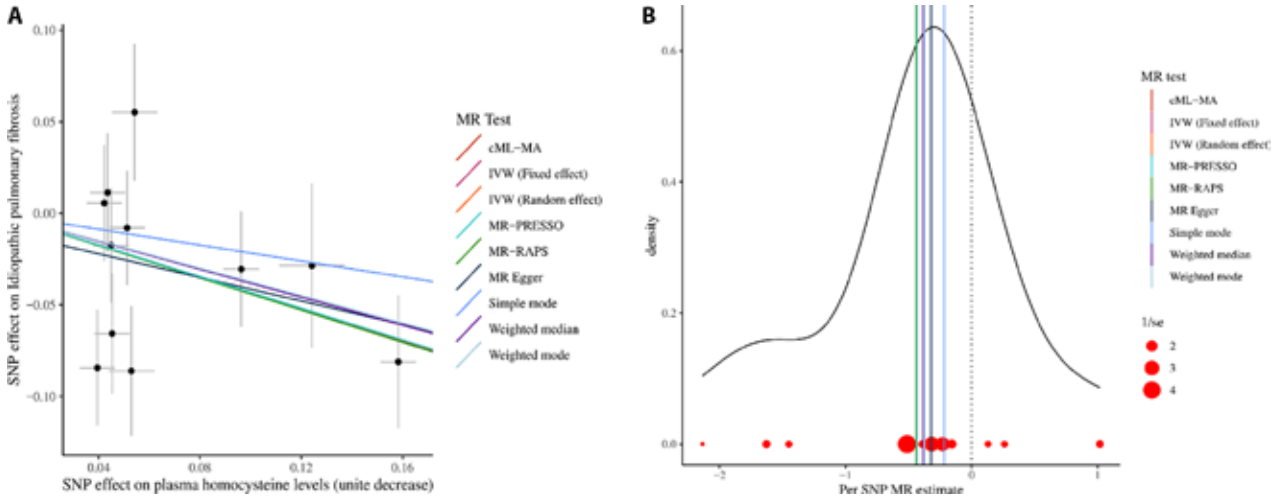

Figure 3. Scatter diagrams of association between Hcy and IPF. (A) different MR methods to assess the potential causal association between Hcy and IPF; (B) Estimate of each selected SNP. A larger circle represents a smaller standard error. Clustering of circles suggests that the results are homogeneous.

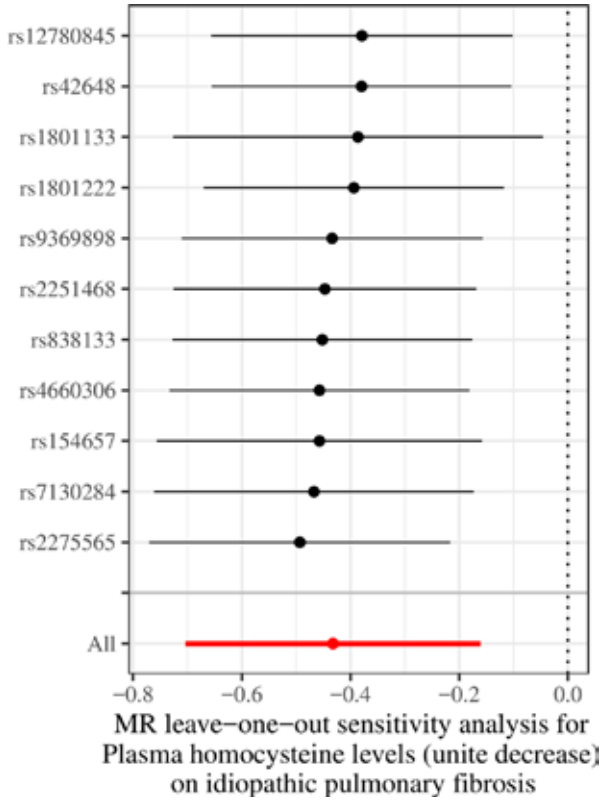

Figure 4. Leave-one-out sensitivity analysis on potential causal association between total plasma Hcy and IPF.

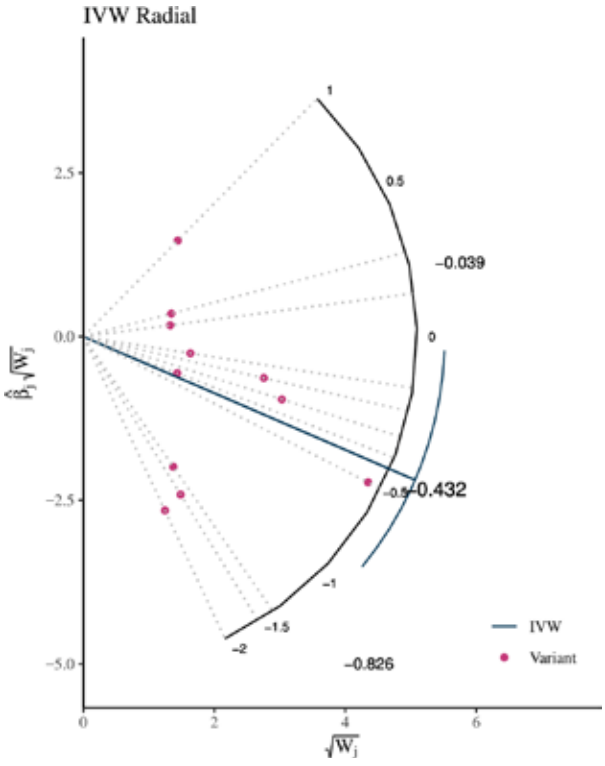

Figure 5. MR-Radial outlier test for potential causal association between total plasma Hcy and IPF.

modulate Hcy levels, such as age and/or racial differences in disease susceptibility, which may influence the association between Hcy and IPF. Consequently, due to the lack of observational studies investigating the effect of Hcy levels on ILD risk, the true impact of Hcy on ILD onset remains unclear and requires further clarification.

The development of IPF involves a complex interaction of multiple cell types and signaling pathways. Specifically, predisposing factors such as aging, environmental, epigenetic, and immunologic factors may contribute to the occurrence of recurrent alveolar epithelial cell injury, leading to cellular senescence, metabolic dysfunction, aberrant epithelial cell activation, and dysregulated epithelial repair (29). Then, the dysregulated epithelial cell interacts with immune, mesenchymal, and endothelial cells through multiple signaling mechanisms to trigger fibroblast and myofibroblast activation. Herein, given this intricate process, it is plausible that Hcy may have a complex and diverse causal association with IPF. Lu et al. (30) found that adenosine and Hcy cause lung vascular endothelial cell apoptosis by inhibiting the carboxyl methylation of the small GTPase, Ras, through the suppression of isoprenylcysteine carboxyl methyltransferase activity. This inhibition leads to the inactivation of Ras and the subsequent disruption of focal adhesion complexes, resulting in cell-extracellular matrix detachment and anoikis. Furthermore, Hcy has been shown to disrupt vascular homeostasis by inhibiting NO synthesis, inducing endothelial dysfunction, and stimulating pro-inflammatory and pro-oxidative pathways in the vascular wall and systemically (31, 32). Therefore, our findings suggest that depressed plasma total Hcy may relieve inflammatory response, oxidative stress and apoptosis of cells associated with fibrosis in the lung interstitium, and further prevents the development of IPF during acute lung injury. Furthermore, Hcy is involved in one-carbon metabolism, which is essential for the production of S-adenosylmethionine, a universal methyl-donor that delivers methyl groups for epigenetic methylations (33). The progression of IPF is marked by the extensive proliferation of macrophages and fibroblasts, which raises the body's need for folate (34). Herein, as a link of one-carbon metabolism, Hcy may be involved in epigenetic regulation of IPF. Nevertheless, due to the complex mechanisms of epigenetics, high levels of homocysteine can alter DNA methylation patterns, potentially contributing to the development and progression of interstitial lung disease through epigenetic mechanisms. For example, Wu et al., highlighted the role of epigenetics in hyperhomocysteinemia (HHcy) and its link to diseases like ILD (35). Wang et al. showed that MBD2 drives pulmonary fibrosis through fibroblast differentiation and TGF-β1-induced Erdr1 repression, suggesting MBD2 modulation or Erdr1 enhancement as therapeutic strategies involving epigenetic methylation (36). To our knowledge, this is the first study to evaluate the causal relationship between Hcy and ILD, providing a valuable reference for future research into the etiology of ILD. As previously mentioned, MR is a more advantageous study design compared to observational studies for clarifying the causal effect of potential influencing factors on diseases of interest. By identifying a potential causal relationship between plasma total Hcy levels and the risk of IPF, this study could inform public health policies and clinical interventions aimed at reducing the incidence and societal burden of ILD.

Limitations

Notably, this study has several limitations. The study's findings may not be generalizable to non-European populations, and further research is needed to confirm the results in diverse populations. Due to the limitation of database, lacking individual study data is impossible to further explore the linear association between Hcy and ILD change. According to IVW estimates, the causal association of Hcy with ILD was not significant, and because of the existence of heterogeneity, the true relationship between was not clear. Since the limitation of the database, information on factors that may influence exposure or outcome, such as cancers and thrombosis, was not available, that limited us to conduct multivariate MR analysis. In addition, although the number of IVs strongly related to Hcy, and cases in the population with partial outcome (IPF-related respiratory insufficiency, systemic autoimmune disease-related IPF) are both small, the F values of the IVs all exceeded 10, indicating the results were relatively robust.

Conclusion

This MR analysis identified a potential causal association between lower total plasma Hcy levels and

reduced odds of IPF in the European population, which may provide some reference for further understanding of the role of Hcy in IPF. However, prospective studies are still needed to clarify the underlying mechanisms and the broader implications of these findings across different populations in the future.

Conflicts of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article".

Authors' Contribution: SL designed the study, XiH, XH, HL and SM collected the data and did the data analysis. SL validated the data and did literature research. All authors participated in the writing and the editing of the manuscript. All authors approved the final version of manuscript.

Availability of Data and Materials: The datasets generated during and/or analyzed during the current study are available in the FinnGen consortium: https://www.finngen.fi/en/access_results.

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