

STROBE-MR checklist of recommended items to address in reports of Mendelian randomization studies^{1 2}

Item No.	Section	Checklist item	Page No.	Relevant text from manuscript
1	TITLE and ABSTRACT	Indicate Mendelian randomization (MR) as the study's design in the title and/or the abstract if that is a main purpose of the study	1	"Causal relationship between circulating glutamine levels and idiopathic pulmonary fibrosis: A two-sample Mendelian randomization study"
INTRODUCTION				
2	Background	Explain the scientific background and rationale for the reported study. What is the exposure? Is a potential causal relationship between exposure and outcome plausible? Justify why MR is a helpful method to address the study question	3~5	"In recent years, the relationship between glutamine metabolism and IPF has attracted increasing attention from researchers." "In the pathological process of IPF, abnormal activation and proliferation of fibroblasts are key factors leading to structural destruction and loss of function of lung tissue. Several studies have suggested that glutamine metabolism may play a crucial regulatory role in IPF disease progression. Glutamine is converted to glutamate by glutaminase (GLS1), which subsequently generates α-ketoglutarate (α-KG), a process that not only provides energy to cells but also participates in the regulation of collagen synthesis and cell signaling. In fibroblasts with IPF, increased glutamine catabolism is closely associated with excessive collagen deposition and increased cell proliferation." "By using alleles as a proxy for actual exposure, MR not only helps to minimize the effects of potential confounders and reverse causation, but also provides more reliable causal inferences. Currently, there are no MR analyses investigating the causal relationship between glutamine levels and the risk of developing IPF."
3	Objectives	State specific objectives clearly, including pre-specified causal hypotheses (if any). State that MR is a method that, under specific assumptions, intends to estimate causal effects	4~5	"In this study, we collected the most recent summary level data on genetic loci and glutamine levels in IPF subjects through a genome-wide association study (GWAS) using a two-sample MR study to analyze the causal relationship between glutamine levels and IPF prevalence." "Mendelian randomization (MR), an innovative approach to genetic epidemiology, is unique in that

it simulates experimental designs using genetic variants (typically single nucleotide polymorphisms), as powerful instrumental variables (IVs), providing a reliable way to assess associations between different factors. This approach exploits the properties of independent segregation and random assignment of genes during meiosis to explore the causal relationship between exposure and outcome.”

METHODS

4	Study design and data sources	Present key elements of the study design early in the article. Consider including a table listing sources of data for all phases of the study. For each data source contributing to the analysis, describe the following:		
	a)	Setting: Describe the study design and the underlying population, if possible. Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection, when available.	6	“It is worth noting that the population we included was of European origin, which to some extent avoids the effects of population stratification. Since the GWAS data for exposure and outcome were obtained from different databases, it can be assumed that there is no population overlap.”
	b)	Participants: Give the eligibility criteria, and the sources and methods of selection of participants. Report the sample size, and whether any power or sample size calculations were carried out prior to the main analysis	5~6	“Summary statistics related to glutamine levels were obtained from the GWAS data, which included 114,751 subjects (https://gwas.mrcieu.ac.uk/). For IPF, we selected data from the FinnGen R10 (published on December 18, 2023) GWAS database (r10.finnngen.fi), which included 2,189 IPF patients and 407,609 healthy controls.”
	c)	Describe measurement, quality control and selection of genetic variants	6~7	“With the three assumptions based on MR analysis, we firstly selected SNPs associated with exposure, and the selected SNPs should be significantly associated with the target exposure at the genome-wide significance threshold ($P < 5 \times 10^{-8}$) to fulfill the first hypothesis. Secondly, the sliding window size was set to 10,000kb, with $r^2 < 0.01$, to ensure the independence of the selected genetic variants. To fulfill the third hypothesis, we set P to less than 5×10^{-5} to filter out SNPs significantly associated with outcome. At the same time, we introduced the Steiger test statistic to test whether the correlation between SNPs and outcome was greater than the correlation with exposure, and SNPs that did not pass the Steiger test were removed, somewhat strengthening the third

				hypothesis and avoiding the existence of reverse causality. Next, F-statistics ($BETA^2/SE^2$) for glutamine levels were calculated for each SNP as a measure of instrumental strength, an F-statistic greater than 10 generally suggests that the selected instrumental variable is sufficiently strong, reducing the risk of bias associated with weak instruments, and thus substantiates the predictive relationship of the instrument with the exposure. SNPs with F-statistics below 10 were considered weak instrumental variables and were removed from subsequent analyses. Finally, we also removed palindromic SNPs with mirror symmetry in the genome to improve data quality.”
	d)	For each exposure, outcome, and other relevant variables, describe methods of assessment and diagnostic criteria for diseases	6	“Regarding the assessment methods and diagnostic criteria for glutamine levels and IPF, we have maintained consistency with the original data of the included study databases.”
	e)	Provide details of ethics committee approval and participant informed consent, if relevant	5	“We used data from published studies or publicly available GWAS statistics, so no additional ethical statements or informed consent were required.”
5	Assumptions	Explicitly state the three core IV assumptions for the main analysis (relevance, independence and exclusion restriction) as well assumptions for any additional or sensitivity analysis	5	“MR analysis was based on three main assumptions: (1) the correlation assumption: the IVs must be strongly associated with the exposure; (2) the independence assumption: the IVs are not related to confounders; and (3) the exclusivity assumption: the IVs affect the outcome only through the exposure and cannot be directly correlated with the outcome.”
6	Statistical methods: main analysis	Describe statistical methods and statistics used		
	a)	Describe how quantitative variables were handled in the analyses (i.e., scale, units, model)		Not applicable.
	b)	Describe how genetic variants were handled in the analyses and, if applicable, how their weights were selected	6~7	“With the three assumptions based on MR analysis, we firstly selected SNPs associated with exposure, and the selected SNPs should be significantly associated with the target exposure at the genome-wide significance threshold ($P < 5 \times 10^{-8}$) to fulfill the first hypothesis. Secondly, the sliding window size was set to 10,000kb, with $r^2 < 0.01$, to ensure the independence of the selected genetic

variants. To fulfill the third hypothesis, we set P to less than 5×10^{-5} to filter out SNPs significantly associated with outcome. At the same time, we introduced the Steiger test statistic to test whether the correlation between SNPs and outcome was greater than the correlation with exposure, and SNPs that did not pass the Steiger test were removed, somewhat strengthening the third hypothesis and avoiding the existence of reverse causality. Next, F-statistics ($BETA^2/SE^2$) for glutamine levels were calculated for each SNP as a measure of instrumental strength, an F-statistic greater than 10 generally suggests that the selected instrumental variable is sufficiently strong, reducing the risk of bias associated with weak instruments, and thus substantiates the predictive relationship of the instrument with the exposure. SNPs with F-statistics below 10 were considered weak instrumental variables and were removed from subsequent analyses. Finally, we also removed palindromic SNPs with mirror symmetry in the genome to improve data quality.”

- c) Describe the MR estimator (e.g. two-stage least squares, Wald ratio) and related statistics. Detail the included covariates and, in case of two-sample MR, whether the same covariate set was used for adjustment in the two samples

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“We used eight methods of analysis, including inverse variance weighted (IVW), the Mendelian Randomization-Egger (MR-Egger), the weighted median (WM), the weighted mode methods, Constrained maximum likelihood (cML-MA), the contamination mixture (ConMix), the Robust adjusted profile score (MR-RAPS), the Debiased inverse-variance weighted method (DIVW). Of these, IVW served as the primary method of analysis because it provided the greatest statistical power in the absence of pleiotropy. We first calculated the effect size of each instrumental variable and its standard error, and then combined these effect sizes using inverse variance weighting to estimate the overall causal effect. This approach assumes that all instrumental variables are valid, i.e. that there is no direct association between them and the outcome, except through exposure factors. We then used the remaining seven methods as complementary methods to verify the robustness as well as the accuracy of the IVW results.”

- d) Explain how missing data were addressed

Not applicable.

	e)	If applicable, indicate how multiple testing was addressed		Not applicable.
7	Assessment of assumptions	Describe any methods or prior knowledge used to assess the assumptions or justify their validity	8	"To ensure the rigor and validity of our study, we have verified this research against the checklist provided by the STROBE-MR guidelines (Additional file 1). For detailed information, please refer to the STROBE-MR website (https://www.strobe-mr.org/)."
8	Sensitivity analyses and additional analyses	Describe any sensitivity analyses or additional analyses performed (e.g. comparison of effect estimates from different approaches, independent replication, bias analytic techniques, validation of instruments, simulations)	7~8	"MR-Egger regression analysis was performed in order to assess the robustness of the results of our main analyses. This method aims to detect and correct for possible horizontal multinomiality in instrumental variables. We report the value of the Egger intercept and the corresponding p-value for the presence of multinomiality. In addition, a global test of MR-PRESSO was also performed to assess the presence of horizontal multinomiality in all instrumental variables. Next was an outlier detection to identify unusual instrumental variables that might influence the results. After detecting pleiotropy or outliers, we manually removed the outliers and revalidated the causal effects and compared the results with those of the original IVW analyses. To confirm the presence of heterogeneity, we assessed it using Cochran's Q test, with $P < 0.05$ indicating the presence of heterogeneity. Finally, a leave-one-out analysis was performed to determine whether the removal of individual SNPs significantly affected the results. Moreover, several studies have suggested that smoking and gastroesophageal reflux disease (GERD) may act as risk factors for IPF, and to prevent potential confounders from biasing our results, we similarly assessed the causal relationship between glutamine levels and smoking and GERD."
9	Software and pre-registration			
	a)	Name statistical software and package(s), including version and settings used	5	"Analyses were performed using the TwoSampleMR (version 0.5.10), Mendelian Randomization (version 0.8.0), and MRPRESSO packages in R language (version 4.3.2)."
	b)	State whether the study protocol and details were pre-registered (as well as when		Not applicable.

and where)

RESULTS

10 Descriptive data

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|----|--|-----|---|
| a) | Report the numbers of individuals at each stage of included studies and reasons for exclusion. Consider use of a flow diagram | 8~9 | <p>“In our study, each SNP considered has successfully undergone the Steiger Test (The last two columns in Table 1). This affirms that the association of each SNP with the exposure variable is more pronounced than with the outcome, thus precluding the possibility of reverse causality and reinforcing the exclusivity assumption of our Mendelian randomization methodology. 32 SNPs significantly associated with glutamine levels ($P < 5 \times 10^{-8}$) were finally included as IVs, and all of them had F-statistics greater than 10 (range: 29.207 to 2201.084), as detailed in Table 1.”</p> <p>“In the preliminary analysis, we screened 33 SNPs for analysis based on the conditions described above, and P was less than 0.05 for IVW and the remaining seven methods; however, when sensitivity analysis was performed, the Cochran's Q test to validate the IVW method showed $P = 0.008$, indicating the presence of heterogeneity. Outliers were detected after performing MR-PRESSO analysis (rs142525555).”</p> |
| b) | Report summary statistics for phenotypic exposure(s), outcome(s), and other relevant variables (e.g. means, SDs, proportions) | 5~6 | <p>“Summary statistics related to glutamine levels were obtained from the GWAS data (Richardson TG, Leyden GM, Wang Q, Bell JA, Elsworth B, Davey Smith G, et al. Characterising metabolomic signatures of lipid-modifying therapies through drug target mendelian randomisation. PLoS Biol. 2022 Feb;20(2):e3001547.), which included 114,751 subjects (https://gwas.mrcieu.ac.uk/). For IPF, we selected data from the FinnGen R10 (published on December 18, 2023) GWAS database (r10.finnngen.fi), which included 2,189 IPF patients and 407,609 healthy controls.”</p> |
| c) | If the data sources include meta-analyses of previous studies, provide the assessments of heterogeneity across these studies | | Not applicable. |
| d) | For two-sample MR:
i. Provide justification of the similarity of the genetic variant-exposure associations between the exposure and outcome samples | 6 | i . “we firstly selected SNPs associated with exposure, and the selected SNPs should be significantly associated with the target exposure at the genome-wide significance threshold ($P < 5 \times 10^{-8}$)” |

	ii. Provide information on the number of individuals who overlap between the exposure and outcome studies		8) to fulfill the first hypothesis.” ii. “Since the GWAS data for exposure and outcome were obtained from different databases, it can be assumed that there is no population overlap.”
11	Main results		
	a) Report the associations between genetic variant and exposure, and between genetic variant and outcome, preferably on an interpretable scale	6	“we firstly selected SNPs associated with exposure, and the selected SNPs should be significantly associated with the target exposure at the genome-wide significance threshold ($P < 5 \times 10^{-8}$) to fulfill the first hypothesis. Secondly, the sliding window size was set to 10,000kb, with $r^2 < 0.01$, to ensure the independence of the selected genetic variants. To fulfill the third hypothesis, we set P to less than 5×10^{-5} to filter out SNPs significantly associated with outcome.” “Next, F-statistics ($BETA^2/SE^2$) for glutamine levels were calculated for each SNP as a measure of instrumental strength, an F-statistic greater than 10 generally suggests that the selected instrumental variable is sufficiently strong, reducing the risk of bias associated with weak instruments, and thus substantiates the predictive relationship of the instrument with the exposure. SNPs with F-statistics below 10 were considered weak instrumental variables and were removed from subsequent analyses.” For specific information, see Table 1.
	b) Report MR estimates of the relationship between exposure and outcome, and the measures of uncertainty from the MR analysis, on an interpretable scale, such as odds ratio or relative risk per SD difference	9	“Table 2 presents the analytical outcomes derived from the eight methodologies employed in our study.”
	c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period		Not applicable.
	d) Consider plots to visualize results (e.g. forest plot, scatterplot of associations between genetic variants and outcome versus between genetic variants and exposure)	9	“Figure 2 shows the scatterplots generated by the eight methods.” “The symmetric and unbiased funnel plot based on the results increased the credibility of the findings (Figure 3).”
12	Assessment of assumptions		

13	Sensitivity analyses and additional analyses	a) Report the assessment of the validity of the assumptions	8	“To ensure the rigor and validity of our study, we have verified this research against the checklist provided by the STROBE-MR guidelines (Additional file 1). For detailed information, please refer to the STROBE-MR website (https://www.strobe-mr.org/).”
		b) Report any additional statistics (e.g., assessments of heterogeneity across genetic variants, such as I^2 , Q statistic or E-value)	9	“and the Cochran's Q test result of $P=0.082$ indicates that there is no heterogeneity. The analysis of pleiotropy using the MR-Egger intercept test indicated the absence of horizontal pleiotropy (intercept = 0.010, $P = 0.373$), and the results of MR-PRESSO also supported the absence of horizontal pleiotropy (OR = 0.750; 95% CI: 0.592 to 0.951; $P = 0.024$) (Table 3).”
		a) Report any sensitivity analyses to assess the robustness of the main results to violations of the assumptions	9	“and the Cochran's Q test result of $P=0.082$ indicates that there is no heterogeneity. The analysis of pleiotropy using the MR-Egger intercept test indicated the absence of horizontal pleiotropy (intercept = 0.010, $P = 0.373$), and the results of MR-PRESSO also supported the absence of horizontal pleiotropy (OR = 0.750; 95% CI: 0.592 to 0.951; $P = 0.024$) (Table 3). The symmetric and unbiased funnel plot based on the results increased the credibility of the findings (Figure 3).”
		b) Report results from other sensitivity analyses or additional analyses	9~10	“Glutamine level was selected as an exposure factor and smoking and GERD as outcomes separately for MR analysis to exclude the effects caused by potential confounders. And as shown in Table 4, there was no causality between glutamine level and these two, which fulfilled the second hypothesis of MR analysis.”
		c) Report any assessment of direction of causal relationship (e.g., bidirectional MR)	8	“In our study, each SNP considered has successfully undergone the Steiger Test (The last two columns in Table 1). This affirms that the association of each SNP with the exposure variable is more pronounced than with the outcome, thus precluding the possibility of reverse causality and reinforcing the exclusivity assumption of our

			Mendelian randomization methodology."
	d) When relevant, report and compare with estimates from non-MR analyses		Not applicable.
	e) Consider additional plots to visualize results (e.g., leave-one-out analyses)	9	"Leave-one-out analysis showed that only one genetic variant, rs2657879, influenced the association between glutamine levels and IPF (Figure 4)."

DISCUSSION

14	Key results	Summarize key results with reference to study objectives	14	"Overall, our study suggests that lower circulating glutamine levels may increase the risk of developing IPF."
15	Limitations	Discuss limitations of the study, taking into account the validity of the IV assumptions, other sources of potential bias, and imprecision. Discuss both direction and magnitude of any potential bias and any efforts to address them	13~14	"Nevertheless, there are still some limitations. First, it may not be possible to directly generalize our conclusions to other races and populations because of genetic differences and environmental factors among races and populations; To address this limitation and enhance the external validity of our findings, we advocate for future research to include diverse populations from varying ethnic backgrounds. Such studies will be instrumental in assessing the consistency of the glutamine-IPF association across different genetic contexts and environments. By doing so, the broader applicability of our results can be established, and a more comprehensive understanding of the global health implications related to glutamine metabolism and IPF can be achieved. Furthermore, we recommend that future studies consider stratification by ethnicity, as well as the integration of genetic ancestry data, to better understand the interaction between genetic variants and environmental exposures. This approach will not only validate our findings but also potentially uncover novel insights into the role of glutamine in IPF pathogenesis across diverse populations. Furthermore, the constraints inherent in the aggregated GWAS data preclude stratified MR analyses based on age, gender, and height, and certain confounding factors, including immune function regulation and cellular energy metabolism, may elude comprehensive validation. Third, despite the absence of detected pleiotropy in our analyses, including assessments via MR-Egger regression and MR-PRESSO, we cannot conclusively rule out

its potential presence. Finally, our study was linear, so it could not accurately capture nonlinear relationships, and larger prospective cohort studies are still needed in the future to validate the complex relationship between the two.

It cannot be overlooked that, although our study establishes a significant inverse correlation between circulating glutamine levels and the risk of idiopathic pulmonary fibrosis (IPF) using Mendelian randomization, further exploration into the mechanistic link between glutamine metabolism and IPF pathogenesis is warranted.”

16 Interpretation

- a) Meaning: Give a cautious overall interpretation of results in the context of their limitations and in comparison with other studies

13~15 “Furthermore, the constraints inherent in the aggregated GWAS data preclude stratified MR analyses based on age, gender, and height, and certain confounding factors, including immune function regulation and cellular energy metabolism, may elude comprehensive validation. Third, despite the absence of detected pleiotropy in our analyses, including assessments via MR-Egger regression and MR-PRESSO, we cannot conclusively rule out its potential presence. Finally, our study was linear, so it could not accurately capture nonlinear relationships, and larger prospective cohort studies are still needed in the future to validate the complex relationship between the two.”

“However, there are still some limitations for the current study and larger samples are needed to confirm our findings.”

- b) Mechanism: Discuss underlying biological mechanisms that could drive a potential causal relationship between the investigated exposure and the outcome, and whether the gene-environment equivalence assumption is reasonable. Use causal language carefully, clarifying that IV estimates may provide causal effects only under certain assumptions

10~11 “Glutamine plays multiple roles in cell biology, including serving as an energy source to support intestinal, immune cell, and renal function; participation in nucleic acid synthesis to promote cell proliferation and repair; and maintenance of intracellular nitrogen balance. It also plays a key role in regulating inflammatory responses and oxidative stress, which are essential for maintaining cellular homeostasis and tissue health. In the context of fibrotic diseases, regulation of glutamine metabolism may be associated with abnormal fibroblast activation and tissue scarring. Glutamine not only plays a part in lung tissue, but is also associated with fibrotic processes in other organs.

For example, in liver and kidney disease, glutamine metabolism disorders are associated with the progression of tissue fibrosis. This amino acid may play a key role in multi-organ fibrosis by influencing extracellular matrix synthesis and cell signaling, which may regulate fibrosis-related cellular activities on a systemic level (Figure 5).

Several studies on IPF have confirmed that glutamine metabolism plays an important part in the development of the disease. Olsen et al. found that transglutaminase 2 (TG2) expression was increased in lung tissue from patients with IPF and correlated with collagen deposition, which is a key component of lung tissue structure. One study showed that glutamine metabolizing enzymes (e.g., LOXs and TG2) regulate fibroblast proliferation and ECM deposition. Lian et al. reported that the glutamine transporter protein ASCT2 inhibited reduced fibroblast activation and collagen deposition, thereby attenuating lung fibrosis. Xiang et al. demonstrated that glutamine metabolism and its derived metabolite α -ketoglutarate (α -KG) were involved in mediating the proliferation, migration, and collagen formation of IPF fibroblasts. Another report found that SLC1A5 was highly expressed in fibrotic lung fibroblasts and lung fibroblasts from IPF patients. TGF- β -induced pro-fibrotic target expression, cell migration, and anchorage-independent growth require SLC1A5 activity. Glutamine plays a key role in TGF- β -induced activation and differentiation of myofibroblasts and is taken up by cells primarily through the cell surface transporter protein SLC1A5. Researchers have targeted SLC1A5 inhibition with the small molecule inhibitor V-9302 and observed improved lung fibrosis in a bleomycin-treated mouse model of lung fibrosis."

- c) Clinical relevance: Discuss whether the results have clinical or public policy relevance, and to what extent they inform effect sizes of possible interventions

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"This study demonstrates a potential causal relationship between circulating glutamine levels and the risk of developing IPF, a finding with important implications for clinical practice. First, the measurement of circulating glutamine levels as a potential biomarker may help in the early identification of individuals at high risk for IPF, especially those with a family history of IPF or other known risk factors. Second, monitoring of

				circulating glutamine levels may provide new perspectives in the management of patients with IPF. Regular testing of glutamine levels may help to better monitor disease progression and assess treatment efficacy. Moreover, our findings provide new theoretical support for the development of glutamine-related drugs for the prevention and treatment of IPF."
17	Generalizability	Discuss the generalizability of the study results (a) to other populations, (b) across other exposure periods/timings, and (c) across other levels of exposure	13	"First, it may not be possible to directly generalize our conclusions to other races and populations because of genetic differences and environmental factors among races and populations," "Furthermore, we recommend that future studies consider stratification by ethnicity, as well as the integration of genetic ancestry data, to better understand the interaction between genetic variants and environmental exposures. This approach will not only validate our findings but also potentially uncover novel insights into the role of glutamine in IPF pathogenesis across diverse populations."
OTHER INFORMATION				
18	Funding	Describe sources of funding and the role of funders in the present study and, if applicable, sources of funding for the databases and original study or studies on which the present study is based	15	"This work was supported by the Natural Science Foundation of Hebei Province (No.H2021108003)."
19	Data and data sharing	Provide the data used to perform all analyses or report where and how the data can be accessed, and reference these sources in the article. Provide the statistical code needed to reproduce the results in the article, or report whether the code is publicly accessible and if so, where	15	"The analysis in this research relied on publicly available datasets, which can be accessed through the IEU Open GWAS Project (https://gwas.mrcieu.ac.uk/) and FinnGen R10 GWAS database (https://r10.finnngen.fi/)."
20	Conflicts of Interest	All authors should declare all potential conflicts of interest	15	"The authors declare that they have no competing interests."

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1. Skrivankova VW, Richmond RC, Woolf BAR, Yarmolinsky J, Davies NM, Swanson SA, et al. Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) Statement. JAMA. 2021;under review.
2. Skrivankova VW, Richmond RC, Woolf BAR, Davies NM, Swanson SA, VanderWeele TJ, et al. Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomisation (STROBE-MR): Explanation and Elaboration. BMJ. 2021;375:n2233.