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	1-2	Mendelian randomization study with mediation analysis
	INTRODUCTION		3	-
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	Recent studies have separately explored the possible relationship between some gut microbiota and the development of NMOSD. However, there is still a need for a comprehensive study that explores this relationship.
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	3	By using the genome-wide association studies (GWAS) summary statistics, Mendelian randomization (MR) can identify causal relationships between exposures and outcomes by utilizing genetic variability as instrumental variables (IVs). MR studies can avoid reverse causation and confounding factors that exist in the vast majority of traditional observational studies. In this study, we comprehensively assessed the possible causal associations of gut microbes, inflammatory proteins, immune cells and NMOSD, and explored whether the mediating effects of inflammatory proteins and immune cells in the pathways between gut microbes and NMOSD to better understand the preventive and therapeutic potential of GM in NMOSD.
	METHODS		4-5	-
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:	4-5	-
	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.	4	Genetic variation in the GM was obtained from the MiBioGen consortium's GWAS, which has the most significant number of published gut microbiome genetics studies to date. The study extracted gene sequencing data from 18,340 individuals from data from cohort studies in 11 countries worldwide. All datasets were streamlined to 10,000 reads per sample and then categorized 103 using direct

			classification bins. The cut-off criteria for the study included cohort size more than or equal to three and valid sample size more than or equal to 3,000 individuals. Within each cohort, binary trait locus mapping (mbBTL) analyses included 196 taxa and 122,110 variant loci. The genetic data for inflammatory proteins and immune cells came from the previously GWAS,91 inflammatory proteins and 731 immune cells.
	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	4	The study extracted gene sequencing data from 18,340 individuals from data from cohort studies in 11 countries worldwide. All datasets were streamlined to 10,000 reads per sample and then categorized 103 using direct classification bins. The cut-off criteria for the study included cohort size more than or equal to three and valid sample size more than or equal to 3,000 individuals. Within each cohort, binary trait locus mapping (mbBTL) analyses included 196 taxa and 122,110 variant loci.
	c) Describe measurement, quality control and selection of genetic variants	4	For this MR study, we used a locus-wide significance threshold ($p < 1\times10\text{-}5$) to obtain the more 116 relevant IVs (35), and an aggregation procedure with a strict threshold ($r^2 < 0.001$, kb = 10,000) was performed to ensure IV independence. If linkage disequilibrium (LD) was present ($r^2 > 0.001$), we used the SNP with the lowest p-value when a high LD was present. In addition, palindromic SNPs were also removed. To avoid the potential of weak instrumental bias, we calculated F-statistic 120 value using the following formula ($F = \text{beta}^2 / \text{se}^2$), with a value >10 indicating sufficient strength.
	d) For each exposure, outcome, and other relevant variables, describe methods of assessment and diagnostic criteria for diseases	4	NMOSD patients in this study were diagnosed based on the 2006 diagnostic criteria, which includes transverse myelitis, optic neuritis, and any two of the following three conditions: (1) longitudinally extensive lesions; (2) brain magnetic resonance imaging inconsistent with multiple sclerosis; (3) seropositive for AQP4-IgG antibody.
	e) Provide details of ethics committee approval and participant informed consent, if relevant	4	This study did not require ethical approval as it was a re-analysis of publicly available GWAS data

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	4	Three fundamental assumptions must be satisfied in MR analysis: (1) the IVs exhibit a robust association with the exposure factors; (2) No correlation between confounding variables and IVs; (3) IVs can affect outcomes only through exposure factors.
6	**Statistical methods: main analysis**		Describe statistical methods and statistics used	5	-
		a)	Describe how quantitative variables were handled in the analyses (i.e., scale, units, model)	5	See under the heading Statistical analysis.
		b)	Describe how genetic variants were handled in the analyses and, if applicable, how their weights were selected	5	See under the heading Selection of IVs.
		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	5	Fixed or random effects models were selected based on the presence of heterogeneity. When horizontal pleiotropy is absent, IVW prevents the confounders' effects and achieves unbiased estimation. Odds ratio (OR) reflects the causal effect of GM and NMOSD, and presents an increased risk of binary outcomes (NMOSD) per SD increase in abundance of GM.
		d)	Explain how missing data were addressed	5	There is no missing data.
		e)	If applicable, indicate how multiple testing was addressed	5	Fixed or random effects models were selected based on the presence of heterogeneity. When horizontal pleiotropy is absent, IVW prevents the confounders' effects and achieves unbiased estimation. Odds ratio (OR) reflects the causal effect of GM and NMOSD, and presents an increased risk of binary outcomes (NMOSD) per SD increase in abundance of GM.
7	**Assessment of assumptions**		Describe any methods or prior knowledge used to assess the assumptions or justify their validity	5	A $p < 0.05$ and $q < 0.1$ were considered to indicate significant causal association; while $p < 0.05$ and $q > 0.1$ were considered to indicate suggestive causal effect.
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)	5	Heterogeneity was assessed by Cochran's Q test using the IVW method (significance level set at 0.05). MR-Egger intercept and MR-PRESSO tests were used to assess the existence of pleiotropy and identify the effects of heterogeneity. Additionally, leave-one-out analysis was conducted

				to assess if a singular outlier exerted a strong influence on the stability of causality.
9	**Software and pre-registration**		5	-
	a)	Name statistical software and package(s), including version and settings used	5	MendelR package (7.8.0) in R software (version 4.3.1).
	b)	State whether the study protocol and details were pre-registered (as well as when and where)	4	We strictly follow the guidelines outlined in "STROBE-MR" (Strengthening the Reporting of Observational Studies in Epidemiology-Mendelian Randomization).
	RESULTS		5-9	-
10	**Descriptive data**		5-9	-
	a)	Report the numbers of individuals at each stage of included studies and reasons for exclusion. Consider use of a flow diagram	5	Additionally, all F-statistics exceeded 10, indicating no weak IV bias. Detailed information of Gut microbiota and selected IVs are shown in Table S1-2.
	b)	Report summary statistics for phenotypic exposure(s), outcome(s), and other relevant variables (e.g. means, SDs, proportions)	5	Detailed information of Gut microbiota and selected IVs are shown in Table S1-2.
	c)	If the data sources include meta-analyses of previous studies, provide the assessments of heterogeneity across these studies	-	-
	d)	For two-sample MR: i. Provide justification of the similarity of the genetic variant-exposure associations between the exposure and outcome samples ii. Provide information on the number of individuals who overlap between the exposure and outcome studies	6	We plotted a heatmap plot of the results based on the MR analysis of 196 GM and NMOSD (Fig 2).
11	**Main results**		5-9	
	a)	Report the associations between genetic variant and exposure, and between genetic variant and outcome, preferably on an interpretable scale	6	Using IVW approach, genetically predicted phylum Tenericutes (OR = 2.73, 95%CI 1.07 to 6.99, p = 0.0357); class Mollicutes (OR = 2.73, 95%CI 1.07 to 6.99, p = 0.0357); genus Eubacterium rectale group (OR = 4.47, 95%CI 1.01 to 19.86, p = 0.0487); genus Barnesiella (OR = 2.95, 95%CI 1.09 to 7.98, p = 0.03); genus Eubacterium xylanophilum group (OR = 3.66, 95%CI 1.08 to 12.41, p = 0.037); and genus Ruminococcus torques group (OR = 5.05, 95%CI 1.32 to 19.31, p

					= 0.0179) were positively associated with the risk of NMOSD.
		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	6	Using IVW approach, genetically predicted phylum Tenericutes (OR = 2.73, 95%CI 1.07 to 6.99, p = 0.0357); class Mollicutes (OR = 2.73, 95%CI 1.07 to 6.99, p = 0.0357); genus Eubacterium rectale group (OR = 4.47, 95%CI 1.01 to 19.86, p = 0.0487); genus Barnesiella (OR = 2.95, 95%CI 1.09 to 7.98, p = 0.03); genus Eubacterium xylanophilum group (OR = 3.66, 95%CI 1.08 to 12.41, p = 0.037); and genus Ruminococcus torques group (OR = 5.05, 95%CI 1.32 to 19.31, p = 0.0179) were positively associated with the risk of NMOSD.
		c)	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-	-
		d)	Consider plots to visualize results (e.g. forest plot, scatterplot of associations between genetic variants and outcome versus between genetic variants and exposure)	6	We plotted a heatmap plot of the results based on the MR analysis of 196 GM and NMOSD (Fig 2).
12	**Assessment of assumptions**				
		a)	Report the assessment of the validity of the assumptions	5	-
		b)	Report any additional statistics (e.g., assessments of heterogeneity across genetic variants, such as I^2, Q statistic or E-value)	6	Series of sensitivity analyses were conducted to see if the results were robust when more than four SNPs were used as IVs.
13	**Sensitivity analyses and additional analyses**			6	
		a)	Report any sensitivity analyses to assess the robustness of the main results to violations of the assumptions	8	MR-Egger intercept test and MR- 188 PROSSO global test reported no pleiotropy in MR estimates.
		b)	Report results from other sensitivity analyses or additional analyses	8	MR-Egger intercept test and MR- 188 PROSSO global test reported no pleiotropy in MR estimates
		c)	Report any assessment of direction of causal relationship (e.g., bidirectional MR)	8	We further investigated the reverse causality using subtypes of NMOSD as exposure and significant GMs as outcomes
		d)	When relevant, report and compare with estimates from non-MR analyses	-	-

		e)	Consider additional plots to visualize results (e.g., leave-one-out analyses)	8	Fig 3. Scatter plots of significant causality of the GM and NMOSD.
	DISCUSSION			9-11	
14	**Key results**		Summarize key results with reference to study objectives	9	This MR analysis reported an increased relative abundance of genes in the specific genera of GM was associated with a lower risk of NMOSD patients. Family Clostridiales vadin BB60 group, genus Eggerthella, and genus Intestinibacter were negatively related to the risk of NMOSD.
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	11	However, some limitations should also be noted.
16	**Interpretation**				
		a)	Meaning: Give a cautious overall interpretation of results in the context of their limitations and in comparison with other studies	11	In this MR study, we comprehensively assessed the causal relationship between GM, inflammatory proteins, immune cells and NMOSD.
		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	9-10	-
		c)	Clinical relevance: Discuss whether the results have clinical or public policy relevance, and to what extent they inform effect sizes of possible interventions	10	This discovery provides a new direction for treating neuroimmune diseases in humans. Therefore, our research focuses on exploring therapeutic potential of the specific GM involved in NMOSD and their mechanisms of action, which may translate into possible prevention for NMOSD
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	11	However, some limitations should also be noted.
	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	11	This work is supported by National Natural Science Foundation of China (No 82171294)
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	17	The data enrolled in the study could be found in the manuscript and the supplementary materials.

		needed to reproduce the results in the article, or report whether the code is publicly accessible and if so, where		
20	**Conflicts of Interest**	All authors should declare all potential conflicts of interest	11	The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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.