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Causal Relationship between Gut Microbiota and Brain Microbleeds: A Comprehensive Mendelian Randomization Study

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Abstract: The background applied in this paper is that considerable evidence has been reported that alterations in gut microbiota composition could cause cerebrovascular diseases. The microbiota-gut-brain axis also hinted at a possible contribution of the gut microbiota to cerebrovascular diseases. However, the causal association between the gut microbiome and the risk of brain microbleeds (BMB) is unclear. The methods applied in this paper is that we performed two-sample bidirectional Mendelian randomization (MR) utilizing the summary-level data of respective genome-wide association study (GWAS) for 211 gut microbial taxa and two BMB phenotypes to reveal the causal association between gut microbiota and BMB. The results applied in this paper is that we identified 7 causal relationships between genetic liability in the gut microbiome and any BMB, including those involving the genus Lachnospiraceae. We found 13 associations between genetic liability in the gut microbiome and lobar BMB. Moreover, we found 6 associations between genetic liability in the gut microbiome and deep infratentorial BMB. The bidirectional, heterogeneity, and pleiotropy analyses confirmed the robustness of MR results. The conclusion applied in this paper is that our MR analysis revealed that the gut microbiota was causally associated with BMB and may be helpful in providing new insights for further mechanistic and clinical studies of microbiota-mediated cerebrovascular diseases.

1. Introduction

Brain microbleeds (BMB), also referred to as cerebral microbleeds or cerebral microhemorrhages correspond to hemosiderin deposits as a result of microscopic hemorrhages that are visible on MRI sequences [1]. In many prospective and epidemiological studies, BMB is highly prevalent in people with clinically manifest cerebrovascular disease including both ischemic stroke and intracerebral hemorrhage, and has been shown to increase the risk of stroke recurrence[2,3]. Once BMB is detected, the impact that they have on the outcomes of patients with them is of great

interest, as they may represent a potential biomarker for an underlying cerebrovascular disease state. Therefore, there is an urgent need to explore the etiology of BMB to find rapid and effective treatment methods and prevent cerebrovascular diseases that may occur later. This has been an important and challenging problem, particularly at the microscopic and molecular levels.

"The gut microbiota" refers to all the microorganisms in the human gastrointestinal tract, with a population reaching trillions[4].Due to the intricate symbiotic relationship between the gut microbiota and the host, it is closely associated not only with gastrointestinal diseases but also with overall human health[5-7].Recently, the complex relationship between the gut microbiota and the occurrence of cerebrovascular diseases through the gut-brain axis has sparked a controversy. So far, an increasing amount of indirect evidence logically supports this hypothesis. Firstly, gut dysbiosis can directly contribute to risk factors for stroke, including hypertension, diabetes, aging, dyslipidemia, obesity, etc[8-10].Furthermore, numerous studies have indicated an association between alterations in gut microbiota composition and the risk of cerebrovascular diseases. For example, enrichment of Enterococcus and a reduction of Prevotella have been associated with an increased risk of intracerebral hemorrhage (ICH)[11,12]. However, it is still unclear whether there is any association between gut microbiota and BMB, and there is a lack of population-level research with a higher level of causal evidence.

Animal model-based or clinical trials examining the onset of BMB following the intervention of gut microbiome are absent and too challenging to accomplish. Disentangling this causality is of great clinical importance, which could help bridge these gaps from traditional epidemiological studies. A better approach to overcome these limitations is Mendelian randomization (MR). MR analysis utilizes the inherent characteristics of common genetic variations to modify the interested environmental exposure and has become a widely used method to explore potential causal relationships between environmental exposures and diseases[13,14]. Moreover, MR results are less likely to be influenced by environmental confounding that could bias causal estimates. Depending on single-nucleotide polymorphisms (SNP) as an instrumental variable, we used MR analyses to infer the causal association between the gut microbiome and BMB.

2. Methods

2.1. Study overview

Using summary statistics of genome-wide association studies (GWAS), we conducted a comprehensive MR study to explore the causal association of the gut microbiome with BMB, including strictly lobar BMB and deep infratentorial BMB. The overall flow chart of this study is shown in **Figure 1**. This MR study strictly followed the three assumptions: (1) genetic variations as SNPs must be significantly associated with gut microbiome (as exposure); (2) SNPs were not related to any confounder factors; (3) outcome can be affected by SNPs only through exposure. Our results were in accordance with the STROBE-MR guidelines[15].

2.2. GWAS Summary Data for gut microbiota

The international consortium MiBioGen was established to study the influence of human genetics on the gut microbiome. The summary human gut microbiome GWAS data that coordinated 16S ribosomal RNA gene sequencing profiles and genotyping data from 18,340 participants from 24 cohorts from the USA, Canada, Israel, South Korea, Germany, Denmark, the Netherlands, Belgium, Sweden, Finland, and the UK to explore the association between autosomal human genetic variants and the gut microbiome[16]. After removing 15 unidentified bacterial taxa, the GWAS data we obtained finally covered a total of 196 taxa (ordered by taxonomy): 9 phyla, 16

classes, 20 orders, 32 families, and 119 genera[16]. Since only a few gut microbiomes had three or more independent SNPs at genome-wide significance levels ($P < 5 \times 10^{-8}$), a higher cut-off ($P < 1 \times 10^{-5}$) was used for obtaining SNPs to obtain more comprehensive results. We also assess the association using $P < 5 \times 10^{-8}$ in supplementary. We clumped these SNPs for linkage disequilibrium (LD) to $r^2 < 0.001$ based on the European 1,000 Genomes panel. Then, we standardized the SNP allele frequencies across studies and eliminated palindromic SNPs (0.42–0.58) with unclear allele frequencies. Finally, to evaluate the strength of weak instrumental bias, we computed the F-statistic of SNPs. It was considered that no bias was caused by weak SNPs if the F-statistic was more than 10[17].

2.3. GWAS Summary Data for BMB

Summary data for BMB were obtained from a meta-analysis of GWASs in 11 population-based cohort studies and three case-control or case-only stroke cohorts[18].BMB were detected in 3,556 of the 25,862 participants (97.1% European ancestry individuals), of which 2,179 were strictly lobar and 1,293 were deep infratentorial BMB. Age and sex were adjusted in each included cohort.

2.4. Statistical analysis

Principal analyses were conducted using the inverse-variance weighted (IVW) MR method. This method assumes that all genetic variants are valid instrumental variables and produces the most accurate estimates[19]. For gut microbiome with only one SNP available, we used the Wald ratio method, as other MR methods require at least 2 SNPs. As robustness validation analyses, the weighted median method and MR-Egger regression were used, and their findings complemented those calculated by the IVW approach[20,21]. Odds ratios (OR) and 95% confidence intervals (CI) were used to present the findings of causal connections. The significance cutoff was established at $p < 0.05$.

The potential heterogeneity was measured and tested using Cochran's Q statistics, and the horizontal pleiotropy was estimated using the MR-Egger intercept test. For significant MR estimates, the MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) test was used to identify outliers for horizontal pleiotropy and heterogeneity. The leave-one-SNP-out analysis was conducted to determine if the aggregated estimate was biased by a high-influence point. Finally, we performed bi-directional MR analyses and MR Steiger directionality test to determine the direction of causation.

All analyses in this study were performed based on R software (version 4.2.3). In our MR study, the "TwoSampleMR" R package (version 0.5.6) and the "MRPRESSO" R package were utilized.

3. Results

3.1. The selection of single nucleotide polymorphisms

First, we identified 2047 SNPs that were associated with both gut microbiota and lobar BMB at a significance level of $p < 1 \times 10^{-5}$. To further investigate the causal effects, we used more strict significance level of $p < 5 \times 10^{-8}$ to select SNPs and identified 22 SNPs that fit this criteria. Then, we screened for SNPs associated with deep infratentorial BMB in the same way. 1783 (genome-wide statistical significance threshold, $p < 1 \times 10^{-5}$) and 22 (genome-wide statistical significance threshold, $p < 5 \times 10^{-8}$) SNPs were selected as SNPs. The *F* statistics of the SNPs were all largely > 10, indicating no evidence of weak instrument bias.

3.2. Causal effects of the gut microbiome on the lobar brain microbleeds

Through the IVW, in the set of SNPs ($p < 1 \times 10^{-5}$), the majority of gut microbiomes were not related to lobar BMB. Meanwhile, we found that the genus Lachnospiraceae NC2004 group, class Negativicutes, order Selenomonadales, family Victivallaceae, and phylum Cyanobacteria were the protective factors against lobar BMB (genus Lachnospiraceae NC2004 group: OR [95% CI]: 0.59 [0.44, 0.80]; class Negativicutes: OR [95% CI]: 0.49 [0.30, 0.81]; order Selenomonadales: OR [95% CI]: 0.49 [0.30, 0.81]; family Victivallaceae: OR [95% CI]: 0.77 [0.62, 0.96] ; phylum Cyanobacteria: OR [95% CI]: 0.69 [0.48, 0.98]). The order Bacillales, as well as the genus Terrisporobacter, phylum Firmicutes, genus Coprobacter, and genus Ruminococcaceae UCG009, were identified as risk factors for lobar BMB (order Bacillales: OR [95% CI]: 1.33 [1.06, 1.67]; genus Terrisporobacter: OR [95% CI]: 1.61 [1.07, 2.40]; phylum Firmicutes: OR [95% CI]: 1.69 [1.08, 2.65]; genus Coprobacter: OR [95% CI]: 1.33 [1.01, 1.75] ; genus Ruminococcaceae UCG009: OR [95% CI]: 1.38 [1.00, 1.90]) (**Figure 2**). We identified the SNPs under a more stringent condition ($p < 5 \times 10^{-8}$), and we found that family Streptococcaceae, genus Streptococcus, and genus Intestinibacter were causally associated with lobar BMB (wald ratio, family Streptococcaceae: OR [95% CI]: 3.87 [1.07, 13.94]; genus Streptococcus: OR [95% CI]: 3.61 [1.07, 12.20]; genus Intestinibacter: OR [95% CI]: 2.99 [1.11, 8.07]).

The same association between the gut microbiome and lobar BMB was still noted in the MR-egger and weighted median analyses. MR-Egger and MR-PRESSO tests showed that there is no horizontal pleiotropy or outliers (**Figure 2**). In addition, the leave-one-out method revealed that no SNP was significantly associated with the outcome. Finally, bi-directional MR analyses showed that there is no evidence that lobar BMB affects gut microbiota. The result of the MR Steiger directionality test also supports above conclusion.

3.3. Causal effects of the gut microbiome on the deep infratentorial brain microbleeds

In the set of SNPs ($p < 1 \times 10^{-5}$), as shown in **Figure 3**, IVW analyses show that phylum Proteobacteria, genus Oscillospira, order Desulfovibrionales and family Desulfovibrionaceae were causally related to an increased risk of deep infratentorial BMB (phylum Proteobacteria: OR [95% CI]: 2.28 [1.10, 4.72]; genus Oscillospira: OR [95% CI]: 2.21 [1.02, 4.80]; order Desulfovibrionales: OR [95% CI]: 1.90 [1.01, 3.58]; family Desulfovibrionaceae: OR [95% CI]: 2.04 [1.01, 4.13]). In contrast, class Bacilli was causally related to a decreased risk of deep infratentorial BMB (OR [95% CI]: 0.40 [0.20, 0.78]). No significant correlation was found between other gut microbiomes and deep infratentorial BMB. In the set of SNPs ($p < 5 \times 10^{-8}$), we found that order Gastranaerophilales was causally associated with deep infratentorial BMB (wald ratio, OR [95% CI]: 0.30 [0.10, 0.88]).

The results of the MR-Egger intercept test and MR-PRESSO global test showed that there were no horizontal pleiotropic effects and The Cochran Q test did not provide any evidence of heterogeneity (**Figure 3**). Results from the weighted median method and MR-egger method also supported principal analyses (IVW), which was not significant. And the results of the leave-one-out analysis showed that no matter which SNP was removed, it would not have a fundamental impact on the results. Finally, through the bi-directional MR analyses and MR Steiger directionality test, we found that there was no reverse causal relationship between the deep infratentorial BMB and gut microbiota associated with it.

4. Discussion

As far as we know, this is the first MR investigation to evaluate whether gut microbiota is causally connected with BMB. In the biggest GWAS of the gut microbiota, robustly related gene

variations were discovered. We explored genetic vulnerability to some gut bacteria that is causally connected with BMB using extensive genomic data from more than 3556 European persons[22]. Interestingly, the genetic susceptibility to the genus Intestinibacter, genus Streptococcus, and family Streptococcaceae was found to causally promote lobar BMB, while order Gastranaerophilales can protect deep infratentorial BMB ($p<1\times10^{-8}$). We also found certain gut microbes that could be BMB risk factors ($p<10^{-5}$). These findings may have ramifications for public health efforts focused on lowering BMB risk.

The reasons why the effect of bacteria features on BMB founding in our study should be discussed. According to locations, BMB could be sorted as lobar BMB mainly caused by cerebral amyloidosis, and deep infratentorial BMB primarily induced by hypertension. The effect of the gut microbiome on BMB is indirect, such as hypertension, metabolite, and pro-inflammatory factors[23,24]. Hypertension is an important inducing factor for BMB, particularly for deep infratentorial BMB. Based on emerging evidence, the progression and pathogenesis of hypertension are closely related to the gut microbiome. Corresponding to the negative causal effect of Streptococcus on deep infratentorial BMB in MR, this bacterial trait was also found to be particularly lower in hypertension patients[25], but Streptococcus mutans Harboring the cnm Gene have been proved to be related to increase the evidence of BMB[26]. This interesting outcome may indicate whether Streptococcus plays an essential role in BMB with hypertension or not. In another study, Firmicutes and Proteobacteria were found to be both significantly changed in ICH patients[27].

Through pro-inflammatory factors produced by gut bacteria entering the bloodstream, the perturbation of gut microbiota could lead to a higher incidence of autoimmunity and allergy in the whole body [28]. The pro-inflammatory factors include inflammatory markers (such as lipopolysaccharides, c-reactive protein, and cytokines. eg), gut microbes, and their derivatives. Especially when the blood-brain barrier is breached, these factors could constantly interact with pattern recognition receptors in intestinal epithelial cells, immune cells in peripheral blood, and even cells in the central neural system[29,30]. Proteobacteria, with highly immune-stimulatory lipopolysaccharides in prevous studies, was calculated to be a risk factor for BMB in our study[31].

Previous researches have shown that gut microbial metabolites such as trimethylamine N-oxide (TMAO), short-chain fatty acids (SCFAs), and equol are associated with various cerebral vascular diseases. Although no clear relationship between TMAO and BMB has been reported, it is of paramount importance as representative research to elucidate the influence of microbial metabolites and to achieve the proof of concept study, "microbial-targeted non-lethal therapeutics"[32, 33]. SCFAs may influence the microbiome-brain-gut axis through direct and indirect pathways [33,34]. Recently, Haak BW et al. showed that the reduction of butyrate-producing bacteria in the gut microbiome was associated with hemorrhagic stroke using 16S ribosomal RNA amplicon sequencing [35].

BMB is not fatal but increases the risk of stroke by about 2-4 times and the risk of ICH by about 5 times [36,37]. Treatment with antithrombotic drugs may lead to an increase in BMB, and the use of warfarin may increase the risk of ICH. The recent AHA/ASA publication provides advice on the risk of ICH in patients with BMB[38]. Therefore, there is an urgent need to explore novel ways to stop the development and clinical disease resulting from BMB[39]. The incomplete understanding of the pathogenesis is a major reason for the lack of more specific preventive and therapeutic strategies for BMB. Encouragingly, accumulating evidence supports the role of gut microbiota in the etiology of BMB[40]. Such advances in the understanding of BMB have provided targets for potential BMB therapies.

There were several limitations to MR selecting the gut microbiome as an exposure. First, the abundance of the gut microbiome may also be affected by diet, gender, medication, time of

sampling, etc., which may reduce the variance explained by genetics. Second, the 16S rRNA gene sequencing from most GWAS typically allows resolution at the genus level, so we cannot estimate whether particular strains or species are correlated with results. Third, the use of different cohorts means that non-linear associations cannot be tested, especially when the gut microbiome may vary between cohorts, and subgroup analysis is not possible. However, causal effects should be generally consistent across cohorts.

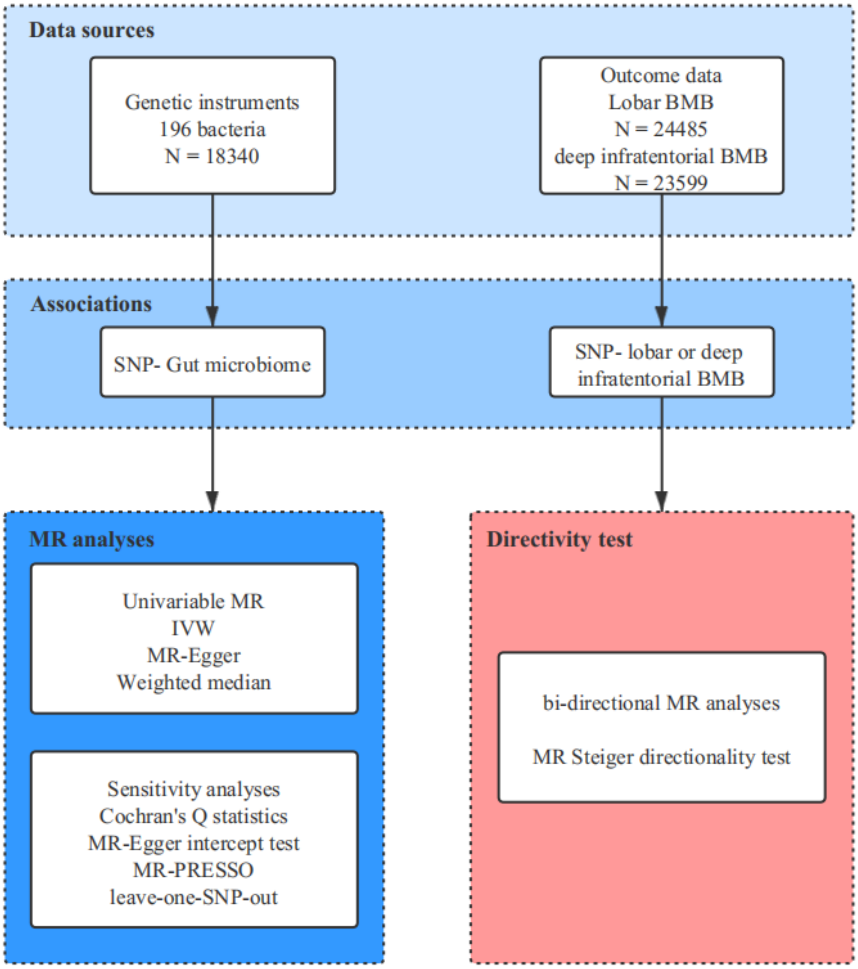

Figure 1: Study design of the Mendelian randomization for the effect of genetically predicted gut microbiome on brain microbleeds.

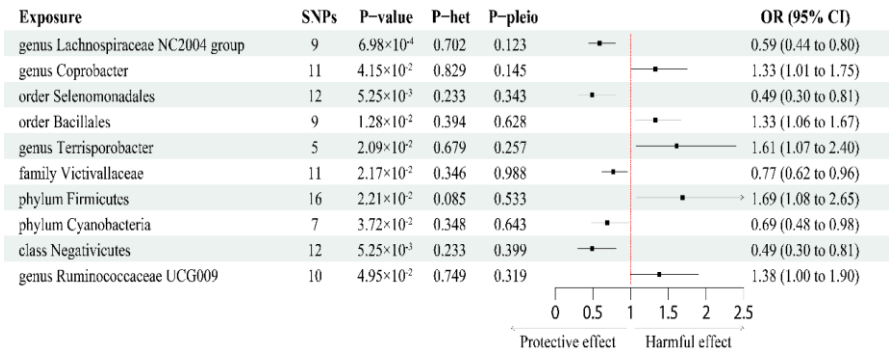

Figure 2: MR associations between genetically determined gut microbiome and the risk of lobar brain microbleeds.

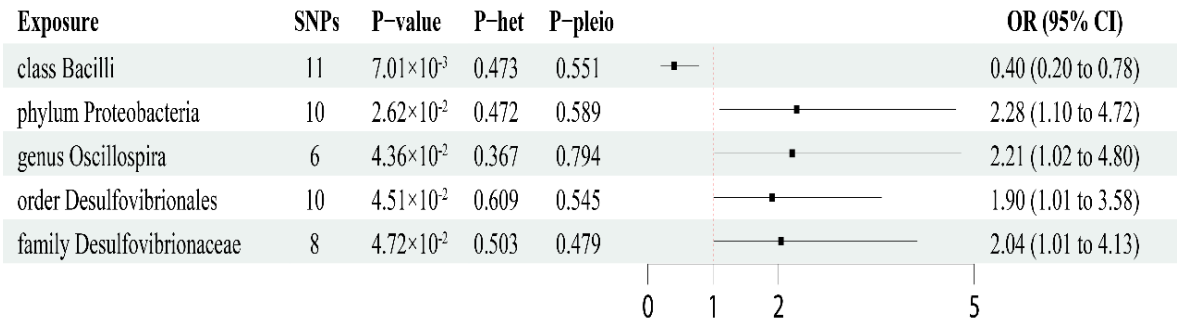

Figure 3: MR associations between the genetically determined gut microbiome and the risk of deep infratentorial brain microbleeds.

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Author contributions

Tianxiang Gao: participated in study design, analyzed data, prepared figures, wrote manuscript. Hanchen Liu: participated in study design, analyzed data, wrote manuscript. Congyan Wu: wrote manuscript. Jianmin Liu and Xiaoxi Zhang: participated in study design, interpreted data, wrote manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Appendix

Abbreviations:
GWAS: large-scale genome-wide association studies
MR, Mendelian randomization
BMB: brain microbleeds
ICH: intracerebral hemorrhage
SNP: single-nucleotide polymorphism
LD: linkage disequilibrium
OR: Odds ratios
MR-PRESSO: MR Pleiotropy Residual Sum and Outlier
CI: confidence intervals
IVW: inverse-variance weighted
TMAO: trimethylamine N-oxide
SCFAs: short-chain fatty acids