Causal relationship between gut microbiota and gastric cancer: A two-sample Mendelian randomization analysis

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Abstract. The gut microbiota is associated with GC; however, the causal association between the gut microbiota and GC remains to be determined. The aim of the present study was to investigate the causal association between gut microbiota and gastric cancer (GC) from the perspective of Mendelian randomization (MR). The present study performed MR analysis using summary statistics from a genome-wide association study of the gut microbiome and GC. Inverse-variance weighted, MR-Egger and weighted median methods were used to investigate the causal relationship between gut microbiota and GC. Heterogeneity tests were performed using Cochrane's Q statistic. Horizontal polytropy was detected using Mendelian Randomization Pleiotropy RESidual Sum and Outlier were eliminated. Estimates from MR indicated that nine gut microorganism remained stable with regard to acceptance of heterogeneity and sensitivity methods. Among them, the genera *Prevotella* 7, *Roseburia* and *Ruminococcaceae* UCG014 were associated with an increased risk of GC; by contrast, the family *Enterobacteriaceae*, the genera *Allisonella*, *Lachnospiraceae* FCS020, *Ruminococcaceae* UCG004 and *Ruminococcaceae* UCG009, and the order Enterobacteriales decreased the risk of GC development. The present study demonstrated the potential importance of modulating the abundance of gut microbiota for the prevention and treatment of GC.

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Introduction

Gastric cancer (GC) is a common primary tumor of the digestive system. In 2020, >1 million new cases of GC were reported worldwide and it accounted for 769,000 deaths, rendering it the fifth most common cancer and the fourth leading cause of cancer-associated death globally (1). Incidence and mortality of GC have increased with changes in living habits and environmental factors (2). Numerous studies have shown that the development of GC is associated with *Helicobacter pylori* infection (2), dietary habits (3), genetic factors (4), and the local and regional environment (5), and these factors are associated with each other. The human gastrointestinal microecosystem is one of the most complex microecosystems in the body, and the relative dynamic balance is closely related to health status. When body functions are disturbed by certain factors such as ulcerative colitis , the dynamic balance of microbiota in the body is disrupted, which can lead to the formation of gastrointestinal microecosystem dysfunction between the host and the flora (6). In addition, tumor progression may occur due to the presence of bacteria that have not yet been detected, whereas the gut microbial community may also shape the microbiota for tumor survival such as induce DNA damage, enhance inflammatory response, and affect the tumor microenvironment to promote tumor growth (7). Therefore, investigation of the association between the changes in intestinal flora and the development of GC is important for the early detection, clinical symptomatic treatment and improvement of survival of patients with GC.

Randomized controlled trials (RCTs) are the gold standard for inferring causality in epidemiology; however, given the ethical constraints and moral limitations such as inappropriate use of placebos, there are difficulties in implementing RCTs (8). Mendelian randomization (MR) studies comprise a statistical method that has been primarily applied to infer causality in epidemiological diseases such as Coronavirus Disease 2019 (9). Different genotypes represent different intermediate phenotypes; when the phenotype represents an exposure characteristic such as intestinal flora, the association effect between a genotype and a disease can represent the influence of exposure factors on the disease. Since alleles follow

the principle of random distribution, traditional epidemiology does not consider confounding and reverse causality (10). With the public release of large-scale gene-wide association data, a large number of reliable genetic variants are available for MR studies (11). Therefore, the present study analyzed the causal association between gut flora and GC to aid the development of novel strategies for the clinical intervention of GC.

Materials and methods

Study population. The present study performed two-sample MR to investigate the causal association between the gut microbiome (fnngen.f/en) and GC (nealelab.is/uk-biobank). Mendelian randomization studies require three core assumptions: i) Extracted instrumental variable single nucleotide polymorphism (SNP) must be closely related to exposure; ii) The instrumental variable SNP should not be associated with any confounding factors [Exposure(gut microbiota) and Outcomes(gastric cancer)] of the expose-outcome relationship; iii) Instrumental variable SNP can only affect results through exposure (Fig. 1). Quality control, such as heterogeneity and genetic pleiotropy tests, were performed to verify the reliability of causal results.

The main exposure factor in the present study was the gut microbiome human genetics. The study of the gut microbiome was based on an international consortium MiBioGen (fnngen.f/en). In the present study, the human gut microbiome genome-wide association study (GWAS) data involved 18,340 individuals from 24 population-based cohorts.

The primary endpoint was GC and the GWAS dataset related to GC was derived from the UK Biobank Project (nealelab.is/uk-biobank). The UK Biobank project collected genetic and phenotypic data from ~500,000 participants across the UK. Genome-wide genotype data for all participants were collected from health and medical records to provide follow-up information.

Single nucleotide polymorphism (SNP) selection. A total of 196 SNPs that were significantly associated with the relative abundance of the gut microbiota were selected as available instrumental variables) IVs. The selection of IVs was based on the results of IVW, MR-Egger and WME methods, which considered $P<1x10^{-5}$ to be significant. The standard of linkage disequilibrium was set as $r^2<0.001$ and genetic distance was 10,000 kb. Highly correlated($P<0.05$) SNPs were excluded to ensure their independence. Finally, SNPs associated with the relative abundance of intestinal flora were projected into data of GC and the corresponding SNPs were extracted. Based on statistical parameters with the same loci in the relative abundance of gut microbes and GWAS results for GC, the data were coordinated so that the exposure and outcome effect values corresponded to the same effect alleles (harmonization).

Statistical analysis. Inverse-variance weighted (IVW), MR-Egger and weighted median (WME) methods were used to estimate the causal association between the gut microbiome and GC. P-value<0.05 used to indicate statistical significance. The IVW method assumes that all genetic variants are valid IVs and the ratio method is used to calculate the causal effect value of the individual IVs. Each estimate is aggregated in weighted linear regression to obtain the total effect value (12). The primary difference between the MR-Egger and IVW methods is that MR-Egger considers the existence of intercept terms (13). The WME method uses the intermediate effects of all available genetic variants and is obtained by weighting the inverse variance of each SNP associated with the result (14). The IVW method has higher test efficiency than the other MR methods. The preferred causal effect estimation method was the IVW method. β-values obtained from the results were converted to odds ratios (ORs) when calculating 95% confidence intervals (CI). The strength of IVs was assessed using the F-statistic. The following formula was used: F=R2(n-K-1)/k(1-R2), where R2 represents the variance explained by IV (for each gut microbiome), n is the sample size and K represents the number of tool variables. R2 was estimated using the minor allele frequency (MAF) and the B-value (effect size of SNPs on exposure factors) was calculated using the following formula: $R2=2 \times MAF \times (1-MAF) \times B^2$.

To assess the stability and reliability of the results, quality control included sensitivity analysis, heterogeneity and gene diversity tests. The leave-one-out method was used for sensitivity analysis and the combined effect value of remaining SNPs was calculated sequentially by deleting individual SNPs(9). SNP heterogeneity was determined by Cochran Q test. The horizontal gene pleiotropy test assessed whether IVs affected the outcome by means other than exposure using the intercept term of the MR-Egger regression and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MRPRESSO) (15). Finally, reverse MR was used to analyze whether a reverse causality was present between GC and significant gut microbiota. MR analysis and quality control were analyzed using R version 4.0.3 (r-project.org) and TwoSample MR Software package version 0.5.6 (github.com/MRCIEU/TwoSampleMR), respectively.

Results

Two-sample MR analysis. The results of the 196 intestinal flora studied in relation to GC are presented in Table SI. The F-statistics of the intestinal flora ranged from 18.667 to 32.374 and all met the threshold of >10, suggesting that they were unlikely to be affected by weak instrumental bias (Table SI).

Gut microbiota and GC. Overall, nine bacterial genera were associated with the risk of developing GC in the primary MR analysis, suggesting that bacterial genera may have an impact on GC (Fig. 2; Table I). Elevated abundances of the genera *Prevotella* 7, *Roseburia* and *Ruminococcaceae* UCG014 were positively associated with an increased risk of developing GC (OR: 1.406, 95% CI: 1.032-1.917, P=0.031 for *Prevotella* 7; OR:1.867, 95% CI=1.011-3.446, P=0.046 for *Roseburia*; and OR:1.791, 95% CI=1.045-3.071, P=0.034 for *Ruminococcaceae* UCG014) whereas the family *Enterobacteriaceae*, the genera *Allisonella*, *Lachnospiraceae* FCS020, *Ruminococcaceae* UCG004 and *Ruminococcaceae* UCG009, and the order Enterobacteriales were increased in abundance with decreasing GC incidence (OR: 0.346, 95% CI: 0.153-0.783, P=0.011 for *Enterobacteriaceae*; OR: 0.676, 95% CI=0.488-0.936, P=0.019 for *Allisonella*; OR: 0.528, 95% CI=0.309-0.903, P=0.020 for *Lachnospiraceae*

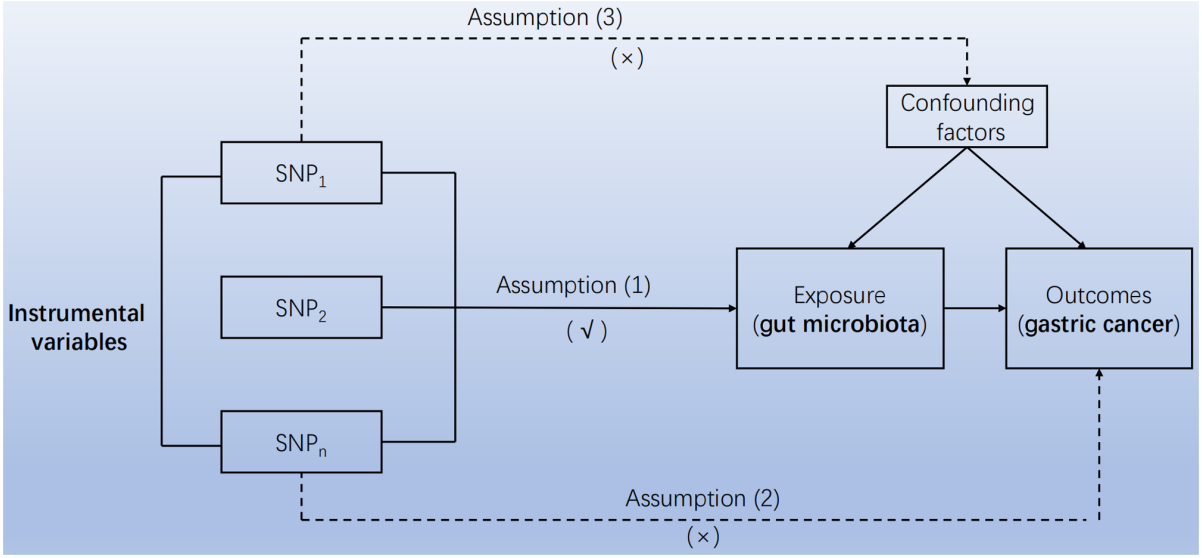

Figure 1. Overview of Mendelian randomization analysis. SNP, single nucleotide polymorphism.

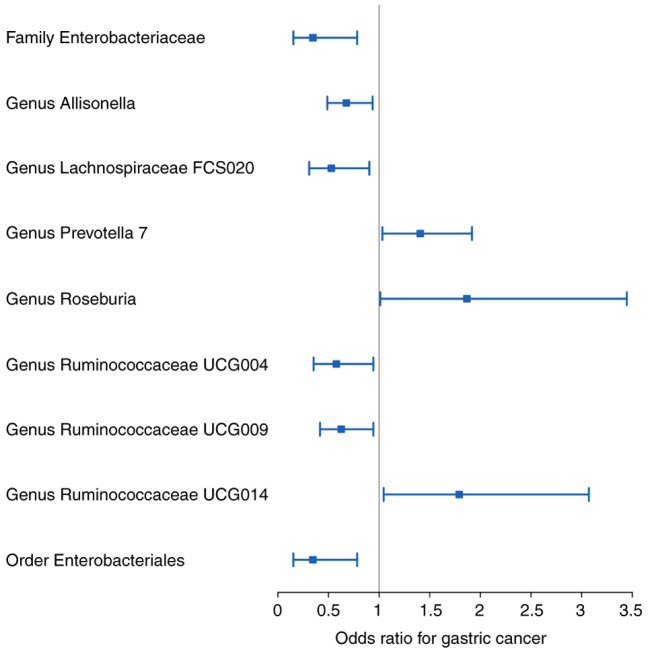

Figure 2. Primary Mendelian randomization results of gastric cancer.

FCS020; OR: 0.577, 95% CI=0.353-0.943, P=0.028 for *Ruminococcaceae* UCG004; OR: 0.626, 95% CI=0.416-0.943, P=0.025 for *Ruminococcaceae* UCG009; and OR: 0.346, 95% CI=0.153-0.783, P=0.011 for Enterobacteriales)(Table I).

The WME method indicated similar results as the IVW method (OR: 0.249, 95% CI=0.084-0.739, P=0.012 for *Enterobacteriaceae*; OR: 0.703, 95% CI=0.461-1.070, P=0.100 for *Allisonella*; OR: 0.456, 95% CI=0.224-0.928, P=0.030 for *Lachnospiraceae* FCS020; OR: 1.275, 95% CI=0.844-1.926, P=0.249 for *Prevotella* 7; OR: 2.363, 95% CI=1.025-5.450, P=0.044 for *Roseburia*; OR: 0.688, 95% CI=0.348-1.363, P=0.284 for *Ruminococcaceae* UCG004; OR: 0.668, 95% CI=0.379-1.179, P=0.164 for *Ruminococcaceae* UCG009; OR: 1.654, 95% CI=0.800-3.422, P=0.175 for *Ruminococcaceae* UCG014; and OR: 0.249, 95% CI=0.092-0.676, P=0.006 for Enterobacteriales; Table II), albeit with wider CIs. In addition, MR-Egger regression intercept showed no heterogeneity in the diversity of these gut microbiota in GC. MRPRESSO regression normality was used and heterogeneity analysis confirmed the accuracy (Table II). Concomitantly, leave-one-out sensitivity analysis confirmed the robustness of the data, indicating a consistent negative association between 9 gut flora and GC risk (Figs. S1-S9).

Reverse MR analysis. In the reverse MR analysis, GC was used as the exposure factor, and gut flora, which was associated with GC, was the outcome variable. The IVW results of MR study did not support a causal relationship between GC and altered gut flora (Table SII).

Discussion

In the present study, the MR method was utilized to explore the causal relationship between the relative abundance of gut microbes and GC. Trillions of symbiotic bacteria colonize the gut and serve a key role in body homeostasis and host defense against pathogenic invasion (16). A healthy microbiota resists colonization and invasion by harmful microorganisms through direct and indirect mechanisms (17,18). For example, short-chain fatty acids (SCFAs), a major metabolite produced by microorganisms, induce the production of antimicrobial peptides by inhibiting the activity of histone deacetylase-3, thereby enhancing the antibacterial activity in infected mouse models (19,20).

Multiple studies have shown decreased diversity of intragastric flora in patients with GC (21-23); however, other studies have suggested a quantitative difference in the composition of the flora between patients with GC and those with dyspepsia (24,25). Aviles-Jimenez *et al* (22) demonstrated a decrease in the abundance of *Porphyromonas*, *Neisseria* and *Streptococcus buglossi*, and an increase in the abundance of *Lactobacillus* spp. and *Trichosporon* spp. during the

Table I. Effect estimates of the associations between 196 bacterial traits and the risk of gastric cancer in MR analysis.

A, Family Enterobacteriaceae (7 SNPs)

Method	OR	95% CI	P-value
MR-Egger	2.314	0.015-368.544	0.759
Weighted median	0.249	0.084-0.739	0.012
Inverse-variance weighted	0.346	0.153-0.783	0.011
Simple mode	0.245	0.064-0.939	0.086
Weighted mode	0.250	0.061-1.015	0.101

B, Genus *Allisonella* (8 SNPs)

Method	OR	95% CI	P-value
MR-Egger	0.341	0.037-3.113	0.377
Weighted median	0.703	0.461-1.070	0.100
Inverse-variance weighted	0.676	0.488-0.936	0.019
Simple mode	0.712	0.387-1.309	0.310
Weighted mode	0.697	0.358-1.355	0.323

C, Genus *Lachnospiraceae* FCS020 (12 SNPs)

Method	OR	95% CI	P-value
MR-Egger	0.291	0.070-1.208	0.120
Weighted median	0.456	0.224-0.928	0.030
Inverse-variance weighted	0.528	0.309-0.903	0.020
Simple mode	0.456	0.142-1.466	0.214
Weighted mode	0.430	0.140-1.325	0.170

D, Genus *Prevotella* 7 (11 SNPs)

Method	OR	95% CI	P-value
MR-Egger	2.009	0.335-12.032	0.464
Weighted median	1.275	0.844-1.926	0.249
Inverse-variance weighted	1.406	1.032-1.917	0.031
Simple mode	1.313	0.652-2.642	0.463
Weighted mode	1.343	0.692-2.606	0.404

E, Genus *Roseburia* (13 SNPs)

Method	OR	95% CI	P-value
MR-Egger	2.809	0.444-17.781	0.296
Weighted median	2.363	1.025-5.450	0.044
Inverse-variance weighted	1.867	1.011-3.446	0.046
Simple mode	3.275	0.795-13.495	0.126
Weighted mode	3.349	0.717-15.633	0.150

Table I. Continued.

F, Genus *Ruminococcaceae* UCG004 (11 SNPs)

Method	OR	95% CI	P-value
MR-Egger	1.057	0.066-17.006	0.970
Weighted median	0.688	0.348-1.363	0.284
Inverse-variance weighted	0.577	0.353-0.943	0.028
Simple mode	0.803	0.257-2.504	0.713
Weighted mode	0.861	0.258-2.869	0.812

G, Genus *Ruminococcaceae* UCG009 (12 SNPs)

Method	OR	95% CI	P-value
MR-Egger	0.859	0.167-4.405	0.859
Weighted median	0.668	0.379-1.179	0.164
Inverse-variance weighted	0.626	0.416-0.943	0.025
Simple mode	0.730	0.304-1.753	0.496
Weighted mode	0.720	0.315-1.649	0.454

H, Genus *Ruminococcaceae* UCG014 (11 SNPs)

Method	OR	95% CI	P-value
MR-Egger	2.112	0.591-7.551	0.280
Weighted median	1.654	0.800-3.422	0.175
Inverse-variance weighted	1.791	1.045-3.071	0.034
Simple mode	1.484	0.484-4.549	0.505
Weighted mode	1.674	0.710-3.942	0.266

I, Order Enterobacteriales (7 SNPs)

Method	OR	95% CI	P-value
MR-Egger	2.314	0.015-368.544	0.759
Weighted median	0.249	0.092-0.676	0.006
Inverse-variance weighted	0.346	0.153-0.783	0.011
Simple mode	0.245	0.063-0.958	0.090
Weighted mode	0.250	0.068-0.917	0.082

MR, Mendelian randomization; SNP, single nucleotide polymorphism.

disease progression of GC. Demiryas *et al* (25) demonstrated that patients with GC with increased homogeneity and diversity of flora compared with healthy controls. A study of 276 patients with GC demonstrated that the abundance of *Streptococcus* spp., *Clostridium* spp., *Crescentomonas* spp., *Propionibacterium* spp. and *Corynebacterium* spp. is increased in cancerous tissues (26). A Korean study concluded that *Prevotella* and *Propionibacterium acnes* are causative

Table II. Sensitivity analysis between gut microbiota and gastric cancer analyzed using the inverse-variance weighted method.

Gut microbiota	Q-value	P-value	Intercept	P-value	MRPRESSO
Family Enterobacteriaceae	6.133	0.293	-0.140	0.490	0.090
Genus Allisonella	4.167	0.654	0.098	0.563	0.808
Genus Lachnospiraceae FCS020	5.240	0.875	0.048	0.396	0.880
Genus Prevotella 7	4.591	0.868	-0.051	0.701	0.945
Genus Roseburia	6.133	0.293	-0.140	0.490	0.852
Genus Ruminococcaceae UCG004	9.301	0.410	-0.052	0.674	0.521
Genus Ruminococcaceae UCG009	7.668	0.661	-0.032	0.705	0.866
Genus Ruminococcaceae UCG0014	5.172	0.819	-0.016	0.786	0.939
Order Enterobacteriales	6.133	0.293	-0.140	0.490	0.079

MRPRESSO, Mendelian randomization Pleiotropy RESidual Sum and Outlier.

agents of GC, whereas *Lactococcus lactis* serves a protective role in the development of GC (27). Furthermore, *H. pylori* infection is a major risk factor for gastric carcinogenesis; however, the majority of infected individuals do not develop GC and significant genomic diversity of strains is associated with virulence factors (28).

Alterations in the gut microbiota may increase the susceptibility to GC through several mechanisms. Gastrointestinal flora produce a number of metabolites and enhance inflammatory responses, antagonize the development of tumors and activate alternative mechanisms via PI3K/AKT, MAPK, JAK-STAT and other signaling pathways, which can lead to disruption of the intestinal flora and further contribute to development of GC (29). The gastric microbiome and metabolome profiles of 37 cases of GC and matched non-tumor tissue were previously characterized by 16S ribosomal RNA technology. The relative abundance of amino acids, carbohydrates, carbohydrate conjugation, glycerophospholipids and nucleosides in GC tissue was revealed to be higher than that in non-tumor tissues (30). Furthermore, the combination of 1-methylnicotinamide and n-acetyl-D-glucosamine 6-phosphate is a reliable biomarker to distinguish GC from normal tissue (31). SCFAs mainly comprise acetic, butyric and propionic acids; these metabolites are important energy sources for gut microbes and epithelial cells, in addition to their different immunomodulatory functions (32). Previous studies have demonstrated that SCFAs serve a tumorigenic role by blocking activation of the NF-κB signaling pathway and inducing the differentiation of regulatory T cells (33,34). Among them, butyrate not only promotes energy metabolism and maintains a low-oxygen environment in the intestinal lumen, but also activates peroxisome proliferator-activated receptor γ in intestinal cells, inhibits expression of the nitric oxide synthase 2 gene and the synthesis of inducible nitric oxide synthase, decreases nitrate production and restricts proliferation of pathogenic anaerobic bacteria, thus inhibiting gastrointestinal tract inflammation and carcinogenesis (35). Bile reflux-generated bile acids are high-risk factors for GC and secondary bile acids promote GC cell proliferation (36). Therefore, the incidence of GC may be decreased by regulating specific types of bile acid.

To the best of our knowledge, the present study is the first to identify a causal association between gut microorganisms and GC, in which elevated abundance of the genera *Prevotella* 7, *Roseburia* and *Ruminococcaceae* UCG014 may increase the risk of GC. In addition, the family *Enterobacteriaceae*, the genera *Allisonella*, *Lachnospiraceae* FCS020, *Ruminococcaceae* UCG004 and *Ruminococcaceae* UCG009, and the order Enterobacteriales decreased the risk of GC. *Ruminococcus* is one of the earliest discovered gastric bacteria and serves a crucial role in metabolism. Cellulose is broken down by rumen bacteria to obtain nutrients. *Ruminococcus* is also capable of fermenting glucose and xylose. In addition to this function, it is able to stabilize the intestinal barrier, prevent diarrhea, reduce the risk of colorectal cancer, reduce kidney stone formation and increase energy (37). *Ruminalococcus* spp. has decreased abundance in ulcerative colitis, allergic disease and cerebral palsy, indicating its function as a beneficial bacterium (38). Notably, the results of the present reverse MR study did not support a causal association between GC and altered intestinal flora.

The causal relationship identified in the present study may provide candidate gut microbiota for subsequent functional studies. However, there are limitations. First, the threshold for screening the gut microbiome IVs was $P<1x10^{-5}$ and although measures were taken to ensure validity by calculating the F-statistic for each SNP, there is the possibility of false-negative errors due to insufficient statistical validity. Second, while the majority of patients in the GWAS pooled data were European, only a small number of gut microbiome data came from other ethnicities, which could lead to biased estimates and could affect generalizability. Third, due to the strict threshold, a number of genetic locus of the gut microbiota were excluded at the IV selection stage, which may have led to some results being missed.

In conclusion, the causal association between intestinal microorganisms and GC was investigated in the present study using MR analysis. The genera *Prevotella* 7, *Roseburia* and *Ruminococcaceae* UCG014 were associated with increased risk of GC, whereas the family *Enterobacteriaceae*, the genera *Allisonella*, *Lachnospiraceae* FCS020, *Ruminococcaceae* UCG004 and *Ruminococcaceae* UCG009, and the order Enterobacteriales reduced the risk of GC development, suggesting that intestinal microorganisms serve a role in the process of GC development and may have potential for the treatment of GC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JZ and CD designed the study. YL analyzed the data and wrote the manuscript. LS and JM collected the data. RH and HW revised the manuscript. JZ and YL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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