

Association Between Serum Total Bilirubin and COPD: Results from a Cross-Sectional Study and a Bidirectional Mendelian Randomization Analysis

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Background: The potential protective role of serum total bilirubin (TB) for chronic obstructive pulmonary disease (COPD) is controversial. We aimed to investigate whether serum TB could prevent airflow limitation (reduce the risk of COPD) and whether these associations were causal or reversely causal.

Methods: We conducted a multi-center and cross-sectional study including 3069 participants. Logistic regression model (LRM) with restricted cubic spline (RCS) and priori defined quintile categories were used to assess the associations of TB with COPD. Besides, ordinary least squares (OLS) regression model with RCS curves were applied to assess the dose-response relationship between serum TB and airflow limitation (FEV₁/FVC). To verify the causal direction between TB and COPD, a bidirectional Mendelian randomization analysis was carried out with GWAS data from European ancestry.

Results: In the cross-sectional study, the relationship between levels of TB and COPD risk was U shaped ($P=0.001$), and the low and high concentrations of TB apparently increasing the risk of COPD (OR 1.40, 95% CI 1.07 to 1.82 for less than 9 $\mu\text{mol/L}$; OR 1.36, 95% CI 1.06 to 1.76 for 9.01–10.88 $\mu\text{mol/L}$; OR 1.50, 95% CI 1.16 to 1.95 for more than 13 $\mu\text{mol/L}$). There was a significant non-linear relationship between TB and FEV₁/FVC (non-linear $p=0.004$). Furthermore, results of bidirectional Mendelian randomization analysis (OR 1.000; 95% CI 0.983 to 1.017 for MR and OR 0.998; 95% CI 0.976 to 1.020 for reversal MR) did not support the causal effects between serum TB and FEV₁/FVC after controlling the effect of potential confounders and revised causality.

Conclusion: Our study reveals that there was non-linear dose-response pattern between serum TB and COPD. However, there was little evidence for the linear causal associations of serum TB with airflow limitation. The relationship of TB with COPD needs further study and careful interpretation.

Keywords: total bilirubin, COPD, airflow limitation, Mendelian randomization analysis

Introduction

Chronic obstructive pulmonary disease (COPD) is manifested as persistent respiratory symptoms and incompletely reversible airflow limitation resulted from the exposures to noxious particles or gases.¹ High prevalence of COPD worldwide results in significant health burden with high rates of morbidity and mortality.^{2,3} The high burden of disease motivates us to pay more attention to the prevention and management in the early stage COPD. Biomarkers are regarded as important approaches to define the high-risk population for COPD and prevent the disease progression.

As we know, one of the most important mechanisms in the pathogenesis of COPD is oxidative stress. Serum total bilirubin (TB), as an end-product of heme degradation, was considered to be a physiologically active and endogenous antioxidant.⁴ And it has been recently confirmed to have protective effects in some aspects by antagonizing oxidative

stress. So far, mild higher serum TB levels has been evidenced to associate with decreased risks of cardiovascular disease via against oxidative stress or inflammation.^{5,6} Likely, the consistent associations were also found in pulmonary diseases including COPD in some animal experiments and prospective studies. For example, a rat model of COPD demonstrated that exogenous administration of bilirubin reduced lung inflammation, suppressed regional oxidative lipid damage and attenuated smoking-induced pulmonary emphysema.⁷ In the Lung Health cohort Study with 11 follow-up years, Scott Apperley etc. found that higher TB concentration was relative to less disease severity and less disease progression in COPD.⁸ Brown etc. showed that higher bilirubin was associated with a significantly lower hazard for AECOPD in the validation MACRO study in moderate-to-severe COPD.⁹ Besides, a significant improving effects of serum TB on FEV₁/FVC in a general population sample were demonstrated as well.^{10,11} Therefore, TB has been strongly advocated to be one of the candidate biomarkers for COPD. However, other studies discovered that serum TB is non-significantly related to FEV₁/FVC even after adjustment for confounding factors.^{12,13} A review published in 2021 also revealed that there is still short of evidence for the influence of raised TB levels on airflow obstruction (FEV₁/FVC ratio).¹⁴ Therefore, it is inconsistent concerning the potential effects of TB on COPD.

Since the controversial conclusions, we designed a multi-center and cross-sectional study to investigate the potential role of TB on COPD. To our best knowledge, it is the first study to apply linear regression model with RCS to assess the non-linear relationship between TB on COPD in the overall population. To overcome problems of residual confounding or reverse causation in the TB-COPD association, a bidirectional Mendelian Randomization analysis was conducted with large published data from genome-wide association studies (GWAS).

Methods

Study Subjects

Our observational study recruited subjects from several communities of five cities (Guangzhou, Zhanjiang, Heyuan, Shaoguan and Huizhou) in Guangdong, China from January 2012 to December 2020. It initially consisted of 3280 participants comprising of COPD patients and people with normal lung function. COPD was diagnosed via a post-bronchodilator FEV₁/FVC <0.7. Among 3280 subjects, 3069 people were included in the final analysis according to the inclusion and exclusion criteria. Participants with completed lung function test and bilirubin measurement and sufficient data on questionnaire were included. Participants were excluded if they had comorbidities that might significantly influence bilirubin concentrations such as hemolytic disorders, hepatobiliary diseases (including malignancy, bile duct blockage, chronic hepatitis, and cirrhosis) or renal insufficiency before bilirubin measurement via self-report. Moreover, TB concentrations >1.75 mg/dL for women and >2.34 mg/dL for men were also excluded from analysis for excluding population with Gilbert syndrome, known as a benign hereditary disease with indirect hyperbilirubinemia caused by the common UGT1A1 genotypes.¹⁵ This study was performed in line with the principles of the Declaration of Helsinki. Written informed consent was attained from all the participants. And the study was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University.

Lung Function Test

Lung function test including pre-bronchodilator and post-bronchodilator (Salbutamol Sulfate Aerosol, 400 ug, 20 min later) measurements was performed by conventional spirometers (CareFusion MasterScreen Pneumo, Germany). The operational manoeuvres and quality control standards were carried out according to the American Thoracic Society and European Respiratory Society.^{16,17} The indicators of lung function included in the article were referred to post-bronchodilator data.

Bilirubin Measurement

All participants were required to be fasting for more than 8 hours before bilirubin measurement. Venous blood samples were collected by well-trained staff, separated by centrifugation at 3000 rpm for 10 minutes at 4 °C, stored at a temperature below -80°C as soon as possible¹⁸ and measured no more than 8 hours at the local clinical laboratory. The reports would be obtained on that day or seldomly the second day. The data on TB (total bilirubin, direct bilirubin,

and indirect bilirubin) were abstracted from the blood test results. The units of bilirubin used in this analysis were $\mu\text{mol/L}$.

Questionnaire

To acquire information about demography, smoke, respiratory symptoms, comorbidities, and the Medical Research Council dyspnea scale, We applied the standardized respiratory epidemiological questionnaire recommended by Zhong et al.¹⁹ The questionnaire was revised from the international Burden of Obstructive Lung Diseases study.²⁰ Smoke index is referred to the mean packs of cigarettes daily multiplied by the years of smoking. Smoking status was defined as never smoking, former smoking and current smoking. We identified never smokers by someone who has smoked totally less than 100 cigarettes up until now. Participants who smoked previously but had quit for at least 6 months are recognized as former smokers, and the others were current smokers.

Statistical Analysis

Continuous variables are presented as means \pm standard deviations (SDs), and categorical data are described as numbers (%) for baseline information of overall population. We fit the logistic regression model with RCS to evaluate the association of serum TB with COPD.

To address the relationship between the best fit and over fitting, we chose the numbers of the knots from three to seven to observe and compare the fitted curve. In accordance with the lowest value for Akaike information criterion, a criterion for goodness of model, we finally selected 3 knots in the main spline curves for COPD. Afterwards, we further applied quintiles to transform a continuous scale of TB into five categories to assess the OR value across different limits, which was performed with Binary logistic regression model. The centiles of continuous bilirubin concentrations were cut at 20th, 40th, 60th and 80th respectively. We took the category of TB levels which covered the lowest OR value for COPD from the non-linear curve as a reference. In order to make the direct comparison of other lung function outcomes (FEV_1/FVC) possible, the same number of knots with RCS based on Ordinary Least Squares regression model was also used to screen the non-linear dose-response association of TB with FEV_1/FVC . All fitted models mentioned above were all adjusted for age, sex, body mass index (BMI) and smoke index. Two-sided $p < 0.05$ were considered statistically significant. The statistical analyses were performed in IBM SPSS Version 25.0 and the statistical software R version 4.0.3.

MR Analysis

Bidirectional MR based on two-sample MR analysis is a method to evaluate the direction of causality between exposure and outcome with Genetic Instrumental Variables. We drew summary data relative to serum TB from GWAS provided by Neale Lab Consortium (ID: ukb-d-30840_irt), which comprises of 342,829 individuals. While the summary data for COPD (FEV_1/FVC) were abstracted mainly from UK Biobank (ID: ebi-A-GCST007431) in a large sample size of 321,047.²¹ The population included in our analysis were of European ancestry. The genotype quality control including missing rate, Hardy-Weinberg principle, as well as minor allele frequency of GWAS data, and sample quality control have been performed by the Neale lab group according to their own standards before the data was public to us. To strengthen again the validity of genetic instruments filtered, we retrieved genotype single nucleotide polymorphisms (SNPs) at the genome-wide significance threshold ($P < 5 \times 10^{-8}$, $R^2 < 0.001$),²² pruned them for linkage disequilibrium (LD) clumping and exclude palindromic ones. Eventually, 98 SNPs were remained to construct instrument variables between TB and FEV_1/FVC . Moreover, we also calculated the R-squared value of 98 SNPs ($R^2 = 0.166$), which explains for averaged 16.6% of the variance of exposure. And the F statistic was 696,²³ far satisfying the required threshold of $F > 10$ and ensuring the powerful validity of instruments. Applying similar selection procedures, the number of genetic instruments constructed for FEV_1/FVC (exposure) and TB (outcome) in MR analysis in the opposite direction was 246 and the F statistic was 33.

In order to get reliable estimates, inverse variance weighted (IVW) model, MR-Egger regression method and the weighted median estimator were all applied to the analyses and test the unbalanced horizontal pleiotropic effects. And then the beta and standard error (SE) coefficients obtained from the MR estimates would be transformed to odds ratios

and 95% confidence interval (CI) for forest plots. The leave-one-out method was conducted as an analysis of sensitivity to check whether the MR estimate is driven by a single SNP. We made a visible funnel plot for heterogeneity by showing the association of the inverse of the SE with the MR estimate. In regard to reverse MR, the procedures mentioned above were repeated via swapping exposure (TB) and FEV₁/FVC with the same database to assess the reverse-causal association of TB and COPD. All the analyses were conducted on the professional platform of MR-base (<http://app.mrbase.org/>), following the protocol provided by Hemani et al,²⁴ and software R version 4.0.3 (Forest plot packages).

Results

Study Population

In accordance with the predefined inclusion and exclusion criteria, 211 participants were excluded out of our observational study and 3069 participants were finally included into the analysis. The reasons for 211 subjects excluded were listed in detail in the flow chart (e-Figure 1, Supplemental materials). In this study, the mean age was 58.5 years old and the ratio of smokers of them was 54.3. The average concentrations for TB was 11.54 µmol/L. Table 1 summarizes Other baseline characteristics in detail after all individuals were categorized into two groups of COPD (n=1173) and non-COPD (n=1896).

Associations of TB Levels with the Risk of COPD and FEV₁/FVC

In our analysis, the exposure-response relationship between TB levels and the risk of COPD incidence was U-shaped after adjustment for sex, age, BMI and smoke index. (non-linear p=0.001; Figure 1A). To assess the non-linear association furtherly, we applied quintiles to classify the TB levels into five categories. Compared with the reference range covering the lowest OR value attained from RCS curve, the multiple adjusted LRM model showed that both higher range (ie, TB >13 µmol/L; 81st-100th centiles) and lower limit (ie, TB <9 µmol/L; 1st-20th centiles) increase the risk of

Table 1 The Baseline Characteristics of Study Population

Characteristics	All (n=3069)	COPD (n=1173)	Non-COPD (n=1896)
Sex, male, n(%)	1941(63.2)	1010(86.1)	931(49.1)
Age, yr	58.5±10.7	64.5±8.0	54.8±10.5
Height, cm	158.0±8.2	160.0±7.4	156.7±8.4
Weight, kg	57.1±10.7	56.4±10.3	57.6±10.9
BMI, kg/m ²	23.0±10.7	22.0±3.4	23.6±13.3
Smoking, n(%)	1668(54.3)	951(81.1)	717(37.8)
Smoking status			
Never smoker, n(%)	1402(45.7)	222(18.9)	1180(62.2)
Former smoker, n(%)	510(16.6)	313(26.7)	197(10.4)
Current smoker, n(%)	1157(37.7)	638(54.4)	519(27.4)
Smoking index, pack*yr	19.6±28.4	31.3±31.9	12.4±23.3
Comorbidities, n(%)	1474(48.0)	770(65.6)	704(37.1)
Respiratory symptoms, n(%)	1356(44.2)	730(62.2)	626(33.0)
mMRC score	0.25±0.57	0.45±0.70	0.14±0.42
TB, µmol/L	11.54±3.37	11.75±4.11	11.36±2.89
DB, µmol/L	3.57±1.85	3.50±2.01	3.55±1.76
IB, µmol/L	7.98±2.96	8.26±3.52	7.81±2.62
Post bronchodilation			
FVC, L	3.09±0.77	3.19±0.79	3.02±0.75
FEV ₁ , L	2.22±0.66	1.89±0.61	2.43±0.60
FEV ₁ /FVC, %	72.3±13.3	58.6±9.8	80.8±6.1
FEV ₁ , % predicted	91.3±21.4	76.5±21.8	100.5±15.2

Notes: Data are shown as mean ± SD for continuous variables and n (%) for categorical variables. BMI was calculated as weight in kilograms divided by the square of height in meters (kg/m²). Smoking index was defined as the packs of cigarettes smoked daily multiplied by total years of smoking (pack*yr). Comorbidities were defined as other diseases diagnosed by clinical doctors except COPD.

Abbreviations: mMRC, modified British Medical Research Council; TB, total bilirubin. DB, direct bilirubin. IB, indirect bilirubin.

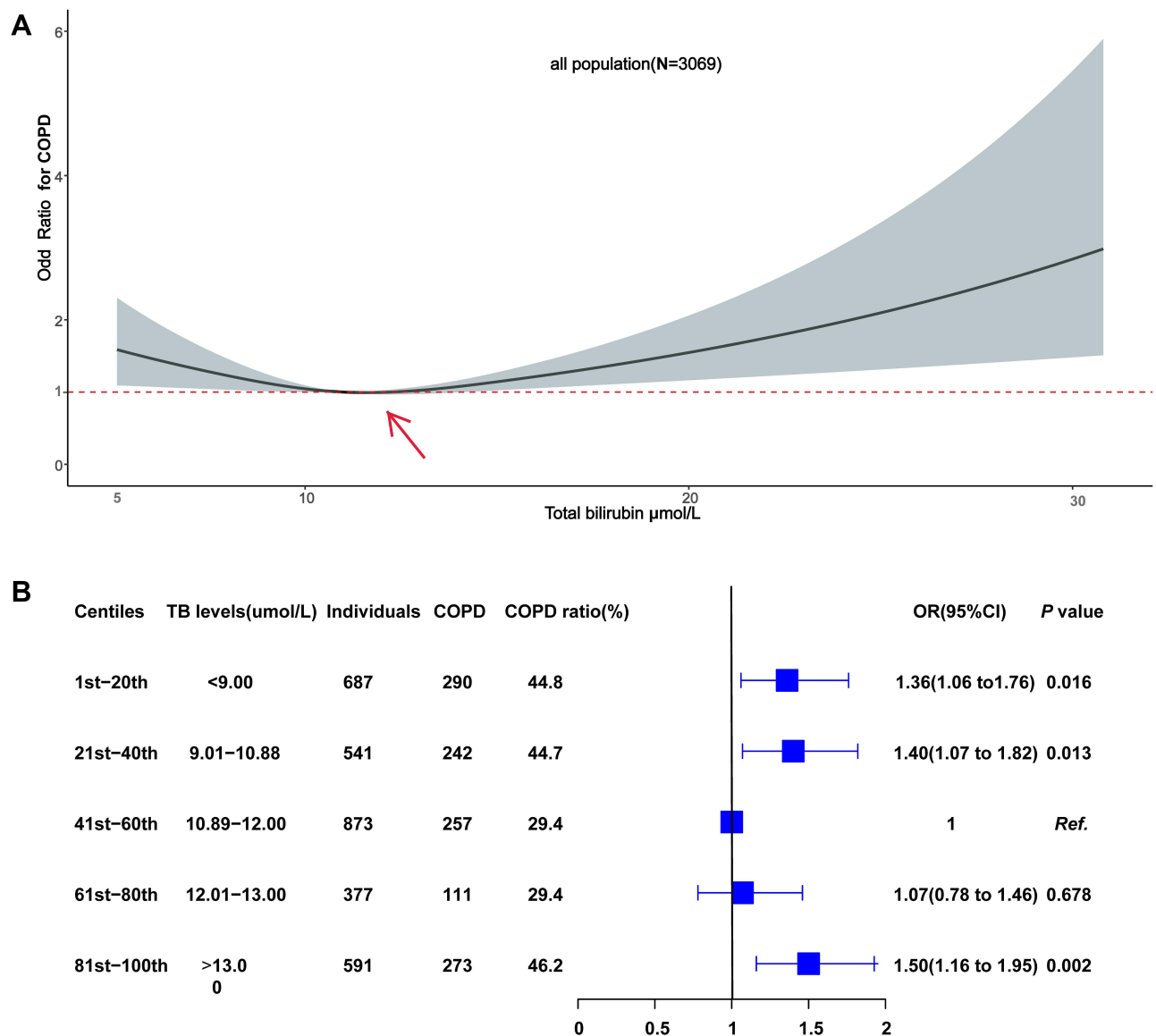


Figure 1 (A) The dose-response effect of TB on COPD as a categorical variable in the overall population. Solid black curves are multivariable-adjusted odds ratios, with light blue area showing 95% confidence intervals derived from restricted cubic spline regressions with three splines based on Logistic regression model. The reference line for no association is indicated by dashed red line at a odds ratio of 1.0. The red arrow represents the approximate cut-off value of bilirubin. The model was adjusted for age, sex, BMI and smoke index. **(B)** Forest plot quantifying associations of TB categorized into five centiles with the risk of COPD. The reference range is defined to cover a limit at a odds ratio of 1 derived from curves in A. The model was adjusted for age, sex, BMI and smoke index.

COPD (OR 1.50, 95% CI 1.16 to 1.95 for higher range; OR 1.36, 95% CI 1.06 to 1.76 for lower range). Besides, the OR value for TB range of 9.01–10.88 $\mu\text{mol/L}$ (21st–40th centiles) is 1.40 (95% CI 1.07 to 1.82). But the TB range of 12.01–13 $\mu\text{mol/L}$ (61st–80th centiles) did not increase the risk of COPD occurrence (OR 1.07, 95% CI 0.78 to 1.46) (Figure 1B). Furthermore, we can get the similar conclusions from Figure 2. The n-shape relationship between TB levels and FEV_1/FVC suggested that only mild higher TB levels (from 5 $\mu\text{mol/L}$ up to 13 $\mu\text{mol/L}$) in physiological range would prevent airflow limitation, thus decrease the risk of COPD (non-linear $p=0.004$). Otherwise, it would be associated with declined FEV_1/FVC beyond that range (>13 $\mu\text{mol/L}$ approximately).

Mendelian Randomization Analyses of TB and FEV_1/FVC

Two GWAS with large-scale population were included in Mendelian Randomization analysis (e-Table 1). The details about Genetic predisposed instruments constructed by 98 SNPs for TB and 246 SNPs for FEV_1/FVC were listed in e-Tables 2 and

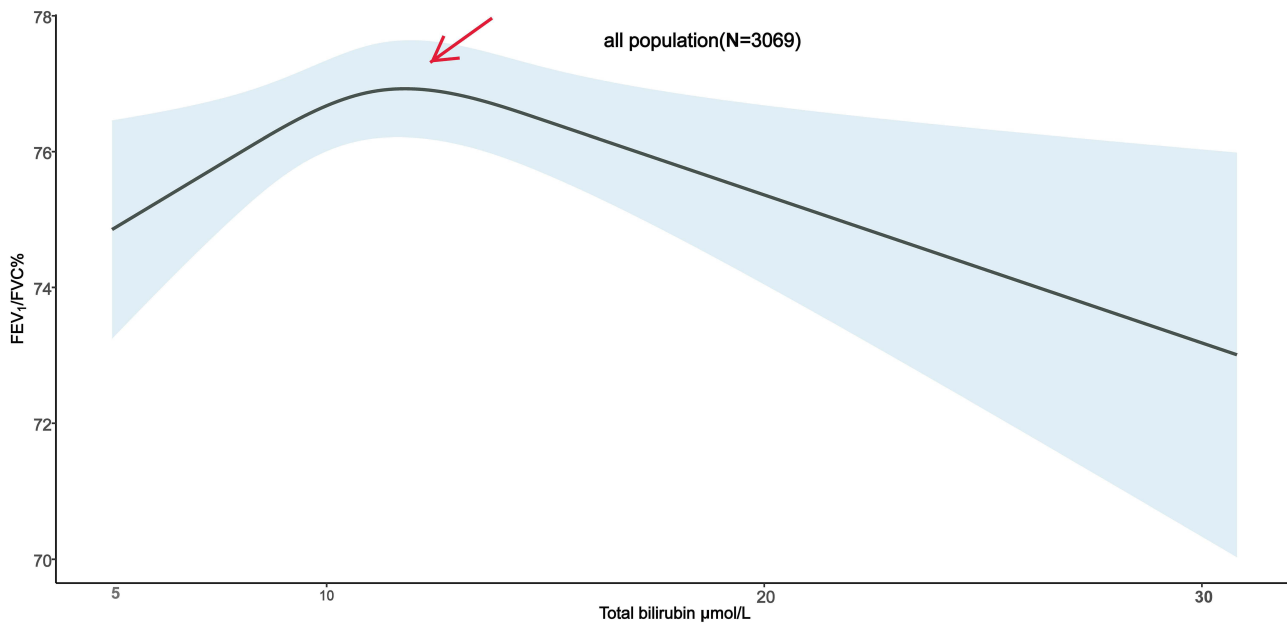


Figure 2 The dose-response effect of TB on FEV1/FVC on a continuous scale in the overall population. Solid black curves show the dose-response effect, with light blue area showing 95% confidence intervals derived from restricted cubic spline regressions with three splines based on Ordinary Least Squares model. The red arrow represents the approximate cut-off value of bilirubin. Analyses were adjusted for age, sex, BMI and smoke index.

3 respectively. The MR analysis with four different methods all showed no evidence for causal or reversely causal effect between TB and FEV₁/FVC (Figure 3: OR 1.000; 95% CI 0.983 to 1.017 for IVW MR and OR 0.998; 95% CI 0.976 to 1.020 for reversal IVW MR). Furthermore, the intercepts of the MR-Egger regression and reversal MR-Egger regression were all not significantly different from zero (e-Table 4, Supplemental materials; intercept 0.00043, 95% CI -0.00128~0.00214 for MR= 0.622; intercept 9.5e-07, 95% CI -0.00128~0.00127 for reversal MR), suggesting that almost none pleiotropy driving the MR results. And findings from leave-one-out sensitivity analysis (e-Figures 2 and 4) and funnel

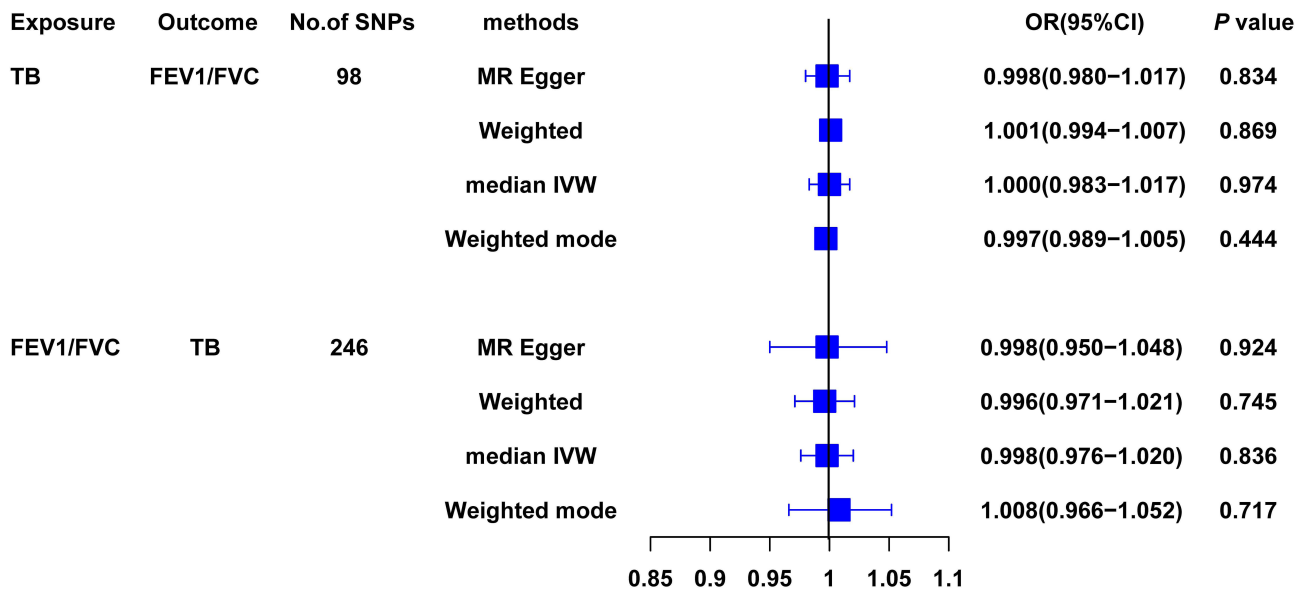


Figure 3 The forest plot for Estimates of causal effects between serum TB and FEV1/FVC. Both estimates coefficients of MR and reverse MR calculated using four methods including MR Egger, Weighted median, Inverse variance weighted and Weighted mode, and transformed for odd ratio and 95% CI. **Abbreviations:** TB, total bilirubin; OR, odds ratio; No., numbers; CI, confidence interval.

plots (e-Figures 3 and 5) also consolidated our conclusions mentioned above by indicating no support for pleiotropic effects and obvious heterogeneity.

Discussion

Our cross-sectional study confirmed the potential protective role of TB in airflow limitation only in a limited range (5–13 $\mu\text{mol/L}$ approximately). However, there was little evidence for a linear causal association of TB with COPD in the bidirectional MR analyses.

In the cross-sectional study, we revealed that only in moderately higher TB levels in normal physiological concentration could be associated with lower occurrence of COPD significantly, which was consistent with conclusions from previous researches.²⁵ For instance, Horsfall et al²⁶ disclosed that moderately higher levels of bilirubin within the normal scale were relative to reduce incidence of COPD in their cohort study among 504,206 subjects. Moreover, the approximate physiological range with protective role of 5–13 $\mu\text{mol/L}$ could be attained from both the RCS fitted curves from Figure 2. To the best of our knowledge, it was only reported that in stratified analyses of male adults abstracted from the UK primary care research database,²⁶ higher bilirubin level (less than 17 $\mu\text{mol/L}$) was significantly related with lower incidence rate of COPD. However, the cut off limit for the all population has not been reported previously. This is because we used LRM and OLS model with RCS to evaluate the dose-response effects of TB on COPD in a continuous scale in the overall population. And it is different from previous studies utilizing TB concentrations as categorical variables. Unlike categorization, the method we used to fit the model took advantage of the full information available in data and flexibly captured dose-response relationships even non-linearity. Despite few investigations about the scale of protective effects of TB on COPD, the related limits or cut-off values of bilirubin were not uncommon in other diseases. For systemic diseases consisting of cardiovascular disease (CVD),²⁷ it has been reported that it would increase the risk of occurrence when less than 7 $\mu\text{mol/L}$.²⁸ Additionally, Vitek etc. also found that the serum bilirubin concentration of 10.0 $\mu\text{mol/L}$ was the cut-off discriminating for the CVD risk.²⁹ In spite of the difference of the cut-off values across various researches, our data also further confirm the protective effects of TB on COPD within the limited extent.

The underlying mechanisms of how moderate levels of bilirubin reducing the risk of COPD are unknown. We could only reasonably speculate about it with some evidence from the current literature. A moderate physiological scale of bilirubin was reported to have powerful cytoprotective properties, including antioxidant, anti-inflammatory, and anti-proliferative effects in lung injured models.^{4,30} COPD is a chronic pathological process accompanied by endogenously or exogenously produced oxidative stress and inflammation.^{31,32} Oxidant insults especially smoking significantly reduces serum bilirubin concentrations, which will rapidly increases shortly after smoking cessation.³³ Bilirubin may be against oxidative stress and suppress inflammation by consuming itself, thus reduce the risk of COPD. If surpass these limits, bilirubin is considered toxic for cell functions³⁴ and may cause pulmonary injury or COPD in potential pathways, which was consistent with what we have found mentioned above.

Another worth highlighting is that the study represented the first attempt to take advantage of bidirectional Mendelian Randomization analysis to test the causality of TB with airflow limitation as well. This approach makes up some potential shortcomings of observational studies such as the presence of confounding variables and reverse causality. No evidence was indicated from the analysis to support the causal effects between TB and COPD. It is suggested that findings from previous investigations and our research might be biased by confounding factors. Except sex, age, BMI and smoke adjusted for our data, risk factors such as alcohol intake, physical activity,³⁴ diet³⁵ and Seasonal variations³⁴ were proved previously to affect TB concentrations. Moreover, it has been found that after adjustment for multiple health indicators, the regression estimate for COPD in male adults was a 6% decrease per 0.1 mg/dL increase in bilirubin level. Similarly, the results were seen for COPD in women as well.²⁶ In line with this, the association may disappear and result in no evidence of direct protective effects of TB on COPD after balancing other unknown potential confounders. Therefore, the actual action bilirubin performed on COPD remains inconclusive in conventional observational studies. Additionally, other several advantages of our study are as follows. A large sample size enough of total 663,876 individuals from WGAS database was used in MR analysis. Notably, both the F-statistics over the threshold and the numbers of SNPs allowed the instruments used to strongly predict exposures.

However, there are two limitations in our study. One is that we lacked specific information about other risk factors including alcohol consumption influenced the levels of bilirubin in the finite original data, which restricted us to comprehensively assess the associations. The other one is that due to the nature of MR, we could not detect assumption II and III.³⁵ So that the results should be explained carefully because of the likely potential existence of pleiotropy and heterogeneity. Nevertheless, we also took multiple measures to ensure the power of our results. For instance, the intercept for MR-Egger was not statistically significant. Likewise, both leave-one-out analysis and funnel plot confirmed the consequences as well. All of these indicate that the genetic instruments we constructed do not exist apparent pleiotropy and distinctive heterogeneity.

Our study findings might affect the extensive debate on the relationship between TB and COPD and the result of cross-sectional study implied that the cut off value provided from the first session suggested that individuals with higher TB concentrations ($>13\mu\text{mol/L}$ approximately) may benefit from closer monitoring of lung function test to facilitate early detection and diagnosis of COPD. However, the results from the MR analysis were inconsistent. Before definite conclusions, more covariable data on TB (such as season or alcohol consumption) are needed in future observational studies or prospective cohort study for a comprehensive assessment. And more targeted validation experiments on animal models are necessary to assess the effect of TB in the future.

In conclusion, we found only a dose-response association of non-linearity between TB and COPD without causality or reverse causality in our study. It is indicated that mild higher TB concentration may not be a direct protective determinant for COPD via itself. The effects of TB on COPD by means of what kind of approaches need to further estimate in animal model experiments and best-design cohort study with enough sample size. So that we should attach more caution to interpreting TB as biomarker of COPD.

Abbreviations

TB, total bilirubin; COPD, chronic obstructive pulmonary disease; LRM, logistic regression model; RCS, restricted cubic spline; OLS, ordinary least squares; MR, Mendelian randomization; GWAS, genome-wide association studies; SNP, single nucleotide polymorphism; OR, odds ratio.

Data Sharing Statement

The datasets used and analyzed in the cross-sectional study are available from the corresponding author on reasonable request. The data in the MR analysis could be attained from GWAS provided by Neale Lab Consortium (ID: ukb-d-30840_irt) and UK Biobank (ID: ebi-A-GCST007431).

Ethics Approval and Informed Consent

This study was performed in line with the principles of the Declaration of Helsinki. All the participants gave written informed consent and the study was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare they have no real or potential competing interests in this work.

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