

The Protective Effect of Bilirubin on MAFLD May Be Mediated by Improving Insulin Re-Sistance and Alleviating Chronic Inflammation

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Background: Bilirubin, as a potent endogenous antioxidant, has demonstrated protective effects in various metabolic and inflammatory diseases. However, the precise role and underlying mechanisms of bilirubin in metabolic-associated fatty liver disease (MAFLD) remain unclear.

Methods: This study involved 3000 participants, categorized into non-MAFLD and MAFLD groups. Using weighted multiple linear regression and mediation effect analysis, this study examined the protective impact of total bilirubin (TBIL), direct bilirubin (DBIL), and indirect bilirubin (IBIL) on MAFLD risk. Additionally, potential mediators-inflammation and insulin resistance (IR) through which bilirubin exerts its protective effects were explored.

Results: TBIL and DBIL levels in the MAFLD group were significantly lower than those in the non-MAFLD group. Multiple linear regression analysis, adjusted for confounding variables, revealed that compared to the lowest tertile group (TBIL < 14.6), the odds ratios (ORs) for the middle tertile (TBIL 14.6–19.2) and the highest tertile (TBIL ≥ 19.3) groups were 0.735 and 0.615. Similarly, compared to the lowest tertile group (DBIL < 3.4), the ORs for the middle tertile (DBIL 3.4–4.4) and the highest tertile (DBIL ≥ 4.5) groups were 0.613 and 0.367. Mediation analysis revealed significant indirect effects of SIRI, PIV, TyG, TyGBMI, METS-IR, and AIP on the relationship between TBIL, DBIL, and MAFLD risk. Specifically, SIRI mediated 4.07% and 1.55% of the TBIL-MAFLD and DBIL-MAFLD associations, respectively; PIV mediated 9.56% and 4.22%; TyG mediated 69.27% and 81.91%; TyGBMI mediated 100% and 78.34%; METS-IR mediated 100% and 81.41%; and AIP mediated 100% for both TBIL-MAFLD and DBIL-MAFLD associations.

Conclusion: Our findings suggest that increased serum levels of TBIL and DBIL are significantly inversely correlated with MAFLD risk, with both serving as independent protective factors against MAFLD occurrence. Further mediation analysis indicates that this protective effect is likely mediated by improvements in IR and the alleviation of systemic chronic inflammation.

Keywords: bilirubin, metabolic-associated fatty liver disease, MAFLD, insulin resistance, IR, systemic chronic inflammation, mediation effect analysis

Introduction

Plasma bilirubin primarily results from the turnover of red blood cells in the spleen. The heme released from red blood cells is converted into biliverdin by the rate-limiting enzyme heme oxygenase-1 (HO-1), which is subsequently reduced to bilirubin by biliverdin reductase (BVR). Total bilirubin (TBIL) is divided into conjugated/direct bilirubin (DBIL) and unconjugated/indirect bilirubin (IBIL) based on its binding to glucuronic acid in the liver. Historically, bilirubin has been viewed as a toxic byproduct of bile, with significantly elevated serum bilirubin levels considered indicative of severe liver disease. However, it is now well-established that bilirubin is not merely a waste product of heme catabolism, but a potent endogenous antioxidant with significant physiological roles. Among various antioxidants, bilirubin demonstrates

the most powerful activity in scavenging oxygen free radicals.¹ Bilirubin directly neutralizes and scavenges reactive oxygen species (ROS) through its conjugated double bond and active hydrogen atom, and inhibits NADPH oxidase activity to reduce the generation of superoxide anion radicals.^{2,3} In addition to its classic antioxidant effect, a large number of basic studies have confirmed that bilirubin can exert its protective effects in various metabolic diseases and chronic inflammatory diseases through multiple molecular mechanisms, such as activating the Peroxisome Proliferator-Activated Receptor α (PPAR α) signaling pathway, enhancing the level of peroxisome proliferator-activated receptor γ (PPAR γ) in the liver, improving insulin signaling pathways in multiple tissues, regulating cholesterol metabolism, inhibiting the expression of pro-inflammatory factors, reshaping mitochondrial activity, and suppressing endoplasmic reticulum stress.⁴⁻⁹

Numerous studies have demonstrated a significant negative correlation between serum bilirubin levels and metabolic syndrome (MetS) and related chronic inflammatory conditions such as atherosclerosis and hypertension. Even a modest increase in serum bilirubin concentration appears to carry substantial biological significance, as each micromolar rise (even within the physiological range) is associated with a marked reduction in the risk of oxidative stress and inflammation-driven diseases. Research has shown that mild elevations in serum bilirubin, such as in Gilbert's syndrome (characterized by the UDP-glucuronosyltransferase 1A1 (UGT 1A1)*28 polymorphism leading to a decrease in enzyme activity), provide significant protection against various diseases, including cardiovascular disease, type 2 diabetes, and MetS.^{10,11} Specific mutations in the UGT 1A1 gene lead to mild chronic hyperbilirubinemia. As the core enzyme in bilirubin metabolism, UGT1A1 is the only enzyme responsible for converting unconjugated bilirubin into water-soluble conjugated bilirubin, facilitating its biliary excretion through glucuronidation.^{12,13}

Therefore, given the role of bilirubin in metabolic diseases and its various beneficial biological mechanisms, research on bilirubin as a potential therapeutic strategy in metabolic and chronic inflammatory diseases has become a hot topic. Supplementing with exogenous bilirubin is undoubtedly the most direct method, but bilirubin has poor water solubility, making it difficult to transmit through traditional mechanisms. In recent years, studies have prepared polyethylene glycol conjugated bilirubin nanoparticles, significantly improving the solubility of bilirubin in aqueous solutions and achieving good results in animal experiments. Bilirubin nanoparticles have shown promise as an adjunctive treatment for inflammatory and metabolic disorders.^{14,15} Another approach is to increase endogenous bilirubin production. At present, a strategy has been proposed to treat or prevent atherosclerotic vascular diseases, that is, to induce "iatrogenic Gilbert syndrome" by reducing the activity of hepatic glucuronidation.^{16,17} This method may also be used for NAFLD. The protective effects of elevated bilirubin within the physiological range in preventing the onset and progression of metabolic diseases underscore the importance of further exploration into its mechanisms.

Metabolic-associated fatty liver disease (MAFLD) is a multifactorial condition with a complex pathogenesis, involving multiple interconnected mechanisms such as insulin resistance (IR), systemic chronic inflammation, obesity, and genetic predisposition. Visceral fat accumulation serves as the initial trigger in the development of MAFLD. Subsequently, factors including increased free fatty acids, IR, and the production of inflammatory mediators contribute to hepatic steatosis. Endoplasmic reticulum stress and oxidative stress, primarily resulting from mitochondrial dysfunction, exacerbate hepatocyte necrosis and apoptosis.¹⁸⁻²¹ Both IR and chronic inflammation serve as crucial pathological links in the development of MAFLD, interacting and mutually reinforcing each other, forming a "bidirectional vicious cycle" in the progression of MAFLD. On the one hand, insulin resistance promotes fat deposition in the liver. Liver lipid overload leads to mitochondrial dysfunction and enhancement of peroxisomal β -oxidation, generating ROS and activating pro-inflammatory signaling pathways (such as NF- κ B, JNK), inducing the release of pro-inflammatory factors such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6).^{22,23} On the other hand, inflammatory factors such as TNF- α and IL-6 inhibit tyrosine phosphorylation of insulin receptor substrates (IRS), block the PI3K/AKT signaling pathway, and further exacerbate liver and peripheral insulin resistance by interfering with the insulin signaling pathway.^{24,25}

Although several studies^{26,27} have reported an inverse relationship between higher serum bilirubin levels within the physiological range and reduced MAFLD risk and all-cause mortality, suggesting that bilirubin may serve as a potential protective factor for MAFLD, the specific subtype of bilirubin responsible for this effect remains a matter of debate. Little is known about whether different bilirubin subtypes (including DBIL and IBIL) exhibit distinct biological activities and functions, and whether they play varying roles in metabolic diseases. Furthermore, previous research findings have

been inconsistent. Some studies^{28,29} have found that DBIL is negatively correlated with the onset of Non-Alcoholic Fatty Liver Disease (NAFLD), while there is no significant correlation between IBIL and NAFLD. Some believe that DBIL is soluble in serum, making it more likely to exist in an active form compared to IBIL. On the contrary, some studies^{30,31} have shown that IBIL is negatively correlated with the incidence or severity of nonalcoholic steatohepatitis (NASH). Some studies, however, have failed to establish a clear causal link between bilirubin and non-alcoholic fatty liver disease (NAFLD) through Mendelian randomization analyses.^{32,33} Therefore, the precise impact of TBIL, including DBIL and IBIL subtypes, on the risk of MAFLD and its underlying mechanisms remain largely unclear, urgently necessitating further research to explore.

This study investigated the association between TBIL and its subtypes (DBIL and IBIL) and the risk of MAFLD in a Chinese cohort. Additionally, their complex relationship with systemic chronic inflammation and IR, as well as the mediating role of these factors in the bilirubin-MAFLD association, were explored. Understanding the role of bilirubin and its molecular mechanisms could provide insights into potential therapeutic targets for MAFLD and related metabolic disorders.

Methods

Inclusion and Exclusion of Research Subjects

This retrospective analysis extracted data from individuals who underwent physical examinations at Fuyang People's Hospital between August and December 2024. A total of 3000 subjects were enrolled and categorized into two groups according to the diagnostic criteria for MAFLD: the non-MAFLD group (1603 participants) and the MAFLD group (1397 participants). Cases with incomplete clinical or laboratory data were excluded.

The diagnostic criteria for MAFLD in the Chinese population adhered to the English version of the "Chinese MAFLD Prevention and Treatment Guidelines".³⁴

The research protocol was approved by the Ethics Committee of Fuyang People's Hospital (Ethics Approval No.: [2024-224]). Due to the retrospective nature of the study, written informed consent from participants was waived.

This retrospective study utilized de-identified patient data approved by the Ethics Committee of Fuyang People's Hospital. No personally identifiable information was retained, and data access complied with institutional security protocols to prevent unauthorized use.

Clinical Data and Laboratory Examination

Clinical and laboratory data were collected through questionnaires and the hospital's electronic medical examination system. Clinical information included participant demographics (gender, age, and body mass index [BMI = weight (kg) / height (m)²]), as well as medical history of hypertension and diabetes. Laboratory data comprised white blood cell count (WBC), neutrophil count (NEU), lymphocyte count (LYM), monocyte count (MONO), platelet count (PLT), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -glutamyltranspeptidase (GGT), fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL-C), and low-density lipoprotein (LDL-C) levels, along with liver and gallbladder ultrasound results. Inflammatory immune biomarkers and surrogate markers of IR were calculated using established formulas. Inflammatory biomarkers derived from complete blood count data included neutrophil-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR), neutrophil-platelet ratio (NPR), systemic immune-inflammation index (SII, platelets*neutrophils/lymphocytes), systemic inflammation response index (SIRI, neutrophils*monocytes/lymphocytes), and pan-immune-inflammation value (PIV, neutrophils*monocytes*platelets/lymphocytes).³⁵⁻³⁷ Alternative IR indicators included the triglyceride-glucose index (TyG), TyGBMI, metabolic score for IR (METS-IR), and atherogenic index of plasma (AIP).³⁸⁻⁴⁰ The formulas used were: $TyG = \ln[TG * (FPG / 2)]$, $TyGBMI = TyG * BMI$, $METS-IR = \ln(2 * FPG + TG) * BMI / \ln(HDL-C)$, and $AIP = \log_{10}[(TG, \text{mol/L}) / HDL-C (\text{mol/L})]$. The units of measurement for FPG, TG, and HDL-C in TyG, TyGBMI, and METS-IR are mg/dL.

Statistical Analysis

Data analysis was conducted using R (version 4.1.3) and Free Statistics software (version 1.9.2). Prior to analysis, measurement data underwent normality testing via the Shapiro–Wilk method. Data following a normal distribution were expressed as mean \pm standard deviation ($\bar{x} \pm s$), with independent samples *t*-test applied for group comparisons. Non-normally distributed data were presented as median and interquartile range (M [P25, P75]), and group comparisons were made using the Mann–Whitney *U*-test. Categorical data were reported as frequencies and proportions, with comparisons performed using the chi-square test or Fisher’s exact test. The relationship between TBIL, DBIL, IBIL, and MAFLD risk was assessed through two weighted multivariable linear regression models. In Model 1, no covariate adjustments were made, while in Model 2, adjustments were made for age, gender, hypertension, diabetes, systolic and diastolic blood pressure, and BMI. Spearman correlation analysis was used to investigate the relationship between TBIL, DBIL, systemic inflammatory immune markers, and surrogate markers of IR.

Mediation models were constructed to assess whether inflammation and IR mediated the association between TBIL, DBIL, and MAFLD risk. The package of R software named “bruceR” was used for mediation analysis. We conducted mediation analysis using Bootstrap method and estimated the confidence interval of indirect effects through repeated sampling 5000 times. The independent variables of the mediation model in this study are TBIL and DBIL, the dependent variable is MAFLD, and the mediator variables include NLR, PLR, NPR, SII, SIRI, PIV, TyG, TyGBMI, METS-IR, and AIP. Through mediation analysis, how much mediation effect needs to be generated can be calculated. Mediation effects were quantified by calculating the mediation percentage, defined as the ratio of the indirect effect to the total effect. It was an ideal strategy to shed light on pathways and to provide statistical evidence for the mechanism analysis. In this study, direct effect represented the association between TBIL, DBIL and MAFLD risk; indirect effect, ie, the association between TBIL, DBIL and MAFLD risk, was mediated by inflammation and IR; the proportion mediated indicated the percentage of the mediating effect. All mediation models were controlled for gender, age, hypertension, T2DM, Systolic blood pressure and diastolic blood pressure. We further conducted sensitivity analysis in the mediation effect analysis by changing the sample range, specifically, we grouped the samples according to some variables (BMI, gender, diabetes status, hypertension status), and then ran the same mediation analysis model in each subsample to conduct mediation analysis respectively, to observe whether the mediation effect is consistent in different subgroups, so as to test the robustness of the results. In addition, we conducted post hoc power analysis on weighted multiple linear regression and mediation effects to justify the sample size, as detailed in the [Supplementary materials](#) and [Supplementary Tables 1](#) and [2](#). Statistical significance was defined as *P* < 0.05.

Results

Differences in Characteristics Between Non-MAFLD and MAFLD Group

This study included a total of 3000 subjects, comprising 1603 Non-MAFLD cases and 1397 MAFLD cases. The participants ranged in age from 18 to 80 years old, with 1741 males accounting for 58% and 1259 females accounting for 42%. As detailed in [Table 1](#), the MAFLD group exhibited a higher proportion of males, older age, and increased prevalence of hypertension and diabetes compared to the Non-MAFLD group. Additionally, the MAFLD group had higher

Table 1 Differences in Characteristics Between Non-MAFLD and MAFLD Group

Variables	Total(n = 3000)	Non-MAFLD(n = 1603)	MAFLD(n = 1397)	χ^2/Z	<i>p</i>
Gender, n(%)				223.945	< 0.001
Male	1741(58)	728(45.4)	1013(72.5)		
Female	1259(42)	875(54.6)	384(27.5)		
Age	46(35,57)	44(34,56)	49(37,58)	−6.153	< 0.001

(Continued)

Table 1 (Continued).

Variables	Total(n = 3000)	Non-MAFLD(n = 1603)	MAFLD(n = 1397)	χ^2/Z	p
Hypertension, n(%)	719(24)	222(13.8)	497(35.6)	192.186	< 0.001
T2DM, n(%)	451(15)	83(5.2)	368(26.3)	260.113	< 0.001
Systolic blood pressure	130(118,143)	123(113,136)	136(126,149)	-18.75	< 0.001
Diastolic blood pressure	81(73,89)	76(70,84)	86(77,93)	-19.989	< 0.001
BMI	24.5(22.21,26.71)	22.63(21.05,24.41)	26.44(24.83,28.53)	-33.39	< 0.001
WBC	5.94(5.03,6.97)	5.55(4.74,6.58)	6.33(5.43,7.47)	-14.034	< 0.001
NEU	3.35(2.73,4.14)	3.11(2.59,3.88)	3.6(2.95,4.44)	-11.884	< 0.001
LYM	1.95(1.62,2.35)	1.86(1.54,2.23)	2.05(1.72,2.48)	-10.026	< 0.001
MONO	0.35(0.28,0.43)	0.32(0.26,0.4)	0.38(0.31,0.46)	-14.017	< 0.001
PLT	239(205,279)	234(202,272)	245(211,284)	-4.626	< 0.001
TBIL	16.7(13.5,20.8)	17(13.95,21.3)	16.4(13.1,20.5)	-3.797	< 0.001
DBIL	3.9(3.18,4.8)	4.1(3.4,5.1)	3.6(2.9,4.5)	-11.014	< 0.001
IBIL	12.8(10.3,16.1)	12.9(10.4,16.2)	12.7(10.2,16)	-1.584	0.113
ALT	20.3(14.2,31)	16.1(12.1,22.1)	27.8(19.5,41.2)	-25.793	< 0.001
AST	21.2(17.6,26.22)	19.7(16.55,23.7)	23.1(19.1,28.7)	-14.499	< 0.001
ALP	69.4(58.5,82.82)	66(54.8,79.2)	73.8(62.6,86.7)	-11.319	< 0.001
GGT	22.6(15.2,37.7)	16.9(13,25.1)	32.6(22,50.9)	-26.786	< 0.001
FPG	5.28(4.96,5.75)	5.12(4.84,5.43)	5.54(5.16,6.2)	-20.419	< 0.001
TC	4.82(4.18,5.47)	4.71(4.08,5.36)	4.98(4.31,5.6)	-6.337	< 0.001
TG	1.27(0.86,1.95)	0.97(0.71,1.34)	1.79(1.27,2.55)	-29.399	< 0.001
HDL-C	1.21(1.03,1.45)	1.36(1.16,1.58)	1.07(0.93,1.24)	-25.334	< 0.001
LDL-C	2.62(2.1,3.11)	2.52(2.05,3.04)	2.7(2.16,3.2)	-5.66	< 0.001
NLR	1.73(1.35,2.19)	1.7(1.32,2.19)	1.77(1.4,2.2)	-2.616	0.009
PLR	121.89(99.41,151.31)	125(102.39,156.35)	117.93(97.05,144.12)	-5.692	< 0.001
NPR	0.01(0.01,0.02)	0.01(0.01,0.02)	0.02(0.01,0.02)	-8.515	< 0.001
SII	408.84(307.3,553.76)	396.59(294.6,539.62)	429.28(324.85,566.09)	-4.151	< 0.001
SIRI	0.6(0.43,0.85)	0.54(0.38,0.79)	0.67(0.48,0.91)	-10.202	< 0.001
PIV	142.6(93.8,215.45)	127.52(85.98,194.77)	160.86(109.56,231.89)	-10.132	< 0.001
TyG	8.61(8.2,9.08)	8.3(7.96,8.64)	9.01(8.66,9.4)	-31.387	< 0.001
TyGBMI	212.48(186.08,240.12)	188.99(170.7,207.61)	238.96(220.77,261.26)	-37.834	< 0.001
METS-IR	36.91(31.67,42.5)	32.37(28.86,36.15)	42.26(38.46,47.07)	-37.473	< 0.001
AIP	0.01(-0.2,0.26)	-0.15(-0.32,0.03)	0.22(0.03,0.4)	-31.363	< 0.001

BMI and systolic and diastolic blood pressure levels. The MAFLD group also displayed significantly elevated levels of WBC, NEU, LYM, MONO, PLT, ALT, AST, ALP, GGT, FPG, TC, TG, and LDL-C compared to the Non-MAFLD group. Conversely, TBIL, DBIL, and HDL-C levels were significantly lower in the MAFLD group, with these differences being statistically significant (all $p < 0.001$), while IBIL levels showed no significant difference between the two groups ($p > 0.05$). Further analysis revealed significant differences in systemic inflammatory immune biomarkers and IR surrogate markers. Specifically, the MAFLD group exhibited significantly higher levels of NLR, NPR, SII, SIRI, and PIV ($p < 0.05$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$), and a significantly lower PLR ($p < 0.001$), while the IR surrogate markers TyG, TyGBMI, METS-IR, and AIP were significantly higher in the MAFLD group (all $p < 0.001$).

Two Weighted Multiple Linear Regression Models to Evaluate the Association Between TBIL, DBIL, IBIL, and MALFD Risk

The role of TBIL and its subtypes (DBIL and IBIL) in MAFLD risk was further investigated by categorizing TBIL, DBIL, and IBIL into three tertiles: the lowest tertile (T1), middle tertile (T2), and highest tertile (T3). As shown in Table 2, the proportions of patients with MAFLD in the T1 (TBIL <14.6), T2 (TBIL 14.6–19.2), and T3 (TBIL ≥ 19.3) groups were 50.9%, 46.0%, and 42.9%, respectively. For DBIL, the proportions in T1 (DBIL <3.4), T2 (DBIL 3.4–4.4), and T3 (DBIL ≥ 4.5) groups were 59.1%, 45.6%, and 36.0%, respectively. For IBIL, the proportions in T1 (IBIL <11.1), T2 (IBIL 11.1–14.8), and T3 (IBIL ≥ 14.9) groups were 47.3%, 47.5%, and 44.9%, respectively. In Model 1 (unadjusted), compared to the T1 group (TBIL <14.6), the odds ratios (ORs) for the T2 (TBIL 14.6–19.2) and T3 (TBIL ≥ 19.3) groups were 0.823 (95% CI 0.69, 0.981) and 0.727 (95% CI 0.608, 0.868), both statistically significant ($p = 0.03$, $p < 0.001$). For DBIL, the ORs for the T2 (DBIL 3.4–4.4) and T3 (DBIL ≥ 4.5) groups were 0.582 (95% CI 0.486, 0.695) and 0.391 (95% CI 0.325, 0.468), both statistically significant ($p < 0.001$, $p < 0.001$). However, for IBIL, the ORs for T2 (IBIL 11.1–14.8) and T3 (IBIL ≥ 14.9) were 1.011 (95% CI 0.848, 1.205) and 0.908 (95% CI 0.761, 1.084) compared to T1 (IBIL <11.1), showing no statistically significant differences (all $p > 0.05$). In Model 2 (adjusted for age, gender, hypertension, diabetes, systolic and diastolic BP, and BMI), compared to the T1 group (TBIL <14.6), the ORs for the T2 (TBIL 14.6–19.2) and T3 (TBIL ≥ 19.3) groups were 0.735 (95% CI 0.581, 0.929) and 0.615 (95% CI 0.483, 0.783), both statistically significant ($p = 0.01$, $p < 0.001$). For DBIL, the ORs for the T2 (DBIL 3.4–4.4) and T3 (DBIL ≥ 4.5) groups were 0.613 (95% CI 0.485, 0.774) and 0.367 (95% CI 0.287, 0.47), both statistically significant ($p < 0.001$, $p < 0.001$). For IBIL, the ORs for T2 (IBIL 11.1–14.8) and T3 (IBIL ≥ 14.9) were 0.854 (95% CI 0.675, 1.079) and 0.837 (95% CI 0.68, 0.936), both not statistically significant ($p = 0.187$, $p = 0.113$).

Table 2 Two Weighted Multiple Linear Regression Models to Evaluate the Association Between TBIL, DBIL, IBIL, and MALFD Risk

Variables	Non-MAFLD(n = 1603)	MAFLD(n = 1397)	Model 1	Model 2
TBIL	n(%)	n(%)	OR,95% CI	OR,95% CI
< 14.6 (T1)	477(49.1)	494(50.9)	Reference	Reference
14.6–19.2 (T2)	556(54.0)	474(46.0)	0.823(0.69,0.981),0.03	0.735(0.581,0.929),0.01
≥ 19.3 (T3)	570(57.1)	429(42.9)	0.727(0.608,0.868), < 0.001	0.615(0.483,0.783), < 0.001
DBIL	n(%)	n(%)	OR,95% CI	OR,95% CI
< 3.4 (T1)	384(40.9)	554(59.1)	Reference	Reference
3.4–4.4 (T2)	566(54.4)	475(45.6)	0.582(0.486,0.695), < 0.001	0.613(0.485,0.774), < 0.001
≥ 4.5 (T3)	653(64.0)	368(36.0)	0.391(0.325,0.468), < 0.001	0.367(0.287,0.47), < 0.001
IBIL	n(%)	n(%)	OR,95% CI	OR,95% CI
< 11.1 (T1)	514(52.7)	461(47.3)	Reference	Reference
11.1–14.8 (T2)	534(52.5)	484(47.5)	1.011(0.848,1.205),0.907	0.854(0.675,1.079),0.187
≥ 14.9 (T3)	555(55.1)	452(44.9)	0.908(0.761,1.084),0.285	0.837(0.68,0.936),0.113

Notes: Model 1: No covariates adjusted; Model 2: Adjusted for age, gender, hypertension, diabetes, systolic BP, diastolic BP, and BMI.

Table 3 Characteristics According to TBIL Tertiles

Variables	Total(n = 3000)	TBIL<14.6(T1, n = 971)	TBIL 14.6–19.2(T2, n = 1030)	TBIL ≥ 19.3(T3, n = 999)	χ^2/H	p
Gender, n(%)					31.299	< 0.001
Male	1741(58)	519(53.5)	572(55.5)	650(65.1)		
Female	1259(42)	452(46.5)	458(44.5)	349(34.9)		
Age	46(35,57)	41(33,53)	47(35,58)	50(37,60)	86.645	< 0.001
MAFLD, n(%)	1397(46.6)	494(50.9)	474(46)	429(42.9)	12.641	0.002
Hypertension, n(%)	719(24)	204(21)	260(25.2)	255(25.5)	6.913	0.032
T2DM, n(%)	451(15)	142(14.6)	153(14.9)	156(15.6)	0.418	0.811
BMI	24.5(22.21,26.71)	24.64(22.34,27.2)	24.55(22.31,26.66)	24.4(22,26.5)	8.334	0.015
WBC	5.94(5.03,6.97)	6.32(5.27,7.35)	5.86(5,6.82)	5.72(4.8,6.69)	81.169	< 0.001
NEU	3.35(2.73,4.14)	3.58(2.92,4.43)	3.32(2.72,4.06)	3.19(2.59,3.92)	68.281	< 0.001
LYM	1.95(1.62,2.35)	2.02(1.7,2.47)	1.92(1.62,2.29)	1.9(1.57,2.3)	33.506	< 0.001
MONO	0.35(0.28,0.43)	0.36(0.3,0.44)	0.35(0.28,0.43)	0.34(0.28,0.42)	21.423	< 0.001
PLT	239(205,279)	254(219,295)	239(206,276.75)	226(194,261)	140.387	< 0.001
ALT	20.3(14.2,31)	20.4(14.2,32.65)	20.3(14.1,30.55)	20.1(14.3,30.1)	0.172	0.918
AST	21.2(17.6,26.22)	20.5(16.9,25.7)	21(17.6,26)	22.1(18.5,26.9)	27.426	< 0.001
ALP	69.4(58.5,82.82)	70.1(59,83.2)	70.3(58.32,84.2)	68.3(58.25,81.65)	2.779	0.249
GGT	22.6(15.2,37.7)	23.7(15.7,40.65)	22.5(15.1,38.4)	22.2(15.2,35.25)	4.601	0.1
FPG	5.28(4.96,5.75)	5.29(4.98,5.74)	5.28(4.95,5.72)	5.27(4.97,5.77)	0.283	0.868
TC	4.82(4.18,5.47)	4.81(4.2,5.36)	4.85(4.18,5.52)	4.83(4.17,5.54)	1.336	0.513
TG	1.27(0.86,1.95)	1.4(0.91,2.05)	1.29(0.9,2.01)	1.16(0.8,1.72)	34.05	< 0.001
HDL-C	1.21(1.03,1.45)	1.17(0.98,1.4)	1.21(1.03,1.45)	1.26(1.09,1.5)	55.13	< 0.001
LDL-C	2.62(2.1,3.11)	2.6(2.1,3.06)	2.63(2.1,3.13)	2.62(2.09,3.14)	0.854	0.652
NLR	1.73(1.35,2.19)	1.76(1.37,2.26)	1.76(1.36,2.18)	1.68(1.33,2.13)	8.181	0.017
PLR	121.89(99.41,151.31)	124.22(101.24,153.93)	122.38(100.43,151.7)	119.37(96.9,146.46)	11.432	0.003
NPR	0.01(0.01,0.02)	0.01(0.01,0.02)	0.01(0.01,0.02)	0.01(0.01,0.02)	1.561	0.458
SII	408.84(307.3,553.76)	446.41(339.54,598.39)	407.88(308.25,546.93)	373.34(281.05,501.73)	74.748	< 0.001
SIRI	0.6(0.43,0.85)	0.65(0.45,0.9)	0.59(0.42,0.82)	0.58(0.4,0.8)	22.199	< 0.001
PIV	142.6(93.8,215.45)	165.48(106.74,244.91)	140.31(92.77,207.62)	128.14(85.76,189.66)	77.481	< 0.001
TyG	8.61(8.2,9.08)	8.7(8.24,9.16)	8.63(8.23,9.1)	8.53(8.12,8.98)	28.489	< 0.001
TyGBMI	212.48(186.08,240.12)	215.25(187.83,245.38)	214.78(187.84,239.7)	208.66(181.31,235.69)	20.14	< 0.001
METS-IR	36.91(31.67,42.5)	37.84(32.48,43.86)	36.96(31.84,42.51)	36.06(30.65,41.33)	31.625	< 0.001
AIP	0.01(−0.2,0.26)	0.07(−0.15,0.3)	0.02(−0.19,0.27)	−0.04(−0.25,0.19)	46.574	< 0.001

0.47), both statistically significant ($p < 0.001$, $p < 0.001$). For IBIL, the ORs for T2 (IBIL 11.1–14.8) and T3 (IBIL ≥ 14.9) were 0.854 (95% CI 0.675, 1.079) and 0.837 (95% CI 0.68, 0.936), with no significant differences (all $p > 0.05$). These results suggest that TBIL and DBIL independently exert a protective effect against MAFLD risk, while IBIL shows no significant association with MAFLD risk.

Characteristics According to TBIL Tertiles

The results presented above indicate that TBIL and DBIL exert a significant protective effect on the risk of MAFLD. To further explore this relationship, TBIL was categorized into three tertiles: the lowest tertile (T1, <14.6), middle tertile (T2, 14.6–19.2), and highest tertile (T3, ≥ 19.3), and compared various characteristics across these groups. The clinical characteristics and laboratory results are summarized in Table 3. Compared to the T2 group, the T3 group exhibited a higher proportion of males, older age, and a higher prevalence of hypertension, along with a lower proportion of MAFLD and lower BMI. These differences were statistically significant ($p < 0.001$, $p < 0.001$, $p < 0.05$, $p < 0.05$, $p < 0.05$). No significant differences were found in the proportion of T2DM across the groups. Regarding laboratory parameters, the WBC, NEU, LYM, MONO, PLT, and TG levels were significantly lower in the T3 group than in both T2 and T1 groups (all $p < 0.001$). Conversely, AST and HDL-C levels were significantly higher in the T3 group (all $p < 0.001$). However, there were no significant differences in ALT, ALP, GGT, FPG, TC, and LDL-C levels among the three groups (all $p > 0.05$). In terms of systemic inflammatory immune biomarkers and IR surrogate markers, the T3 group showed significantly lower levels of NLR, PLR, SII, SIRI, and PIV compared to the T2 and T1 groups ($p < 0.05$, $p < 0.05$, $p < 0.001$, $p < 0.001$, $p < 0.001$). No significant differences in NPR were observed between the groups ($p > 0.05$). Additionally, the TyG, TyGBMI, METS-IR, and AIP were significantly lower in the T3 group (all $p < 0.001$). These results suggest that as TBIL levels increase within a certain range, the degree of systemic chronic inflammation and IR gradually decreases.

Characteristics According to DBIL Tertiles

DBIL was stratified into three tertiles: the lowest (T1, < 3.4), middle (T2, 3.4–4.4), and highest (T3, ≥ 4.5). Differences in clinical characteristics and laboratory results among the groups are summarized in Table 4. Compared to the T2 group, the T3 group exhibited significantly higher age, a lower proportion of MAFLD, and lower BMI (all $p < 0.001$). No significant differences in hypertension or T2DM prevalence were observed among the groups (all $p > 0.05$). The male proportion in the T3 group was higher than in T2 but lower than in T1 ($p = 0.001$). Regarding laboratory indicators, WBC, NEU, LYM, MONO,

Table 4 Characteristics According to DBIL Tertiles

Variables	Total(n = 3000)	DBIL < 3.4 (T1, n = 938)	DBIL 3.4–4.4 (T2, n = 1041)	DBIL ≥ 4.5 (T3, n = 1021)	χ^2/H	p
Gender, n(%)					13.28	0.001
Male	1741(58)	587(62.6)	568(54.6)	586(57.4)		
Female	1259(42)	351(37.4)	473(45.4)	435(42.6)		
Age	46(35,57)	41(34,52)	47(35,57)	50(37,61)	103.065	< 0.001
MAFLD, n(%)	1397(46.6)	554(59.1)	475(45.6)	368(36)	104.668	< 0.001
Hypertension, n(%)	719(24)	229(24.4)	243(23.3)	247(24.2)	0.354	0.838
T2DM, n(%)	451(15)	145(15.5)	143(13.7)	163(16)	2.196	0.334
BMI	24.5(22.21,26.71)	25.17(22.9,27.55)	24.52(22.34,26.59)	24.03(21.59,26.09)	68.289	< 0.001
WBC	5.94(5.03,6.97)	6.38(5.34,7.49)	5.91(5.08,6.87)	5.55(4.7,6.58)	139.214	< 0.001
NEU	3.35(2.73,4.14)	3.62(2.95,4.45)	3.35(2.76,4.09)	3.11(2.55,3.88)	107.315	< 0.001

(Continued)

Table 4 (Continued).

Variables	Total(n = 3000)	DBIL < 3.4 (T1, n = 938)	DBIL 3.4–4.4 (T2, n = 1041)	DBIL ≥ 4.5 (T3, n = 1021)	χ^2/H	p
LYM	1.95(1.62,2.35)	2.07(1.73,2.52)	1.94(1.63,2.32)	1.84(1.53,2.23)	80.647	< 0.001
MONO	0.35(0.28,0.43)	0.37(0.3,0.45)	0.35(0.29,0.42)	0.33(0.27,0.42)	44.121	< 0.001
PLT	239(205,279)	253(217,295)	241(210,281)	224(193,260)	141.581	< 0.001
ALT	20.3(14.2,31)	21.9(15.5,35.18)	20.4(14.1,30.1)	18.8(13.5,28.2)	34.198	< 0.001
AST	21.2(17.6,26.22)	21.2(17.52,26.5)	20.9(17.5,26)	21.4(17.8,26.1)	2.473	0.29
ALP	69.4(58.5,82.82)	71.75(60.73,85.4)	68.9(58.1,82.2)	68.1(56.9,81.4)	18.581	< 0.001
GGT	22.6(15.2,37.7)	27.55(17.3,46.27)	22.3(15.5,36.7)	20.6(14.4,30.2)	87.959	< 0.001
FPG	5.28(4.96,5.75)	5.34(5.03,5.79)	5.26(4.96,5.71)	5.23(4.91,5.77)	15.916	< 0.001
TC	4.82(4.18,5.47)	5.04(4.45,5.62)	4.79(4.2,5.4)	4.62(3.93,5.35)	91.072	< 0.001
TG	1.27(0.86,1.95)	1.65(1.13,2.5)	1.3(0.89,1.93)	1.02(0.72,1.42)	334.765	< 0.001
HDL-C	1.21(1.03,1.45)	1.12(0.95,1.3)	1.21(1.03,1.43)	1.34(1.12,1.58)	237.96	< 0.001
LDL-C	2.62(2.1,3.11)	2.8(2.28,3.21)	2.62(2.12,3.11)	2.45(1.92,3.01)	77.621	< 0.001
NLR	1.73(1.35,2.19)	1.75(1.38,2.2)	1.75(1.35,2.25)	1.69(1.33,2.16)	4.407	0.11
PLR	121.89(99.41,151.31)	120.69(99.63,149.34)	124.88(100.86,152.06)	120.09(97.86,151.72)	4.124	0.127
NPR	0.01(0.01,0.02)	0.01(0.01,0.02)	0.01(0.01,0.02)	0.01(0.01,0.02)	2.574	0.276
SII	408.84(307.3,553.76)	439.92(336.91,583.79)	416.53(311.34,567.09)	372.24(280.97,497.62)	66.877	< 0.001
SIRI	0.6(0.43,0.85)	0.67(0.46,0.89)	0.6(0.42,0.84)	0.56(0.4,0.79)	30.545	< 0.001
PIV	142.6(93.8,215.45)	165.2(108.68,240.99)	145.41(95.79,211.15)	125.42(85.25,181.81)	88.461	< 0.001
TyG	8.61(8.2,9.08)	8.9(8.48,9.36)	8.64(8.21,9.06)	8.37(8.8,77)	292.724	< 0.001
TyGBMI	212.48(186.08,240.12)	226.13(198,252.98)	212.68(186.99,238.4)	200.08(175.45,227.45)	176.192	< 0.001
METS-IR	36.91(31.67,42.5)	39.83(34.41,45.27)	36.91(31.83,42.35)	34.26(29.57,39.68)	203.01	< 0.001
AIP	0.01(−0.2,0.26)	0.17(−0.05,0.39)	0.02(−0.19,0.25)	−0.12(−0.31,0.08)	370.798	< 0.001

PLT, ALT, ALP, GGT, FPG, TC, TG, and LDL-C levels were significantly lower in the T3 group than in T2 and T1 (all $p < 0.001$). Conversely, HDL-C levels were significantly higher in T3 than in T2 and T1 ($p < 0.001$). No significant difference in AST was found among the groups ($p > 0.05$). For systemic inflammatory and IR markers, the SII, SIRI, and PIV in the T3 group were significantly lower than in the T2 and T1 groups (all $p < 0.001$). NLR, PLR, and NPR did not differ statistically among the groups (all $p > 0.05$). TyG, TyGBMI, METS-IR, and AIP levels were also significantly lower in T3 compared to T2 and T1 (all $p < 0.001$). These results suggest that within a specific range, increasing DBIL levels are associated with reduced systemic chronic inflammation and improved insulin sensitivity.

Correlation Between TBIL, DBIL, Systemic Inflammatory Immunity Index, and Insulin Resistance Surrogate Index

Spearman correlation analysis was conducted to assess the relationships between TBIL, DBIL, systemic inflammatory immune markers, and IR surrogates, aiming to elucidate their complex interactions. As shown in Figure 1, TBIL was negatively correlated with PIV ($r = -0.172$, $p < 0.05$), SII ($r = -0.169$, $p < 0.05$), AIP ($r = -0.128$, $p < 0.05$), METS-IR (r

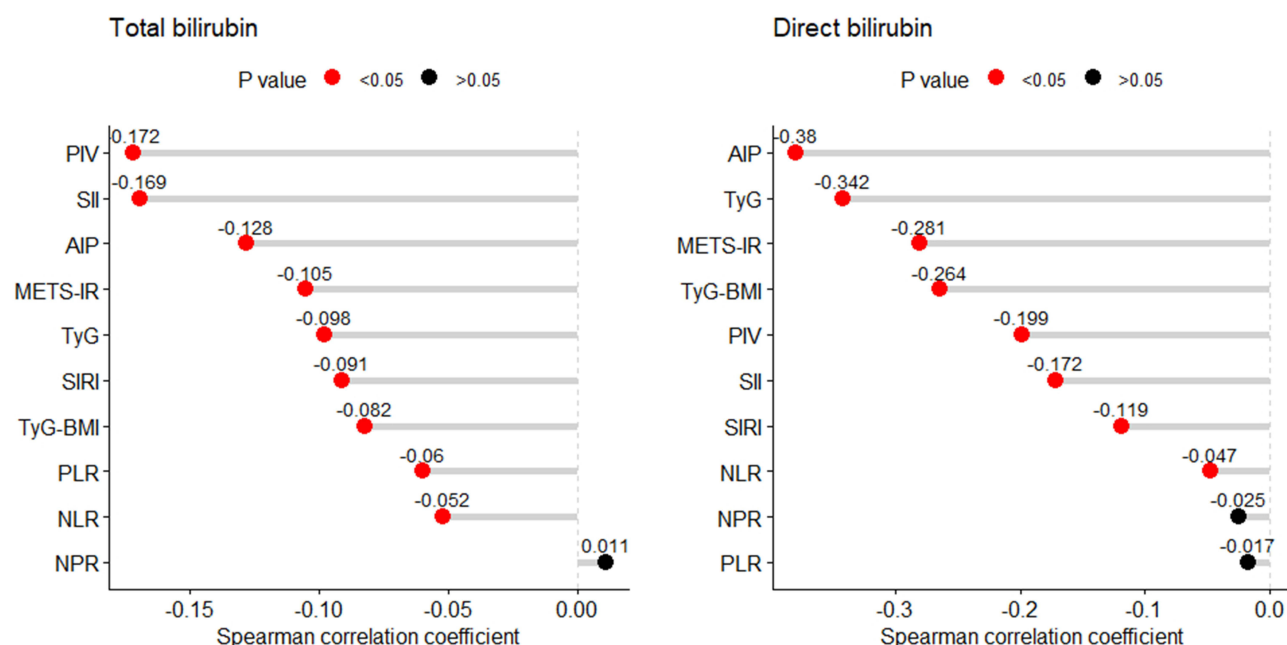


Figure 1 Lollipop charts illustrating the correlations between TBIL, DBIL, systemic inflammatory immune index, and insulin resistance surrogate index. The horizontal line length represents the magnitude of the correlation coefficient.

$r = -0.105$, $p < 0.05$), TyG ($r = -0.098$, $p < 0.05$), SIRI ($r = -0.091$, $p < 0.05$), TyGBMI ($r = -0.082$, $p < 0.05$), PLR ($r = -0.06$, $p < 0.05$), and NLR ($r = -0.052$, $p < 0.05$), though no correlation with NPR was observed ($p > 0.05$). DBIL also demonstrated significant negative correlations with AIP ($r = -0.38$, $p < 0.05$), TyG ($r = -0.342$, $p < 0.05$), METS-IR ($r = -0.281$, $p < 0.05$), TyGBMI ($r = -0.264$, $p < 0.05$), PIV ($r = -0.199$, $p < 0.05$), SII ($r = -0.172$, $p < 0.05$), SIRI ($r = -0.119$, $p < 0.05$), and NLR ($r = -0.047$, $p < 0.05$), with no significant correlation with NPR or PLR ($p > 0.05$ for both). These results suggest that both TBIL and DBIL are significantly negatively correlated with systemic chronic inflammation and IR.

Potential Mediation Effects of Systemic Inflammatory Response and Insulin Resistance on the Association Between TBIL and Prevalence of MAFLD

After adjusting for potential confounders (including age, gender, hypertension, diabetes, and systolic/diastolic blood pressure), significant natural indirect effects of SIRI, PIV, TyG, TyGBMI, METS-IR, and AIP on the relationship between TBIL and MAFLD prevalence were identified, as shown in Table 5 and Figure 2. Specifically, SIRI mediated 4.07% of the TBIL-MAFLD association, PIV mediated 9.56%, and TyG accounted for 69.27%. TyGBMI, METS-IR, and AIP each mediated 100.00% of the TBIL-MAFLD association.

Potential Mediation Effects of Systemic Inflammatory Response and Insulin Resistance on the Association Between DBIL and Prevalence of MAFLD

After adjusting for potential confounding factors (including age, gender, hypertension, diabetes, and systolic/diastolic blood pressure), significant natural indirect effects of SIRI, PIV, TyG, TyGBMI, METS-IR, and AIP on the association between DBIL and MAFLD prevalence were observed, as shown in Table 6 and Figure 2. Specifically, SIRI mediated 1.55% of the DBIL-MAFLD association, PIV mediated 4.22%, and TyG accounted for 81.91%. TyGBMI mediated 78.34%, METS-IR mediated 81.41%, and AIP mediated 100.00% of the DBIL-MAFLD association.

This mediation analysis suggests that both TBIL and DBIL may exert a protective effect against MAFLD by improving IR and reducing systemic chronic inflammation. Notably, enhancing IR appears to play a more significant intermediary role in this protective mechanism than alleviating systemic chronic inflammation.

Table 5 Potential Mediation Effects of Systemic Inflammatory Response and Insulin Resistance on the Association Between TBIL and Prevalence of MAFLD

	Type	Effect	95% CI	SE	Z	P	Proportion of Mediation
NLR	Indirect(ab)	0.000	(-0.000,0.000)	0.000	0.624	0.533	–
	Direct(c')	-0.008	(-0.010,-0.006)	0.001	-6.566	< 0.001	
	Total(c)	-0.008	(-0.010,-0.006)	0.001	-6.534	< 0.001	
PLR	Indirect(ab)	0.000	(-0.000,0.000)	0.000	1.545	0.122	-
	Direct(c')	-0.008	(-0.010,-0.006)	0.001	-6.460	< 0.001	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.370	< 0.001	
NPR	Indirect(ab)	0.000	(-0.000,0.000)	0.000	-0.633	0.527	-
	Direct(c')	-0.008	(-0.010,-0.005)	0.001	-6.359	< 0.001	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.441	< 0.001	
SII	Indirect(ab)	0.000	(-0.001,-0.000)	0.000	-1.847	0.065	-
	Direct(c')	-0.008	(-0.010,-0.005)	0.001	-5.938	< 0.001	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.243	< 0.001	
SIRI	Indirect(ab)	0.000	(-0.001,-0.000)	0.000	-2.260	0.024	4.07%
	Direct(c')	-0.008	(-0.010,-0.005)	0.001	-6.118	< 0.001	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.431	< 0.001	
PIV	Indirect(ab)	-0.001	(-0.001,-0.000)	0.000	-3.456	0.001	9.56%
	Direct(c')	-0.007	(-0.009,-0.005)	0.001	-5.686	< 0.001	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.401	< 0.001	
TyG	Indirect(ab)	-0.005	(-0.006,-0.004)	0.001	-9.962	< 0.001	69.27%
	Direct(c')	-0.002	(-0.005,-0.000)	0.001	-2.008	0.045	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.355	< 0.001	
TyGBMI	Indirect(ab)	-0.006	(-0.008,-0.005)	0.001	-9.269	< 0.001	100.00%
	Direct(c')	-0.002	(-0.004,0.000)	0.001	-1.508	0.132	
	Total(c)	-0.008	(-0.010,-0.006)	0.001	-6.748	< 0.001	
METS-IR	Indirect(ab)	-0.007	(-0.009,-0.006)	0.001	-10.655	< 0.001	100.00%
	Direct(c')	-0.001	(-0.003,0.001)	0.001	-0.659	0.510	
	Total(c)	-0.008	(-0.010,-0.006)	0.001	-7.221	< 0.001	
AIP	Indirect(ab)	-0.006	(-0.007,-0.005)	0.001	-10.638	< 0.001	100.00%
	Direct(c')	-0.001	(-0.004,0.001)	0.001	-1.220	0.222	
	Total(c)	-0.008	(-0.010,-0.005)	0.001	-6.148	< 0.001	

Notes: Type: Indirect(ab): mediation effect; Direct(c'): direct effect; Total(c): total effect; Effect: The estimated value of the effect variable, indicating the strength of the influence of the independent variable on the dependent or mediating variable; Proportion of mediation=Indirect(ab)/ Total(c)*100%.

Abbreviations: 95% CI, 95% confidence interval; SE, Standard Error; Z, test statistic.

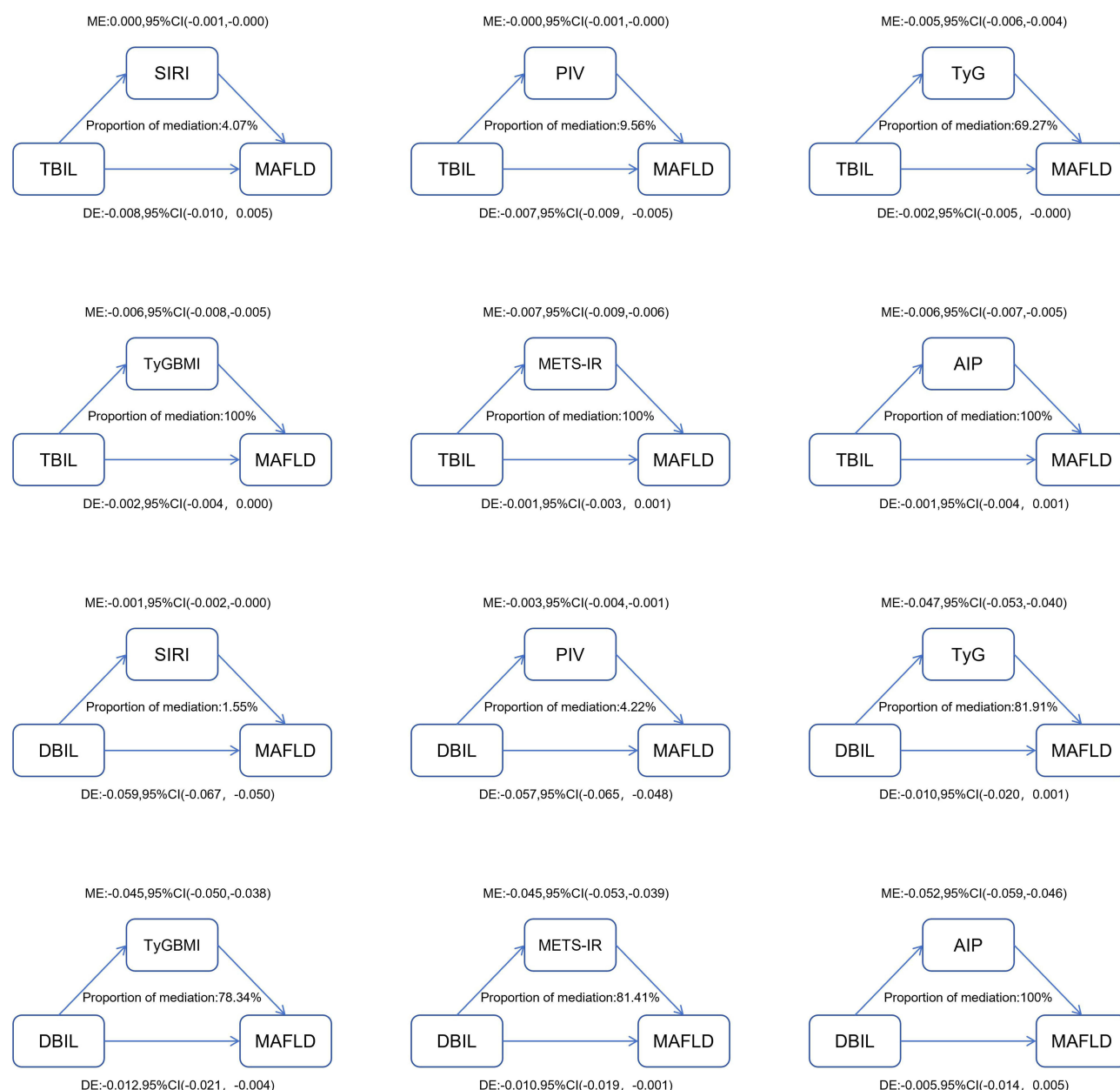


Figure 2 Mediation models exploring the relationship between TBIL, DBIL, and MAFLD risk, mediated by systemic inflammatory immune markers (SIRI, PIV) and IR surrogate indicators (TyG, TyGBMI, METS-IR, AIP).

Abbreviations: ME, Mediation effect; DE, Direct effect.

Additionally, We conducted sensitivity analysis (robustness test) in the mediation effect analysis by changing the sample range, specifically, we grouped the samples according to some variables (BMI, gender, diabetes status, hypertension status), and then ran the same mediation analysis model in each subsample to conduct mediation analysis respectively, this study validated the robustness of the main results: In different genders (males, females), different BMI ranges (<24 , ≥ 24), non-diabetes and different hypertension states (with or without), the mediating effect of inflammation and insulin resistance in bilirubin-MAFLD risk is stable and significant (as shown in). [Supplementary Tables 3–18](#)

Table 6 Potential Mediation Effects of Systemic Inflammatory Response and Insulin Resistance on the Association Between DBIL and Prevalence of MAFLD

	Type	Effect	95% CI	SE	Z	P	Proportion of mediation
NLR	Indirect(ab)	0.000	(−0.000,0.001)	0.000	0.572	0.567	–
	Direct(c')	−0.060	(−0.068,−0.052)	0.004	−14.178	0.000	
	Total(c)	−0.060	(−0.067,−0.051)	0.004	−14.182	0.000	
PLR	Indirect(ab)	0.001	(0.000,0.002)	0.000	1.682	0.093	-
	Direct(c')	−0.060	(−0.068,−0.052)	0.004	−14.563	0.000	
	Total(c)	−0.060	(−0.067,−0.051)	0.004	−14.366	0.000	
NPR	Indirect(ab)	0.000	(−0.001,0.001)	0.000	0.210	0.834	-
	Direct(c')	−0.060	(−0.067,−0.051)	0.004	−14.203	0.000	
	Total(c)	−0.060	(−0.067,−0.051)	0.004	−14.146	0.000	
SII	Indirect(ab)	−0.001	(−0.002,0.000)	0.001	−1.413	0.158	-
	Direct(c')	−0.059	(−0.067,−0.050)	0.004	−13.283	0.000	
	Total(c)	−0.060	(−0.067,−0.051)	0.004	−13.797	0.000	
SIRI	Indirect(ab)	−0.001	(−0.002,−0.000)	0.000	−2.068	0.039	1.55%
	Direct(c')	−0.059	(−0.067,−0.050)	0.004	−13.799	0.000	
	Total(c)	−0.060	(−0.068,−0.051)	0.004	−14.170	0.000	
PIV	Indirect(ab)	−0.003	(−0.004,−0.001)	0.001	−3.261	0.001	4.22%
	Direct(c')	−0.057	(−0.065,−0.048)	0.005	−12.671	0.000	
	Total(c)	−0.060	(−0.068,−0.051)	0.004	−13.769	0.000	
TyG	Indirect(ab)	−0.047	(−0.053,−0.040)	0.003	−14.532	0.000	81.91%
	Direct(c')	−0.010	(−0.020,0.001)	0.005	−1.983	0.047	
	Total(c)	−0.057	(−0.065,−0.048)	0.004	−13.451	0.000	
TyGBMI	Indirect(ab)	−0.045	(−0.050,−0.038)	0.003	−14.823	0.000	78.34%
	Direct(c')	−0.012	(−0.021,−0.004)	0.005	−2.670	0.008	
	Total(c)	−0.057	(−0.065,−0.048)	0.004	−12.940	0.000	
METS-IR	Indirect(ab)	−0.045	(−0.053,−0.039)	0.003	−13.573	0.000	81.41%
	Direct(c')	−0.010	(−0.019,−0.001)	0.005	−2.092	0.036	
	Total(c)	−0.056	(−0.065,−0.048)	0.004	−12.797	0.000	
AIP	Indirect(ab)	−0.052	(−0.059,−0.046)	0.003	−15.555	0.000	100.00%
	Direct(c')	−0.005	(−0.014,0.005)	0.005	−0.963	0.336	
	Total(c)	−0.056	(−0.065,−0.048)	0.004	−13.352	0.000	

Notes: Type: Indirect(ab): mediation effect; Direct(c'): direct effect; Total(c): total effect; Effect: The estimated value of the effect variable, indicating the strength of the influence of the independent variable on the dependent or mediating variable. Proportion of mediation=Indirect(ab)/ Total(c)*100%.

Abbreviations: 95% CI, 95% confidence interval; SE, Standard Error; Z, test statistic.

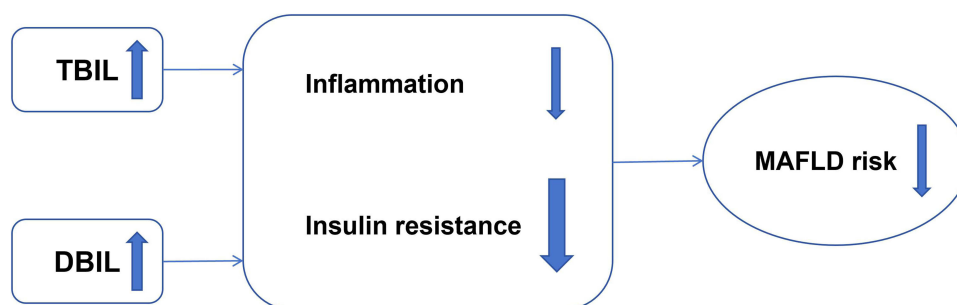


Figure 3 Potential mechanisms by which TBIL and DBIL protect against MAFLD through the improvement of insulin resistance and the reduction of systemic chronic inflammation.

Discussion

This study is the first to comprehensively investigate the associations between TBIL and its subtypes (DBIL and IBIL), six systemic inflammatory immune markers, four IR surrogate indicators, and the risk of MAFLD. Our findings reveal a significant negative correlation between TBIL, DBIL levels and the risk of MAFLD. Both TBIL and DBIL are also notably negatively correlated with the degree of systemic inflammation and IR. Furthermore, TBIL and DBIL function as independent protective factors against the development of MAFLD, while IBIL does not show a significant correlation with MAFLD risk. Mediation analysis further suggests that the protective effects of TBIL and DBIL may be mediated through the improvement of IR and the reduction of systemic chronic inflammation. Notably, the mediating role of improved insulin resistance appears to be stronger than that of reduced systemic inflammation (Figure 3). These findings highlight the potential of bilirubin and its molecular mechanisms as therapeutic targets for MAFLD and related metabolic disorders.

Prior studies have primarily explored the relationship between serum bilirubin levels and NAFLD, with inconsistent and controversial results. Moreover, limited research has focused on which specific form of bilirubin exerts a protective effect or its underlying mechanisms. Mendelian randomization studies have failed to establish a causal relationship between bilirubin levels and NAFLD.^{32,33} Other observational studies have shown that DBIL is negatively correlated with NAFLD incidence, while TBIL and IBIL show no such correlation. Some researchers hypothesize that DBIL, being soluble in serum, is more likely to exist in an active form compared to IBIL.^{28,29} Conversely, some studies have reported that IBIL is negatively correlated with the incidence or severity of NASH.^{30,31} The discrepancies in research findings may stem from variations in population characteristics, study methodologies, sample sizes, and factors such as age, gender, ethnicity, and underlying diseases. Our study provides clear evidence of a significant negative correlation between TBIL and DBIL levels and the risk of MAFLD, further supporting the clinical relevance of bilirubin in MAFLD prevention. Differences in metabolic pathways and bioavailability between DBIL and IBIL may be potential factors underlying their generating distinct effects in metabolic diseases. DBIL, as a conjugated bilirubin, has a longer half-life. Its high water solubility facilitates its entry into bile through hepatocyte membrane transporters, allowing for stable excretion through bile and reducing toxicity risks. IBIL, as unconjugated bilirubin, has a short half-life. It is primarily produced in the spleen and bone marrow through the decomposition of hemoglobin and then transported to the liver via the bloodstream. In the liver, IBIL is conjugated into DBIL by the UGT1A1 enzyme, becoming a water-soluble substance that is subsequently excreted through bile.^{41,42} This implies that DBIL may exhibit higher bioavailability in the liver, while IBIL may exist briefly in the bloodstream but due to its instability, its action time is short, potentially leading to lower bioavailability. Its effect indirectly depends on the activity of the UGT1A1 enzyme, thereby limiting its role in metabolic diseases. Furthermore, due to its unconjugated state, the free form of IBIL may more easily penetrate cell membranes but may also cause oxidative stress to cells, posing potential toxicity risks. Combining the results of this study with previous research conclusions, we speculate that DBIL may exhibit a stronger protective effect on MAFLD due to its liver targeting, stable metabolic pathway, and high bioavailability. Conversely, IBIL has weak effects and is highly controversial due to its low bioavailability and metabolic instability.

Spearman correlation and mediation model analyses were employed to explore the intricate relationships between TBIL, DBIL, systemic chronic inflammation, and IR, as well as the mediating roles of systemic chronic inflammation and IR in the association between bilirubin and MAFLD risk, aiming to elucidate the underlying mechanism by which bilirubin confers protection against MAFLD. Spearman correlation analysis revealed significant negative correlations between TBIL, DBIL, and both systemic inflammatory response and IR. Mediation analysis indicated substantial indirect effects of SIRI, PIV, TyG, TyGBMI, METS-IR, and AIP on the relationship between TBIL, DBIL, and MAFLD risk. Specifically, SIRI mediated 4.07% and 1.55% of the TBIL-MAFLD and DBIL-MAFLD associations, respectively; PIV mediated 9.56% and 4.22%; TyG mediated 69.27% and 81.91%; TyGBMI mediated 100% and 78.34%; METS-IR mediated 100% and 81.41%; and AIP mediated 100% for both associations. These findings suggest that the protective effect of bilirubin may be mediated through improvements in IR and the alleviation of systemic chronic inflammation, with the enhancement of insulin sensitivity playing a more prominent role than the reduction of systematic chronic inflammation. The findings of our study align closely with previous research. Animal studies have shown that bilirubin can improve insulin resistance by activating the PPAR α signaling pathway and improving insulin signaling pathways in multiple tissues. Bilirubin directly binds to and activates the nuclear receptor PPAR α , promoting β -oxidation and ketone body production, inhibiting lipid synthesis, thereby improving insulin sensitivity in the liver and adipose tissue.^{7,8,43} Additional research has shown that bilirubin treatment can improve insulin signaling pathways (phosphorylation of protein kinase B (PKB/Akt)) in skeletal muscle, adipose tissue, and liver of obese mice, reduce the expression of inflammatory cytokines (including TNF- α , interleukin 1 β , and monocyte chemoattractant protein-1), and endoplasmic reticulum stress markers (including 78 kDa glucose-regulated protein (GRP78), CCAAT/enhancer binding protein (C/EBP) homologous protein, X-box binding protein (XBP-1), and activating transcription factor 4), thereby improving hyperglycemia and obesity by increasing insulin sensitivity.⁹ Another prior study⁶ demonstrated that bilirubin treatment improves blood glucose levels and insulin sensitivity in obese model mice. This effect was attributed to bilirubin's regulation of cholesterol metabolism, enhancement of PPAR γ levels in the liver, and inhibition of the NF- κ B inflammatory signaling pathway, which together contribute to its anti-inflammatory and IR ameliorating effects. Other studies⁴⁴ have also shown that bilirubin can improve IR by reducing reactive oxygen species (ROS) and repairing mitochondrial dysfunction, positioning bilirubin as an active insulin sensitizer. Furthermore, bilirubin displays robust anti-inflammatory activity. Research has confirmed a negative correlation between serum bilirubin and C-reactive protein levels.⁴⁵ Both bilirubin and its rate-limiting enzyme, heme oxygenase-1 (HO-1), reduce the expression of various inflammatory markers, including tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) under stress conditions, exerting a significant protective effect in various chronic inflammatory diseases.^{46–48} In addition, HO-1 exerts a protective effect on endotoxic shock by inhibiting NADPH oxidase NOX4 through the production of carbon monoxide, reducing ROS generation, and alleviating inflammation.³

Building on the current and prior research, it is hypothesized that modest elevation of endogenous bilirubin levels or controlled supplementation with exogenous bilirubin could offer a novel therapeutic strategy for MAFLD. This potential therapeutic effect may be mediated through the improvement of IR and the reduction of systemic chronic inflammation. Existing studies have shown that bilirubin nanoparticles hold promise as adjunctive tools for treating inflammatory and metabolic disorders.^{14,15} Moreover, a strategy for treating atherosclerotic diseases suggests that increasing endogenous bilirubin levels by reducing hepatic glucuronidation activity could induce “iatrogenic Gilbert syndrome”,^{16,17} a method potentially applicable to MAFLD as well. Additionally, regulating key enzymes involved in bilirubin metabolism emerges as a promising therapeutic target. Natural compounds such as resveratrol, curcumin, and statins, which act as HO-1 inducers, can elevate bilirubin levels and exert antioxidant effects both in vitro and in vivo.⁴⁹ Targeting multiple stages of bilirubin metabolism to sustain mild elevations in bilirubin may become an effective approach for preventing and treating various metabolic diseases.

There are several limitations in this study. The retrospective study design may have confounding factors that are not fully controlled, such as diet, exercise, drug, etc. Our study did not involve genetic factors that may affect bilirubin metabolism, such as UGT1A1 polymorphism (for example Gilbert syndrome), which are known to affect serum bilirubin levels and may confuse the relationship between bilirubin and MAFLD. Secondly, the mediating effect is based on statistical models, but has not been experimentally validated (such as animal models or cell experiments), and the

molecular mechanism has not been fully confirmed yet. Furthermore, although it has been suggested that increasing bilirubin levels may have a protective effect, its actual efficacy and safety have not been validated through intervention studies such as supplementing exogenous bilirubin or drug trials. But this study provides important clues for the metabolic protective effect of bilirubin through rigorous statistical analysis and large-scale clinical cohorts. These results lay the foundation for further basic and translational research, and in the future, it is necessary to fill the current gap through basic experiments and intervention studies to promote the development of precise prevention and treatment strategies for MAFLD.

Conclusions

Our findings demonstrate significantly reduced serum TBIL and DBIL levels in the Chinese MAFLD population, accompanied by an increase in systemic chronic inflammation and IR. A significant negative correlation was observed between TBIL, DBIL levels and the risk of MAFLD. Both TBIL and DBIL also showed notable negative correlations with the degree of systemic inflammation and IR, acting as independent protective factors against the development of MAFLD. In contrast, IBIL was not significantly correlated with MAFLD risk. Mediation analysis suggests that the protective effect of bilirubin may be mediated through the improvement of IR and the reduction of systemic chronic inflammation, with the enhancement of insulin sensitivity appearing to play a more dominant role. Regarding the hypothesis that bilirubin's protective mechanism in MAFLD may involve these processes, further research, including animal models and in vitro studies, is necessary to validate this hypothesis and provide insights into the underlying molecular signaling pathways of the disease. Additionally, as this study is a clinical retrospective analysis, other critical physiological effects of bilirubin, such as its antioxidant properties and its impact on mitochondrial function, were not investigated. These aspects will be addressed in future research endeavors.

Informed Consent Statement

Due to the retrospective nature of the study, written informed consent from participants was waived.

Institutional Review Board Statement

The study was conducted in accordance with the Helsinki Declaration of 2013. The research protocol was approved by the Ethics Committee of Fuyang People's Hospital (Ethics Approval No.: [2024-224]).

Author Contributions

Mengying Yang and Jing Liu contributed equally to this paper and should be considered as co-first authors. 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 no conflicts of interest.

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