Associations between different bilirubin subtypes and diabetic microvascular complications in middle-aged and elderly individuals

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Abstract

Aims: Some studies have reported associations between bilirubin and diabetic microvascular complications. However, these studies focused only on total bilirubin (TBIL) without distinguishing different bilirubin subtypes. In this study, we aimed to investigate the associations of TBIL, direct bilirubin (DBIL) and indirect bilirubin (IBIL) levels with albuminuria/creatinine ratio (ACR) and the prevalence of diabetic retinopathy (DR) among diabetic adults.

Methods: We analyzed 4368 individuals out of 4813 diabetic participants enrolled from seven communities in 2018 in a cross-sectional study. Participants underwent several checkups, including the measurement of anthropometric parameters, blood pressure, glucose, lipid profile, TBIL, DBIL, IBIL and ACR. DR was detected by high-quality fundus photographs and was remotely read by ophthalmologists.

Results: Compared with the first quartile of DBIL, participants in the fourth quartile had a lower prevalence of high ACR (odds ratio (OR) 0.76; 95% confidence interval (CI) 0.59, 0.99) (p for trend < 0.05). Neither TBIL nor IBIL was associated with the prevalence of high ACR. In DR, higher DBIL and TBIL by one standard deviation was associated with a 19% (OR 0.81; 95% CI 0.69, 0.94) and a 12% (OR 0.88; 95% CI 0.78, 0.99) lower frequency of DR, respectively (both p for trend < 0.05). However, IBIL was not associated with the prevalence of DR. These associations were adjusted for potential confounding factors.

Conclusion: DBIL had a stronger association with high ACR and DR than TBIL or IBIL did in diabetic adults. The effect of DBIL on diabetic complications should be noted and investigated in further studies.

Keywords: albuminuria/creatinine ratio, diabetic microvascular complications, diabetic retinopathy, direct bilirubin, indirect bilirubin, total bilirubin

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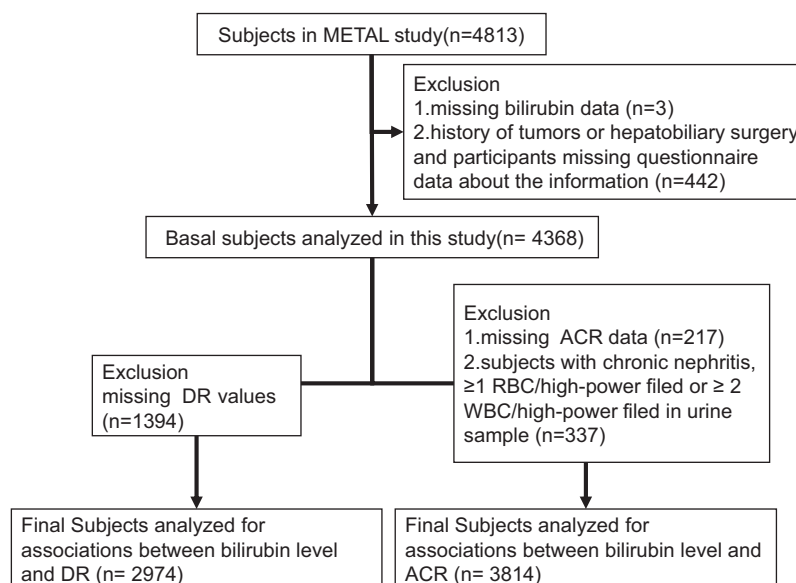Institute and Department of Endocrinology and Metabolism, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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Introduction

Type 2 diabetes mellitus (T2DM), a chronic, life-threatening disease, has become a serious threat to human health and global economies.[1,2] In 2017, there were an estimated 451 million people with diabetes worldwide, and it was expected to increase to 693 million by 2045,[3] affecting the incidence and prevalence of microvascular complications directly. Approximately one in three patients with T2DM will develop diabetic retinopathy (DR), and one in four will develop diabetic kidney disease, leading to blindness and kidney failure.[4] Although the rates of diabetic complications in developed nations have stabilized as the result of improved management of risk factors, the actual numbers of individuals

Figure 1. Flowchart of the sampling frame and participants selected from the METAL study.
ACR, albumin/creatinine ratio; DR, diabetic retinopathy; METAL, Environmental Pollutant Exposure and Metabolic Diseases in Shanghai; RBC, red blood cell; WBC, white blood cell.

affected by diabetes complications is still increasing because of the increasing absolute number of new diabetes cases.[5,6] Thus, it is paramount to find novel therapies to prevent and/or treat diabetic complications.

Bilirubin is generated in the catabolism of heme, in which biliverdin, as an intermediate product, is converted into indirect bilirubin (IBIL) by biliverdin reductase rapidly.[7] Further processing of bilirubin occurs in hepatocytes, where IBIL is conjugated by uridine diphosphate-glucuronosyltransferase to direct bilirubin (DBIL).[8] Total bilirubin (TBIL) is the sum of IBIL and DBIL, and IBIL accounts for more than 95% of TBIL in healthy adults.[9] Although bilirubin was long considered to be a marker of liver disease and to be supportive evidence for hemolytic anemia, bilirubin is actually an antioxidant.[10] In fact, moderate elevations in serum total bilirubin have been associated with reduced susceptibility to such diseases as diabetic kidney disease, DR and so on.[11,12] However, these studies merely focused on TBIL, without distinguishing between IBIL and DBIL, which have different clinical significance. To the best of our knowledge, studies reporting the associations of TBIL, DBIL and IBIL with diabetic microvascular complications in the same population are lacking.

Thus, in this study, from a large community-based sample, we aimed to investigate the associations of three serum bilirubin (TBIL, DBIL and IBIL) levels with the albuminuria/creatinine ratio (ACR) and the prevalence of DR among diabetic adults.

Materials and methods

Study design and participants
We designed a cross-sectional study named the METAL study (Environmental Pollutant Exposure and Metabolic Diseases in Shanghai, www.chictr. org.cn, ChiCTR1800017573). The present study is primary analysis of the METAL study. The detailed protocols have been described in previously published papers.[13–15] In brief, Chinese citizens ≥18 years old who have been previously diagnosed with diabetes and have lived in their current area for ≥6 months were enrolled, using a simple random method, from the registration platform of the healthcare centers in seven communities in Huangpu and Pudong District, Shanghai, China, from May to August 2018. A total of 4813 subjects with diabetes who were 23–99 years old received examination. Participants who had history of tumors or hepatobiliary surgery or missing questionnaire data about the information or bilirubin data ($n=445$) were excluded. A total of 4368 participants were involved in the basal analyses (Figure 1).

The study protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital,

Shanghai Jiao Tong University School of Medicine (2017-210). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the appropriate institutional review committee. Written consent was obtained from all participants included in the study.

Measurements

A questionnaire about sociodemographic characteristics, medical history, family history and lifestyle factors was adopted during the interview. The same group of trained and experienced personnel as in the SPECT-China study[16] conducted the interviews and clinical examinations, including weight, height and blood pressure, according to a standard protocol. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Current smoking was defined as having smoked at least 100 cigarettes in one's lifetime and currently smoking cigarettes.[17]

Peripheral venous blood samples were collected between 6:00 am and 9:00 am after fasting for at least 8 h in K-EDTA anti-coagulation tubes (Gaode, Zhejiang, China) for testing glycated hemoglobin (HbA1c), sodium fluoride tubes (Gaode, Zhejiang, China) for fasting plasma glucose (FPG) and separation gel vacuum procoagulant tube (Gaode, Zhejiang, China) for serum bilirubin (including TBIL, DBIL and IBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, total cholesterol and high- (HDL) and low-density lipoprotein (LDL). Then, the blood samples were refrigerated immediately and, in 2h, they were centrifuged, and the sera were aliquoted and frozen in a central laboratory. HbA1c was measured by high-performance liquid chromatography (MQ-2000PT, Medconn, Shanghai, China). Serum bilirubin, FPG, serum creatinine, ALT, AST, triglycerides, total cholesterol, HDL and LDL were performed with a Beckman Coulter AU 680 (Brea, USA).

Morning fasting spot urine samples were collected, refrigerated immediately and frozen at –20°C in 2h. The concentrations of urine albumin and creatinine were measured with a Beckman Coulter AU 680 (Brea, USA) using a turbimetric immunoassay and an enzymatic method in a single spot urine sample, and urine ACR was calculated.

DR screening was done by mydriatic binocular indirect ophthalmoscopy (Topcon TRC-NW400 Non-Mydriatic Retinal Camera, Oakland, USA). Fundus photographs were read by an ophthalmologist specialized in the retina as before.[18]

Outcome definition

Hypertension was defined as systolic blood pressure ≥140mmHg, diastolic blood pressure ≥90mmHg, or a self-reported previous diagnosis of hypertension by a physician. Dyslipidemia was defined as total cholesterol ≥6.22mmol/l (240mg/dl), triglycerides ≥2.26mmol/l (200mg/dl), LDL ≥4.14mmol/l (160mg/dl), HDL<1.04mmol/l (40mg/dl), or self-reported previous diagnosis of hyperlipidemia by a physician, according to the modified National Cholesterol Education Program–Adult Treatment Panel III.

The estimated glomerular filtration rate (eGFR) was calculated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation for 'Asian origin'.[19] High ACR was defined as ACR ≥30mg/g.

The internationally accepted DR classification by the 'Global Diabetic Retinopathy Project Group' in 2003 was used.[20] The classification was as follows: no diabetic retinopathy (NDR) (DR stage 0, no abnormalities), non-proliferative DR (NPDR) (DR stage 1–3, intraretinal microaneurysms, hemorrhages, venous beading, prominent microvascular abnormalities) and proliferative DR (PDR) (DR stage 4, neovascularization or vitreous/preretinal hemorrhages).

Statistical analysis

Data analyses were performed using IBM SPSS Statistics, Version 22 (IBM Corporation, Armonk, NY, USA). A *p* value < 0.05 indicated significance (two sided). Continuous variables were summarized as the mean ± standard deviation (SD) or median (interquartile range) and categorical variables as percentages (%). Linear or logistic regression analysis was used to test for trend of the variable changes across the bilirubin level quartiles, providing *p* values adjusted for age, sex, current smoking, education and duration of diabetes.

Bilirubin levels were divided into quartiles, with the first quartile representing the lowest and the

fourth quartile representing the highest. Linear (continuous dependent variables) and logistic regression (categorical dependent variables) were used to measure the association between bilirubin and diabetic microvascular complication outcomes. Data are expressed as regression coefficient or odds ratio (95% confidence interval) [OR (95% CI)].

For the association of bilirubin with eGFR, linear regression analysis was used. Binary logistic regression analysis was used for the association of bilirubin with the prevalence of high ACR. Associations of bilirubin with the prevalence of DR were calculated by binary logistic regression analysis. For the association between bilirubin and DR ranging from NDR to PDR, ordinal logistic regression was used. The restricted cubic spline analysis for the dose-relationship between DBIL and the prevalence of DR and high ACR were completed by the statistical package R (http://www.R-project.org, The R Foundation) and Empower (R) (www.empowerstats.com; X&Y Solutions, Inc., Boston, MA, USA). The model was adjusted for age, sex, duration of diabetes, education, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia and hypertension and the usage of insulin, angiotensin-converting enzyme inhibitor (ACEI) or angiotensin receptor antagonist (ARB) and statins.

Sensitivity analyses were performed. We evaluated the general characteristics of the participants with or without DR data shown in the Supplemental Material Table 1 online, the associations of bilirubin level quartiles with the prevalence of DR after adjusting for FPG instead of HbA1c shown in Supplemental Table 2. Multiple imputation analysis was done, assuming the main missing data about DR are missing at random. Ten imputed datasets were created, and the relative efficiency of the substantive regression analysis was at least 99%. Fully conditional specification was applied with logistic regression models. The imputation procedure included all variables from the regression model (age, sex, duration of diabetes, education, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia, hypertension and the usage of insulin, ACEI or ARB and statins, DBIL, IBIL, TBIL and DR for the DR regression analysis) (Supplemental Table 3). Furthermore, the associations of DBIL with DR and High ACR in different subgroups by the median of age, duration of diabetes, FPG and HbA1c and the

interaction of these variables were calculated (Supplementary Figure 1).

Results

Characteristics of the participants by bilirubin level quartile

The general demographic and characteristics of the study population are shown in Table 1. We analyzed 4368 diabetic participants with a mean age of 67 years [SD 9, maximum (max.) 99, minimum (min.) 23]. The mean TBIL, DBIL and IBIL levels were 14.1 μmol/l (SD 5.5, max. 139.1, min. 3.1), 2.5 μmol/l (SD 1.2, max. 38.3, min. 0.2) and 11.6 μmol/l (SD 4.5, max. 113.7, min. 2.5), respectively. Compared with the participants in the lowest DBIL quartile, those in the highest quartile had significantly lower HbA1c, total cholesterol, triglycerides, LDL, prevalence of high ACR, NPDR and dyslipidemia and higher HDL and eGFR (all p for trend < 0.05). (Table 1).

Association of bilirubin level quartiles with high ACR and eGFR

Table 2 indicates that higher DBIL level, rather than TBIL or IBIL, was significantly associated with the lower prevalence of high ACR, although higher TBIL, DBIL and IBIL were all associated with higher eGFR significantly. It is worth noting that higher DBIL by one SD had the highest β associated with eGFR (β 1.12, 95% CI 0.64, 1.60), compared with TBIL (β 0.99, 95% CI 0.52, 1.46) and IBIL (β 0.93, 95% CI 0.47, 1.40), which indicated that DBIL had a stronger association with eGFR than TBIL or IBIL.

In addition, participants in the fourth DBIL quartile had a significantly lower prevalence of high ACR (OR 0.76; 95% CI 0.59, 0.99) (p for trend < 0.05), and higher DBIL by one SD was significantly associated with a 14% lower frequency of high ACR events (OR 0.86; 95% CI 0.78, 0.95). A non-linear inverse association ($p_{\text{for non-linearity}}$ < 0.01) between DBIL and the prevalence of high ACR was detected (Figure 2). However, neither DBIL nor IBIL was significantly associated with high ACR. These associations were all adjusted for age, sex, duration of diabetes, education, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia and hypertension and the usage of insulin, ACEI or ARB and statins.

Table 1. General characteristics of all participants by DBIL level quartiles.

Characteristic	DBIL level, μmol/l				
	Quartile 1 (≤1.8)	Quartile 2 (>1.8, ≤2.3)	Quartile 3 (>2.3, ≤2.9)	Quartile 4 (>2.9)	*p* for trend
n	1156	1169	1062	981	–
Age, years	65.92 ± 9.15	66.65 ± 8.42	67.70 ± 8.52	68.26 ± 8.50	–
Men, %	32.3	41.7	57.4	63.9	–
Duration of diabetes, years	8 (3, 15)	10 (4, 15)	8 (3, 15)	8 (3, 15)	–
Current smoking, %	18.9	20.0	20.7	16.8	–
Beyond high school education, %	49.4	51.4	54.3	52.8	–
BMI, kg/m^2	24.93 ± 3.64	24.96 ± 3.57	25.04 ± 3.48	24.94 ± 3.51	0.558
FPG, mmol/l	7.82 ± 2.65	7.78 ± 2.37	7.79 ± 2.53	7.76 ± 2.28	0.781
HbA1c, %	7.58 ± 1.42	7.50 ± 1.45	7.49 ± 1.37	7.41 ± 1.31	0.010
Total cholesterol, mmol/l	5.66 ± 1.23	5.26 ± 1.13	4.84 ± 1.07	4.51 ± 1.02	<0.001
Triglycerides, mmol/l	1.92 (1.34, 2.85)	1.56 (1.12, 2.15)	1.44 (1.05, 2.00)	1.31 (0.96, 1.74)	<0.001
HDL, mmol/l	1.19 ± 0.27	1.23 ± 0.29	1.18 ± 0.29	1.20 ± 0.31	<0.001
LDL, mmol/l	3.50 ± 0.84	3.26 ± 0.82	3.00 ± 0.79	2.75 ± 0.74	<0.001
AST, U/l	20 (17, 25)	21 (18, 25)	21 (18, 26)	22 (19, 28)	<0.001
ALT, U/l	18 (14, 24)	19 (14, 25)	19 (14, 26)	20 (15, 30)	<0.001
TBIL, μmol/l	9.4 (8.3, 10.7)	12.1 (11.0, 13.3)	14.5 (13.2, 16.3)	19.4 (17.1, 22.5)	<0.001
IBIL, μmol/l	7.9 (6.8, 9.1)	10.0 (8.9, 11.2)	11.9 (10.6, 13.6)	15.9 (13.8, 18.7)	<0.001
High ACR, %	28.5	25.9	24.3	22.5	0.006
eGFR, ml/min per 1.73 m^2	91.17 ± 19.89	92.93 ± 15.99	91.22 ± 16.49	91.31 ± 15.31	<0.001
NPDR, %	19.0	16.9	17.7	14.6	0.033
PDR, %	0.6	0.5	0.3	0.4	0.365
Hypertension, %	78.6	77.3	81.5	77.9	0.223
Dyslipidemia, %	71.7	61.3	60.2	54.2	<0.001
Using insulin, %	14.0	12.6	13.7	13.9	0.517
Taking ACEI or ARB, %	22.2	21.5	25.7	21.6	0.468
Taking statins, %	12.8	16.0	15.8	14.8	0.086

The data are summarized as the mean ± SD or median (interquartile range) for continuous variables or as a numerical proportion for categorical variables. *p* for trend was calculated by regression tests and was adjusted for age, sex, current smoking, education and duration of diabetes. High ACR was defined as ACR ⩾30 mg/g.
ACEI, angiotensin-converting enzyme inhibitor; ACR, albumin/creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor antagonist; AST, aspartate aminotransferase; BMI, body mass index; DBIL, direct bilirubin; eGFR, estimated glomerular infiltration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; IBIL, indirect bilirubin; LDL, low-density lipoprotein; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; TBIL, total bilirubin

Table 2. Associations between bilirubin level quartiles and high ACR and eGFR.

	Bilirubin level, μmol/l				*p* for trend	1-SD increment of bilirubin
	Quartile 1	Quartile 2	Quartile 3	Quartile 4		
TBIL						
High ACR	Ref.	0.92 (0.73, 1.16)	0.99 (0.79, 1.25)	0.81 (0.64, 1.03)	0.170	0.89 (0.81, 1.01)
eGFR	Ref.	2.27 (0.98, 3.56)	1.91 (0.61, 3.21)	3.21 (1.89, 4.54)	<0.001	0.99 (0.52, 1.46)
DBIL						
High ACR	Ref.	1.03 (0.82, 1.29)	0.84 (0.66, 1.06)	0.76 (0.59, 0.99)	0.015	0.86 (0.78, 0.95)
eGFR	Ref.	2.06 (0.78, 3.34)	2.08 (0.75, 3.40)	3.30 (1.90, 4.70)	<0.001	1.12 (0.64, 1.60)
IBIL						
High ACR	Ref.	0.90 (0.72, 1.13)	1.01 (0.81, 1.27)	0.81 (0.64 1.03)	0.204	0.90 (0.82, 1.02)
eGFR	Ref.	2.43 (1.14, 3.71)	2.02 (0.72, 3.33)	3.19 (1.86, 4.51)	<0.001	0.93 (0.47, 1.40)

Data are expressed as regression coefficient or odds ratio (95% confidence interval). Linear and logistic regression analyses were used for the association of bilirubin with continuous and categorical variables, respectively. Some 217 subjects had missing ACR values, and 337 had chronic nephritis, $\geq$1 RBC/high-power field or $\geq$2 WBCs/high-power field in urine samples, so they were excluded. High ACR was defined as ACR $\geq$30 mg/g. In total, 3814 subjects were involved in the regression analyses. The model was adjusted for age, sex, duration of diabetes, education, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia, hypertension and the usage of insulin, ACEI or ARB and statins.
ACEI, angiotensin-converting enzyme inhibitor; ACR, microalbumin/creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor antagonists; AST, aspartate aminotransferase; BMI, body mass index; DBIL, direct bilirubin; eGFR, estimated glomerular infiltration rate; HbA1c, glycated hemoglobin; IBIL, indirect bilirubin; RBC, red blood cell; Ref., reference; TBIL, total bilirubin; WBC, white blood cell.

Association of bilirubin level quartiles with the prevalence and severity of DR

Table 3 shows that increased DBIL and TBIL, but not IBIL, were significantly associated with a decreased prevalence of DR and the severity of DR. Compared with the first DBIL or TBIL quartile, participants in the fourth quartile had a lower prevalence of DR [(OR 0.70; 95% CI 0.51, 0.95) and (OR 0.72; 95% CI 0.54, 0.97)] (both *p* for trend < 0.05). Higher DBIL by one SD was associated with a 19% lower prevalence of DR (OR 0.81, 95% CI 0.69, 0.94), whereas higher TBIL by one SD was associated with a 12% lower frequency of DR (OR 0.88, 95% CI 0.78, 0.99), which indicated that DBIL had a stronger association with the prevalence of DR than TBIL. However, IBIL was not significantly associated with the prevalence of DR. Furthermore, in ordinal logistic regression, the ORs (95% CIs) of DR ranging from NDR to PDR for DBIL and TBIL by one SD were respectively 0.98 (0.96, 0.99) and 0.98 (0.97, 1.00) (*p* for trend < 0.05). In the restricted cubic spline model, a non-linear inverse association ($p_{\text{for non-linearity}} < 0.01$) between DBIL and the prevalence of DR was detected (Figure 2). The associations were all adjusted for age, sex, education, duration of diabetes, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia and hypertension and the usage of insulin, ACEI or ARB and statins.

Discussion

To the best of our knowledge, this is the first study to evaluate the association of TBIL, DBIL and IBIL with eGFR, high ACR and DR in the same large-scale population simultaneously. In this study, among over 4000 community-dwelling Chinese type 2 diabetic adults, we found that increased DBIL, rather than TBIL or IBIL, was significantly associated with a decreased prevalence of high ACR, and higher DBIL and TBIL, but not IBIL, were associated with a lower prevalence of DR, although DBIL, TBIL and IBIL were positively associated with eGFR. Importantly, our results showed that DBIL may have stronger associations with high ACR, eGFR and DR compared with TBIL and IBIL in diabetic adults. These associations were strong, dose dependent and consistent even after adjusting for potential confounding factors.
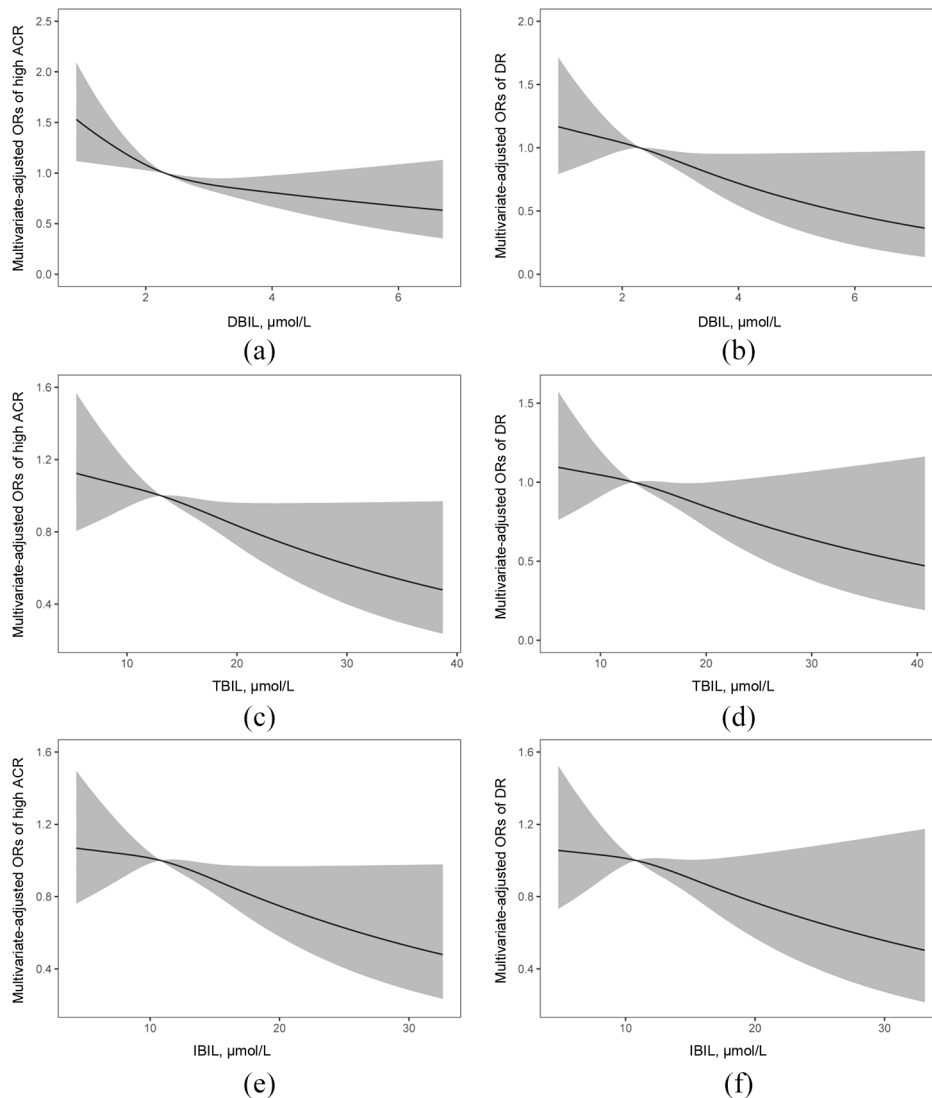
Figure 2. Dose–response relationship between bilirubin level with the prevalence of high **ACR** and DR.
(a) DBIL and high ACR; (b) TBIL and high ACR; (c) IBIL and high ACR; (d) DBIL and DR; (e) TBIL and DR; (f) IBIL and DR.
The model was adjusted for age, sex, duration of diabetes, education, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia, hypertension and the usage of insulin, ACEI or ARB and statins.
ACEI, angiotensin-converting enzyme inhibitor; ACR, albumin/creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor antagonists; AST, aspartate aminotransferase; BMI, body mass index; DBIL, direct bilirubin; DR, diabetic retinopathy; HbA1c, glycated hemoglobin; IBIL, indirect bilirubin; TBIL, total bilirubin

DBIL production is the final step in the complex chain of heme degradation, which occurs in hepatocytes.[8,21] Compared with IBIL, the clinical impact of DBIL seems to be minimal due to its hydrophilic and less antioxidant properties.[10,22] In such cases, the clinical role of DBIL has often been overlooked. However, in reality, some studies have demonstrated the benefits of DBIL. One cohort study of 5900 Korean men found that higher serum DBIL level was significantly associated

with a lower risk of developing non-alcoholic fatty liver disease.[23] Moreover, other epidemiologic studies have also suggested that higher DBIL was significantly associated with a lower prevalence of various diseases, such as metabolic syndrome,[24] coronary artery calcium[25] and stroke severity.[26] One more recent study suggested that DBIL is more negatively correlated with levels of small, dense LDL compared with IBIL and TBIL.[27] Specifically, these authors all suggested that lower

Table 3. Association of bilirubin level quartiles with the prevalence and severity of DR.

	Bilirubin level, μmol/l				*p* for tend	1-SD increment of bilirubin
	Quartile 1	Quartile 2	Quartile 3	Quartile 4		
TBIL						
DR	Ref.	0.96 (0.73, 1.27)	0.90 (0.68, 1.19)	0.72 (0.54, 0.97)	0.031	0.88 (0.78, 0.99)
DR (stage NDR to PDR)	Ref.	1.00 (0.95, 1.04)	0.99 (0.95, 1.03)	0.95 (0.91, 0.99)	0.018	0.98 (0.97, 1.00)
DBIL						
DR	Ref.	0.84 (0.64, 1.11)	0.88 (0.66, 1.16)	0.70 (0.51, 0.95)	0.038	0.81 (0.69, 0.94)
DR (stage NDR to PDR)	Ref.	0.97 (0.93, 1.02)	0.98 (0.94, 1.02)	0.95 (0.91, 0.99)	0.025	0.98 (0.96, 0.99)
IBIL						
DR	Ref.	0.98 (0.75, 1.29)	0.96 (0.73, 1.27)	0.76 (0.57, 1.02)	0.084	0.89 (0.79, 1.01)
DR (stage NDR to PDR)	Ref.	0.99 (0.95, 1.04)	0.99 (0.95, 1.04)	0.95 (0.91, 1.00)	0.044	0.98 (0.97, 1.00)

Data are expressed as odds ratio (95% confidence interval). Ordinal logistic regression analysis was used for the association between bilirubin and DR (stage NDR to PDR). The association between bilirubin and the prevalence of DR was calculated by binary logistic regression analysis. A total of 1394 subjects with missing DR values were excluded. A total of 2974 subjects were involved in the regression analyses. The model was adjusted for age, sex, duration of diabetes, education, current smoking, BMI, HbA1c, ALT, AST, dyslipidemia, hypertension and the usage of insulin, ACEI or ARB and statins. ACEI, angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin receptor antagonist; AST, aspartate aminotransferase; BMI, body mass index; DBIL, direct bilirubin; DR, diabetic retinopathy; HbA1c, glycated hemoglobin; IBIL, indirect bilirubin; NDR, non-diabetic retinopathy; PDR, proliferative diabetic retinopathy; Ref., reference; TBIL, total bilirubin

DBIL level was more strongly associated with a high prevalence of the disease than were TBIL and IBIL levels. However, no studies have reported the effects of individual bilirubin sub-types on diabetic microvascular complications.

Our study found that in type 2 diabetic adults, a higher DBIL level, rather than TBIL or IBIL, was associated with a decreased prevalence of high ACR, although TBIL, DBIL and IBIL were all positively associated with eGFR. In fact, the association between TBIL and ACR has been reported in several studies, but the results are inconsistent.[25–28] In a cross-sectional study with 93,909 subjects (aged 18–96 years, 53.0% male), the association between high serum bilirubin and decreased prevalence of diabetic kidney disease was not found in men.[28] Another cohort study with normal serum bilirubin subjects showed no significant association between serum bilirubin levels and the progression from normoalbuminuria to microalbuminuria.[29] Inversely, two cohort studies found low TBIL concentration could be a risk factor for the development of albuminuria in patients with type 2 diabetes.[30,31] However, no studies have reported the association between DBIL and albuminuria. It is noteworthy that

studies about the association between bilirubin and eGFR have resulted in similar conclusions, but not all reports concur.[26,29] One cohort study showed that not only baseline TBIL but also follow-up changes were negatively associated with eGFR in male diabetic patients;[32] however, in another cohort study, recruiting subjects with normal serum bilirubin, no association between TBIL and eGFR was found.[29] Our study found that TBIL, DBIL and IBIL were all positively associated with eGFR. As the mechanism involved is poorly understood, possible explanations for these inconsistent findings might be discrepancies in the considered confounders, the relatively wide range of bilirubin levels measured and population characteristics. More studies are required to further explore this association.

Similar to high ACR, we found that the incidence and severity of DR were associated with lower DBIL and TBIL levels, but not IBIL level. Indeed, previous studies about the association between TBIL and DR are inconsistent. Some cross-sectional studies reported a significant negative relationship between TBIL and DR,[33–35] which was consistent with our results. Furthermore, a five-year cohort study that consisted of 5323 Chinese

male diabetic patients suggested that serum TBIL had a U-shaped relationship with DR incidence.[36] Another previous study found no association between TBIL and DR.[37] The controversy in the studies may be due to adjustments for different covariates, variations in population sampling and the quality of the assessment of retinopathy. Interestingly, we found that DBIL was negatively associated with the incidence and severity of DR. As far as we know, our study is the first to reveal a negative and significant association between DBIL and DR. Thus, whether lower DBIL might be a clinical marker for predicting the occurrence of DR should be further investigated.

Although the mechanisms linking bilirubin subtype and diabetic microvascular complications are still unclear, some possible explanations can be proposed. One is the different binding affinities of the different bilirubin forms for albumin. DBIL, which binds weakly to albumin, plays an important role in preventing lipid peroxidation as a chain-breaking antioxidant.[38] An epidemiological study found that, compared with IBIL and TBIL, DBIL was more strongly correlated with a level of small, dense LDL[27] and metabolic syndrome,[24] which are risk factors for diabetic microvascular complications. DBIL-mediated inhibition of immune reactions and inflammatory processes, and as a marker reflecting heme oxygenase-1 activity, may be the other reason. DBIL may possess cytoprotective effects, mediated through albumin-bound bilirubin protecting plasma and mitochondrial membranes from damage by removal of reactive oxygen species at physiological concentrations.[39] Evidence from population studies suggested that increased serum concentrations of DBIL, rather than TBIL, may participate in the protection of cardiovascular function.[25] Furthermore, in the Eisai hyperbilirubinemia rat, a model of Dubin–Johnson syndrome that usually manifests as recurrent jaundice with conjugated hyperbilirubinemia, findings have suggested that increased concentrations of DBIL had a protective effect against focal ischemia, possibly participating in heme oxygenase-1-induced neuroprotection.[40] More studies are required to elucidate the specific discrepancies between DBIL and IBIL with respect to their molecular mechanisms of action.

Though our study had some strengths, including its relatively large sample of community-dwelling participants and strong quality control, there were also some limitations. First, owing to the nature of cross-sectional design of the current study, causal relationships cannot be confirmed linking bilirubin with diabetic microvascular complications. The present findings should be cautiously interpreted and further prospective studies are needed. Second, Han Chinese were the ethnic group investigated, so the results may not be generalizable to other ethnicities. Third, the inability to account for all confounders related to bilirubin or diabetic microvascular complications might have limited our multivariate approach.

Conclusion

We reported that DBIL had a stronger association with ACR, eGFR and DR compared with TBIL and IBIL in Chinese diabetic adults. The effect of DBIL on diabetic complications should be noted, and the association between bilirubin subtype and diabetic microvascular complications should be assessed in additional follow-up studies. Future research should be performed to clarify the different roles of DBIL and IBIL in diabetic microvascular complications. Further prospective studies in external populations are still required to verify our findings.

Author contribution(s)
Heng Wan: Formal analysis; Investigation; Methodology; Writing original draft; Writing-review & editing.

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Availability of data and materials

The data supporting the findings of this study are available on reasonable request from the corresponding authors. All data analyzed during this study are included in this published article. Data are available upon reasonable request to any scientist who wishes to use them without breaching participant confidentiality.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethics statement

The study protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine and conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the appropriate institutional review committee.

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Informed consent

Written informed consent was obtained from all participants included in the study.

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Supplemental material

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