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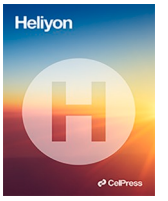

Research article

High-normal serum bilirubin is a useful indicator to assess the risk of diabetic retinopathy in type 2 diabetes: A real-world study

Man-Rong Xu [1], Jun-Wei Wang [1], Yi-Lin Ma [1], Yu-Jie Wang, Meng-Han Li, Jun-Xi Lu, Lian-Xi Li [*]

Department of Endocrinology and Metabolism, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai Clinical Center for Diabetes, Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Key Clinical Center for Metabolic Disease, 600 Yishan Road, Shanghai, 200233, China

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ABSTRACT

Background: To investigate the association of serum bilirubin within normal range, especially unconjugated bilirubin (UCB), with diabetic retinopathy (DR) in patients with type 2 diabetes mellitus (T2DM).

Methods: In this cross-sectional, real-world study, 7617 T2DM patients were stratified into quartiles based on serum UCB levels. DR was determined by digital fundus photography and further classified into non-proliferative diabetic retinopathy (NPDR) and PDR. The associations of serum bilirubin levels and UCB quartiles with DR were investigated by logistic regression analysis.

Results: After controlling for age, sex, and diabetes duration, the DR prevalence was significantly decreased across the serum UCB quartiles (40.4 %, 33.4 %, 29.7 %, 26.6 % for each quartile, respectively, $p < 0.001$ for trend). The subjects with DR had lower serum total bilirubin (TB) and UCB, rather than conjugated bilirubin (CB), compared with those without DR ($p = 0.003$ for TB, $p < 0.001$ for UCB, and $p = 0.528$ for CB, respectively), while all three types of serum bilirubin in the subjects with PDR were obviously lower than those with NPDR ($p = 0.006$ for TB, and $p < 0.001$ for UCB and CB, respectively). After adjustment for confounding factors, logistic regression demonstrated negative associations of serum TB and UCB levels, rather than CB, with the presence of DR (OR: 0.844, 95%CI: 0.774–0.920, $p < 0.001$ for TB; OR: 0.828, 95%CI: 0.763–0.899, $p < 0.001$ for UCB; and OR: 0.984, 95%CI: 0.900–1.074, $p = 0.713$ for CB, respectively). Additionally, a fully-adjusted analysis revealed a negative correlation between UCB quartiles and DR ($p < 0.001$).

Conclusion: High-normal serum TB and UCB were closely associated with the decreased odds of DR, while all types of serum bilirubin were negatively correlated with the severity of DR in T2DM patients. Serum bilirubin may be used as a potential indicator to assess the risk and severity of DR in T2DM.

* Corresponding author.
E-mail address: lilx@sjtu.edu.cn (L.-X. Li).
[1] These authors contributed equally to this work.

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1. Background

Unconjugated bilirubin (UCB), also known as indirect bilirubin (I-BIL), is one of the types of serum bilirubin and has long been known to have oxidative toxicity and neurotoxicity [1]. However, serum bilirubin especially UCB has also been characterized in several recent studies as having significant antioxidant and anti-inflammatory effects [2,3], which play a significant protective role in atherosclerosis [4]. Additionally, in a retrospective study, low UCB levels were observed to be associated with moderate-to-severe inflammation and fibrosis of liver in patients with non-alcoholic fatty liver disease [5]. Furthermore, diabetic patients with Gilbert's syndrome, characterized by mildly elevated serum UCB levels, showed a low prevalence of hypertension and a significantly reduced levels of CRP [6]. Interestingly, the anti-oxidative stress effect of UCB has been experimentally demonstrated to improve insulin sensitivity in patients with type 2 diabetes mellitus (T2DM) [7]. Therefore, UCB may possess a protective effect against metabolic diseases related to chronic inflammation such as diabetes, hypertension, atherosclerosis and liver fibrosis.

As one of the diabetic microangiopathy, diabetic retinopathy (DR) has become one of the leading causes of blindness with more than a third of diabetic patients suffering from DR [8]. Some clinical parameters such as duration of diabetes (DD), glycosylated hemoglobin A1C (HbA1c) levels, and systolic blood pressure (SBP) have been identified as critical risk factors for DR [8,9], but the exact pathogenic mechanisms of DR have not been completely elucidated so far. However, the early pathogenesis of DR has recently been shown to involve neurodegeneration and its interaction with microangiopathy [10], in which the molecular mechanisms include glutamate excitotoxicity, mitochondrial dysfunction, and antioxidant defense system damage and relate to oxidative stress damage [11]. For example, the dysregulation of polyamine metabolic pathway can lead to the production of reactive oxygen species (ROS) and thus the subsequent retinal neurodegeneration [11]. Therefore, the high glucose-mediated oxidative stress and subsequent inflammatory response are key mechanisms leading to diabetic microangiopathy such as retinopathy and neuropathy [12,13]. Given that oxidative stress and inflammation are closely associated with the onset and progression of DR, it is reasonable to hypothesize that UCB may exert a protective effect on the development and progression of DR through its anti-oxidative stress and anti-inflammatory effects.

Recently, several clinical studies have revealed that serum bilirubin may have a protective effect on diabetic microangiopathy such as diabetic nephropathy and retinopathy [14,15]. For example, a negative non-linear association between serum bilirubin levels and the risk of diabetic microvascular complications including diabetic nephropathy, retinopathy and neuropathy has been uncovered in a meta-analysis of 27 studies with a total of 132,240 diabetic subjects [15]. Furthermore, several observational clinical studies also suggested that mildly evaluated serum total bilirubin (TB) might be independently and negatively associated with the presence of DR in T2DM patients [16,17]. Likewise, Su et al. observed that there was a significant difference in TB levels between the patients with proliferative diabetic retinopathy (PDR) and those with non-proliferative diabetic retinopathy (NPDR), and TB could delay the progression of DR to PDR by reducing the levels of risk factors-associated with DR such as SBP, low-density lipoprotein cholesterol (LDL-C) and 24h urinary albumin excretion (UAE) [18].

Interestingly, when conjugated bilirubin (CB) and UCB were distinguished from TB, the different types of serum bilirubin exhibited a distinct relationship with DR [19,20]. For example, Dave et al. found a significantly inverse correlation between all three types of bilirubin levels and DR [19]. However, another cross-sectional study including 4318 middle-aged and elderly diabetic patients observed that one standard deviation higher CB and TB was respectively associated with 19 % and 12 % reduction in DR prevalence [21]. In contrast, UCB was found to have no significant association with the prevalence of DR [21].

Therefore, the existing studies on the relationship between serum bilirubin and DR mainly focused on TB and its association with DR. Furthermore, the association of three types of serum bilirubin with DR in T2DM population remains controversial, and few clinical investigations explored the relationship between UCB levels within normal limits and DR in T2DM subjects. Accordingly, the aim of the present study in a relatively large sample of Chinese T2DM patients was to investigate the real association between high-normal serum bilirubin, especially UCB, and the prevalence and progression of DR.

2. Patients and methods

2.1. Subjects and study design

This cross-sectional, real-world study was approved by the ethics committee of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (approval number: 2018-KY-018(K)) and complied with the tenets of the Declaration of Helsinki. Written informed consents were obtained from all studied subjects.

From January 2003 to December 2012, a total of 11,805 T2DM patients hospitalized in our department were consecutively recruited in this study. The criteria for exclusion included: 1) lack of complete clinical data; 2) without retinal photographs due to technical difficulties; 3) severe liver diseases other than non-alcoholic fatty liver disease; 4) serious systemic diseases or infectious diseases; 5) abnormal serum bilirubin (defined as TB $>$ 18 μmol/L or/and CB $>$ 6 μmol/L in our hospital). Ultimately, 7617 T2DM patients were included in the present study.

All subjects were interviewed to obtain information about a history of hypertension, medication history including antihypertensive agents (AHAs), anticoagulant agents (ACAs), lipid-lowering drugs (LLDs), metformin, sulfonylureas (SU), and insulin or insulin analogues (IIAs), alcohol consumption, and smoking status. The definitions of hypertension, obesity, smoking, and alcohol consumption had been described in our previous studies [22,23].

2.2. Physical examination and laboratory measurements

The methods of physical examination and laboratory measurements had been mentioned in our previous studies [22,23]. Briefly, physical examinations included weight, height, waist circumference (WC), hip circumference, SBP and diastolic blood pressure (DBP). Waist-to-hip ratio (WHR) was equal to waist circumference divided by hip circumference. Body mass index (BMI) was calculated as weight divided by height squared (kg/m2).

Venous blood samples were obtained after an overnight fasting and 2 h after breakfast. Laboratory parameters including HbA1c, fasting plasma glucose (FPG), 2 h postprandial plasma glucose (2h PPG), fasting C-peptide (FCP), 2h postprandial C-peptide (2h PCP), total triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), LDL-C, aspartate aminotransferase (ALT), γ-glutamyl transferase (γ-GT), glycated albumin (GA), creatinine (Cr), serum uric acid (SUA), and C-reactive protein (CRP) were measured by standard protocols [24,25]. The homeostasis model assessment for insulin resistance (HOMA2-IR), UAE, as well as estimated glomerular filtration rate (eGFR) was computed according to our recent protocols [26].

As described in detail in our previous studies, the concentrations of serum TB and CB were determined using an automated analyzer, LABOSPECT 008 AS (Hitachi High-Tech Co., Tokyo, Japan), and serum UCB levels were calculated by subtracting CB from

Table 1
Characteristics of the subjects according to UCB quartiles.

Variables	Q1 (n = 1890)	Q2 (n = 1896)	Q3 (n = 1395)	Q4 (n = 2436)	p value	[a]p value
UCB (umol/l))	<7.0	7.0–8.39	8.40–9.99	>9.99	–	–
#TB (umol/l))	8.0 (6.9–9.0)	10.0 (9.0–11.0)	12.0 (11.0–13.0)	14.9 (13.1–16.0)	<0.001	<0.001
#CB (umol/l))	2.3 (1.8–3.1)	2.1 (2.0–3.1)	3.0 (2.0–3.8)	3.0 (2.6–4.0)	<0.001	<0.001
Male (n, %)	910 (48.1 %)	940 (49.6 %)	714 (51.2 %)	1425 (58.5 %)	<0.001	<0.001
Age (years)	60 ± 12	60 ± 12	59 ± 11	58 ± 12	<0.001	<0.001
#DD (months)	120 (60–180)	96 (36–156)	84 (36–144)	72 (12–120)	<0.001	<0.001
Smoking (n, %)	551 (29.2 %)	535 (28.2 %)	406 (29.1 %)	746 (30.6 %)	0.369	<0.001
Alcohol consumption (n, %) (n, %)	260 (13.8 %)	268 (14.1 %)	217 (15.6 %)	460 (18.9 %)	<0.001	0.349
HTN (n, %)	1063 (56.2 %)	1052 (55.5 %)	735 (52.7 %)	1265 (51.9 %)	0.014	0.393
Obesity (n, %)	853 (45.5 %)	856 (46.1 %)	662 (47.8 %)	1114 (46.4 %)	0.637	0.659
AHAs (n, %)	1002 (53.0 %)	970 (51.2 %)	671 (48.1 %)	1176 (48.3 %)	0.005	0.130
ACAs (n, %)	973 (51.5 %)	934 (49.3 %)	687 (49.2 %)	1141 (46.8 %)	0.026	0.193
LLDs (n, %)	794 (42.0 %)	698 (36.8 %)	525 (37.6 %)	907 (37.2 %)	0.003	0.003
Metformin (n, %)	1159 (61.3 %)	1129 (59.5 %)	836 (59.9 %)	1418 (58.2 %)	0.226	0.145
SU (n, %)	1265 (66.9 %)	1304 (68.8 %)	964 (69.1 %)	1651 (67.8 %)	0.495	0.151
IIAs (n, %)	1384 (73.2 %)	1319 (69.6 %)	937 (67.2 %)	1611 (66.1 %)	<0.001	<0.001
SBP (mmHg)	133 ± 17	133 ± 18	132 ± 17	132 ± 17	0.776	0.537
DBP (mmHg)	79 ± 9	80 ± 9	81 ± 10	81 ± 10	<0.001	<0.001
BMI (kg/m^2)	24.88 ± 3.60	24.91 ± 3.52	24.85 ± 3.45	24.94 ± 3.41	0.876	0.961
WC (cm)	89.7 ± 10.8	90.0 ± 10.6	89.1 ± 10.3	89.8 ± 10.0	0.139	0.137
WHR	0.92 ± 0.07	0.92 ± 0.07	0.91 ± 0.06	0.92 ± 0.06	0.033	0.007
#FPG (mmol/l)	7.3 (5.9–9.4)	7.7 (6.2–9.7)	7.7 (6.2–9.6)	8.2 (6.6–10.3)	<0.001	<0.001
#2h PPG (mmol/l)	12.4 (9.5–15.8)	13.1 (10.0–16.5)	13.3 (10.0–16.5)	14.2 (10.9–17.5)	<0.001	<0.001
#GA (%)	21.1 (18.0–27.2)	23.2 (19.0–31.0)	23.0 (19.0–30.0)	25.0 (19.8–32.0)	<0.001	<0.001
HbA1C (%)	8.7 ± 2.25	8.96 ± 2.25	8.81 ± 2.14	9.01 ± 2.17	<0.001	0.003
#FCP (ng/mL)	1.83 (1.13–2.67)	1.81 (1.11–2.64)	1.79 (1.14–2.52)	1.78 (1.20–2.54)	0.799	0.802
#2h C–P (ng/mL)	3.91 (2.25–6.11)	3.94 (2.14–5.93)	4.14 (2.42–6.12)	4.04 (2.45–6.00)	0.102	0.057
#HOMA2-IR	1.56 (0.94–2.27)	1.56 (0.95–2.34)	1.54 (0.98–2.18)	1.58 (1.04–2.25)	0.337	0.309
#TG (mmol/l)	1.39 (0.99–2.10)	1.46 (1.00–2.16)	1.39 (0.99–2.09)	1.55 (1.06–2.29)	<0.001	<0.001
TC (mmol/l)	4.67 ± 1.28	4.77 ± 1.10	4.84 ± 1.06	4.99 ± 1.08	<0.001	<0.001
HDL-C (mmol/l)	1.08 ± 0.29	1.13 ± 0.31	1.16 ± 0.32	1.18 ± 0.34	<0.001	<0.001
LDL-C (mmol/l)	2.84 ± 0.94	3.04 ± 0.90	3.16 ± 0.94	3.25 ± 0.93	<0.001	<0.001
#ALT (U/l)	18 (13–28)	18 (13–28)	19 (14–29)	21 (15–33)	<0.001	<0.001
#γ-GT (U/l)	23.0 (16.0–35.0)	23.0 (16.0–36.0)	23.0 (16.0–36.0)	24.0 (17.0–37.0)	<0.001	<0.001
#Cr (μmol/l)	66 (55–81)	65 (54–79)	65 (54–78)	66 (55–77)	0.208	<0.001
#SUA (μmol/l)	315 (263–377)	310 (255–373)	311 (259–374)	312 (259–374)	0.283	0.034
#UAE (mg/24h)	14 (7–54)	13 (7–39)	11 (7–24)	11 (7–26)	<0.001	<0.001
#eGFR (ml/min/1.73 m^2)	109 (87–132)	111 (90–134)	110 (91–134)	112 (95–134)	<0.001	<0.001
#CRP (mg/L)	1.25 (0.55–3.41)	1.30 (0.52–3.18)	1.11 (0.47–2.57)	1.02 (0.47–2.35)	<0.001	<0.001

Values are expressed as the mean ± S.D, or median with interquartile range, or percentages.
P-value: The p-values were not adjusted for sex for the trend.
*P-value: The *p-values were adjusted for sex for the trend.
TB: total bilirubin; CB: conjugated bilirubin; DD: diabetes duration; HTN: hypertension; IIAs: insulin or insulin analogues; LLDs: lipid-lowering drugs; AHAs: antihypertensive agents; ACAs: anticoagulant agents; SU: sulfonylureas; SBP/DBP: systolic/diastolic blood pressure; BMI: body mass index; WC: waist circumference; WHR: waist-to-hip ratio; FPG: fasting plasma glucose; 2hPPG: 2hpost-prandial plasma glucose; GA: glycated albumin; FCP: fasting C peptide; 2 hC-P: 2hpost-prandial C-peptide; HOMA2-IR: updated homoeostasis model assessment of insulin sensitivity/insulin resistance; TG: total triglycerides; TC: total cholesterol; HDL-C/LDL-C: high-density/low-density lipoprotein cholesterol; ALT: alanine aminotransferase; γ-GT:γ-glutamyl transpeptidase; Cr: creatinine; SUA: Serum uric acid; UAE: urinary albumin excretion; CRP: C-reactive protein.
[a] The Mann-Whitney U–test was applied.

serum TB [27,28].

2.3. Digital nonmydriatic fundus photography and image analysis

Retinal photographs of each subject were taken according to our previously described standard protocol [29]. According to the AAO Preferred Practice Pattern (PPP) published by the American Academy of Ophthalmology (2019) [30], DR was categorized into two major types including non-proliferative diabetic retinopathy (NPDR) and PDR. NPDR is characterized by microaneurysms, retinal hemorrhages, hard exudates, cotton wool spots, dilated and beaded veins, and retinal microvascular abnormalities [31]. The diagnosis of PDR is based on neovascularization or preretinal/vitreous hemorrhage, and tractional retinal detachment observed in advanced cases [31]. In addition, the subjects who had received fundus laser treatment or/and were blind due to fundus lesions were classified as PDR in our study.

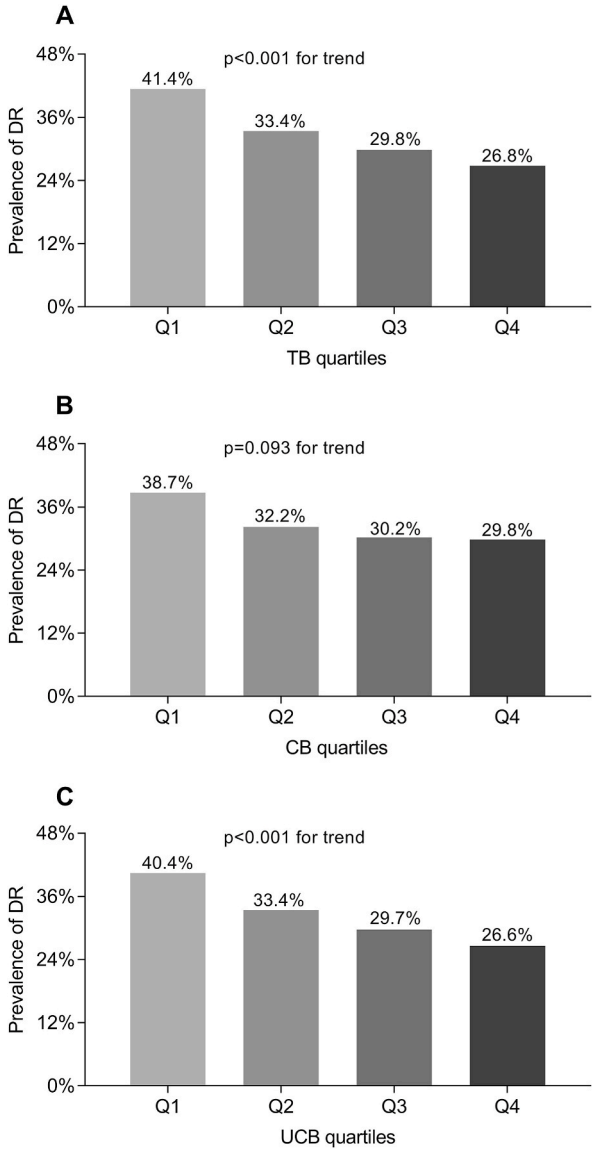

Fig. 1. Comparisons of DR prevalence across serum TB, CB, and UCB quartiles
(A) Comparison of the DR prevalence stratified by serum TB quartiles after adjusting for age, sex and DD ($p < 0.001$ for trend). (B) Comparison of the DR prevalence stratified by serum CB quartiles after adjusting for age, sex and DD ($p = 0.093$ for trend). (C) Comparison of the DR prevalence stratified by serum UCB quartiles after adjusting for age, sex and DD ($p < 0.001$ for trend).

2.4. Statistical analysis

Statistical analyses were performed with SPSS 15.0 software. Normally distributed variables were expressed as mean ± SD, and independent sample t-tests or one-way ANOVA with LSD was utilized to determine the differences among groups. While non-normally distributed data were expressed as median and interquartile range (25 %–75 %), and the Mann-Whitney U and Kruskal-Wallis H tests were applied to compare the difference among different groups. The χ^2 test was performed to compare the difference in the prevalence of categorical variables. Univariate linear regression was applied to compare continuous variables between different groups when controlling for other factors. Additionally, categorical variables were controlled with binary logistic regression when controlling for other confounders. Binary logistic regression was used to evaluate the associations of serum TB, UCB, and CB levels and UCB quartiles with the DR presence. P value < 0.05 was statistically significant.

To assess the relationships of serum TB, UCB, and CB levels and UCB quartiles with DR by logistic regression, five models were constructed: model 1 included adjustments for age, gender, DD; model 2 included further adjustments for hypertension, obesity, smoking, and alcohol consumption; model 3 included further adjustments for the use of LLDs, ACAs, IIAs, metformin, SU and AHA; model 4 had additional adjustments for SBP, DBP, WC, WHR and BMI; and model 5 had additional adjustments for ALT, γ-GT, HDL, LDL, TG, TC, eGFR, Cr, SUA, UAE, FPG, 2h PPG, CRP, HbA1C, GA, HOMA2IR, FCP and 2h CP.

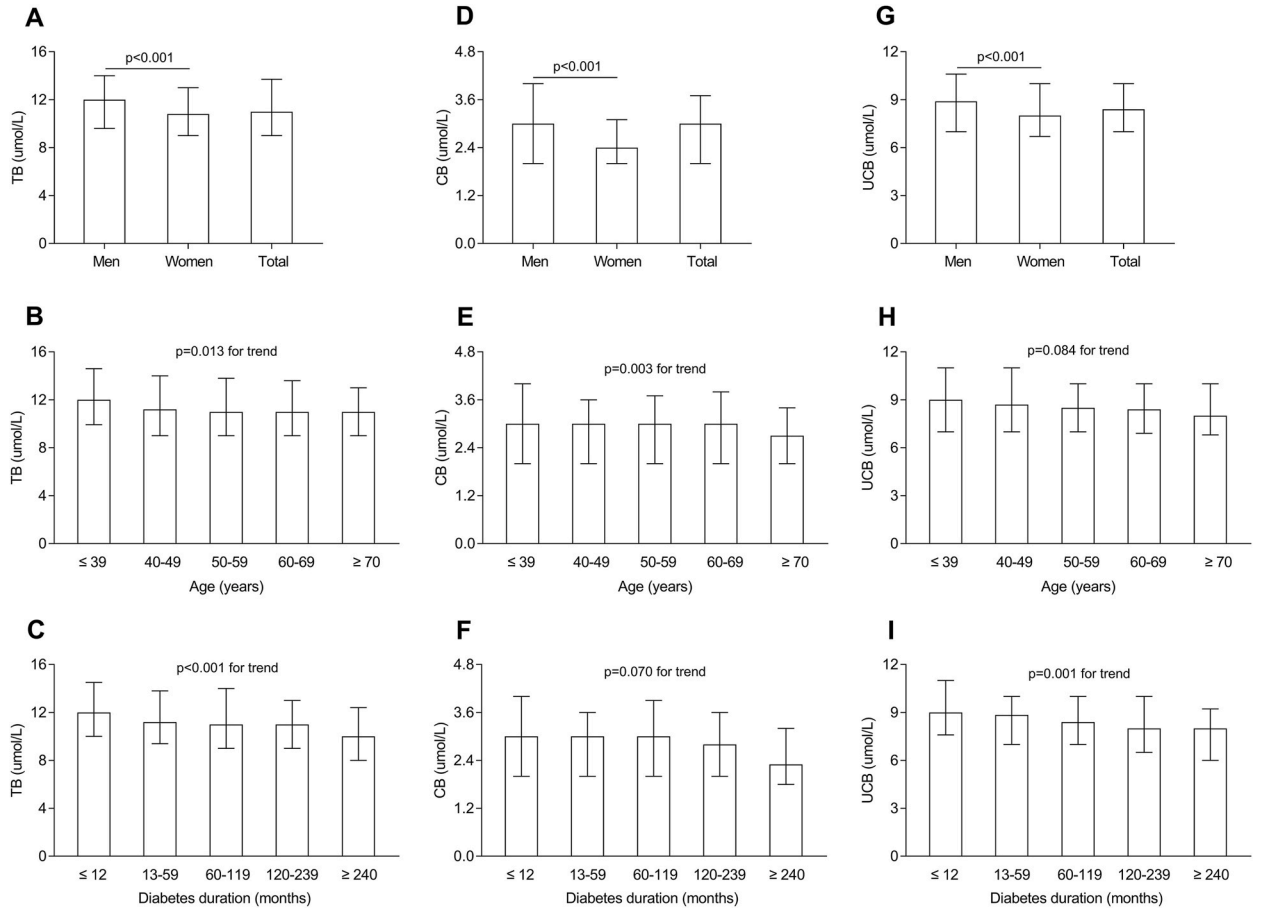

Fig. 2. Comparisons of TB, UCB and CB levels stratified by sex, age, and DD.
(A) Comparison of serum TB levels between men and women T2DM patients after adjusting for age and DD ($p < 0.001$). (B) Comparison of serum TB levels stratified by age after adjusting for sex and DD ($p = 0.013$ for trend). (C) Comparison of serum TB levels stratified by DD after adjusting for age and sex ($p < 0.001$ for trend). (D) Comparison of serum CB levels between men and women T2DM patients after adjusting for age and DD ($p < 0.001$). (E) Comparison of serum CB levels stratified by age after adjusting for sex and DD ($p = 0.003$ for trend). (F) Comparison of serum CB levels stratified by DD after adjusting for age and sex ($p = 0.070$ for trend). (G) Comparison of serum UCB levels between men and women T2DM patients after adjusting for age and DD ($p < 0.001$). (H) Comparison of serum UCB levels stratified by age after adjusting for sex and DD ($p = 0.084$ for trend). (I) Comparison of serum UCB levels stratified by DD after adjusting for age and sex ($p = 0.001$ for trend).

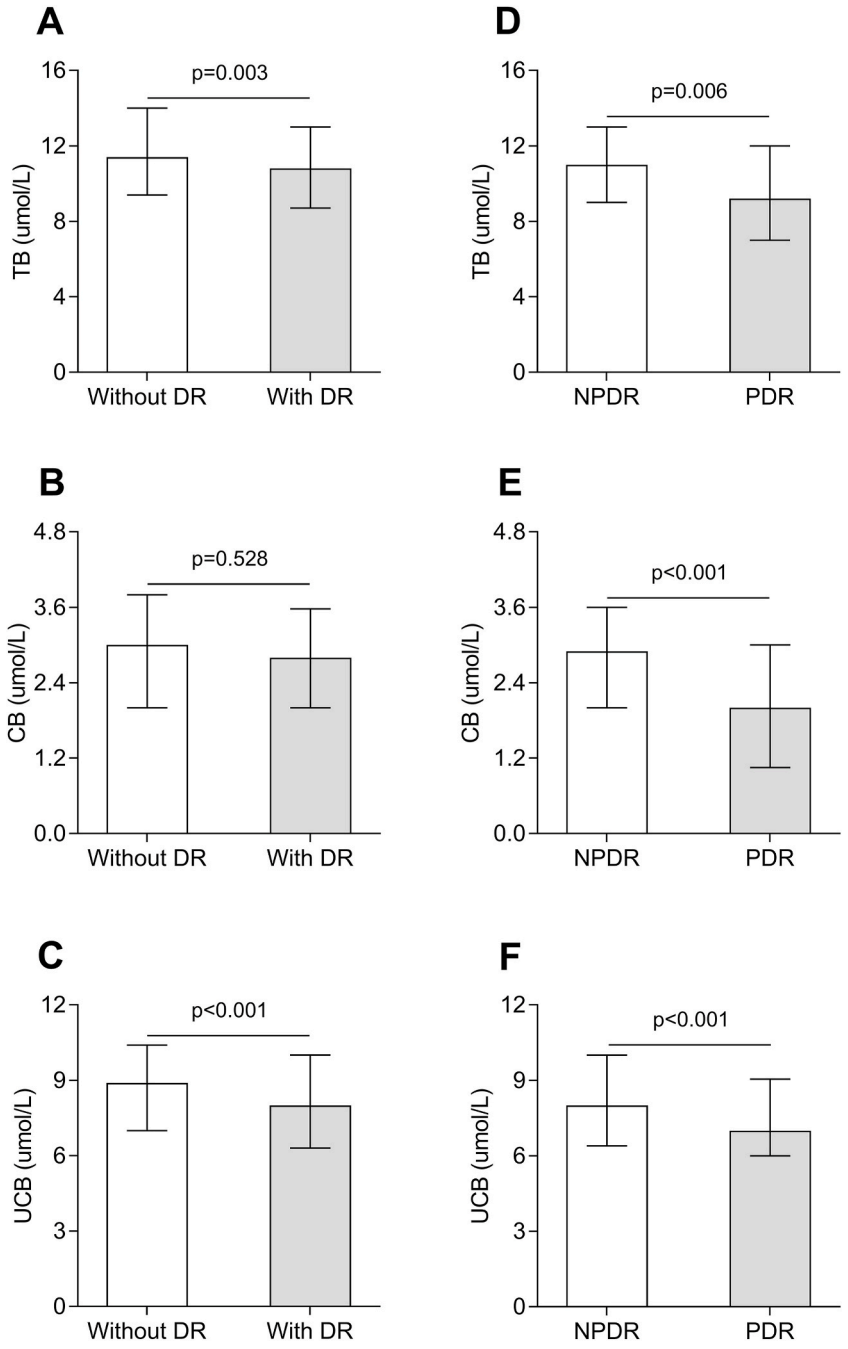

Fig. 3. Comparisons of TB, UCB and CB levels among different groups.
(A) Comparison of serum TB levels between the patients with and without DR after adjusting for gender, age, and DD ($p = 0.003$). (B) Comparison of serum CB levels between the patients with and without DR after adjusting for gender, age, and DD ($p = 0.528$). (C) Comparison of serum UCB levels between the patients with and without DR after adjusting for gender, age, and DD ($p < 0.001$). (D) Comparison of serum TB levels between the patients with NPDR and with PDR after adjusting for gender, age, and DD ($p = 0.006$). (E) Comparison of serum CB levels between the patients with NPDR and with PDR after adjusting for gender, age, and DD ($p < 0.001$). (F) Comparison of serum UCB levels between the patients with NPDR and with PDR after adjusting for gender, age, and DD ($p < 0.001$).

3. Results

3.1. Characteristics of the subjects according to UCB quartiles

The clinical characteristics of the studied subjects are illustrated in Table 1. According to serum UCB levels, T2DM patients were stratified into UCB quartiles with the cutoffs of <7.0, 7.0–8.39, 8.40–9.99, >9.99 μmol/l. Both age and gender had remarkably difference among the UBC quartiles. After controlling for age and gender, higher levels of TB, CB, DBP, FPG, 2h PPG, TC, LDL-C, HDL-C, ALT, γ-GT, lower CRP levels and shorter DD were observed across the UBC quartiles (all $P < 0.05$). The obvious differences in the percentage of smoking subjects, the use of IIAs and LLDs, and the values of WHR, TG, GA, HbA1c, UAE, SUA, Cr, eGFR were also observed among the four groups after controlling for age and gender (all $P < 0.05$). Inversely, the percentage of alcohol consumption patients, prevalence of hypertension and obesity, the use of metformin, AHAs, ACAs and SU, and the values of SBP, BMI, WC, FCP, 2h C–P, HOMA-IR had no significant difference among the four groups after adjustment for age and gender (all $P > 0.05$).

3.2. Comparisons of characteristics between the patients with and without DR

The clinical characteristics between the patients with and without DR are compared in Table S1. The significant differences in both age and gender were observed between the two group patients. After adjustment for age and gender, T2DM patients with DR had significantly higher DD, SBP, DBP, GA, HbA1C, HDL, Cr and UAE. In contrast, the participants with a normal fundus had markedly higher UCB, TB, FCP, 2h C–P, HOMA2-IR, TG, ALT, γ-GT and eGFR; higher prevalence of hypertension; and the higher proportion of the use of metformin, AHAs, IIAs and SU.

3.3. Comparisons of DR prevalence across serum TB, CB, and UCB quartiles

Fig. 1 presents the comparisons of DR prevalence across serum TB, CB, and UCB quartiles. After adjusting for age, gender and DD, a remarkably decreased prevalence of DR was displayed along with evaluated serum TB and UCB quartiles (41.4 %, 33.4 %, 29.8 %, 26.8 % for each TB quartile; 40.4 %, 33.4 %, 29.7 %, 26.6 % for each UCB quantile, respectively, $p < 0.001$ for both trends, Fig. 1A and C). However, although the prevalence of DR decreased across serum CB quartiles, this decrease was not statistically significant (38.7.4 %, 32.2 %, 30.2 %, 29.8 % for each CB quantile, respectively, $p = 0.093$ for trend, Fig. 1B).

3.4. Comparisons of TB, UCB and CB levels stratified by sex, age, and DD

Fig. 2 displays the comparison of TB, UCB and CB levels stratified by sex, age, and DD in T2DM patients. The levels of TB, CB, and UCB displayed significant differences between genders, with males showing higher levels than females ($P < 0.001$ for all) (Fig. 2A, D, and 2G). Serum TB levels significantly decreased with increase of age ($P = 0.013$ for trend) and prolongation of DD ($P < 0.001$ for trend) (Fig. 2B and C). Serum CB levels only relatively decreased with increase of age ($P = 0.003$ for all trend), and UCB levels only remarkedly decreased with prolongation of DD ($P = 0.001$ for all trend) (Fig. 2E and I).

3.5. Comparisons of TB, UCB and CB levels among different groups

Fig. 3 illustrates the comparisons of TB, UCB and CB levels among different groups. The levels of TB ($P = 0.003$) and UCB ($P < 0.001$) were remarkably reduced in the patients with DR compared to those without DR (Fig. 3A and C), while the levels of CB showed no significant difference in the patients with and without DR (Fig. 3B). In addition, the levels of TB, UCB and CB were all observed to be significantly lower in the patients with PDR than in those with NPDR ($P = 0.006$, Fig. 3D; $P < 0.001$, Fig. 3E; $P < 0.001$, Fig. 3F)

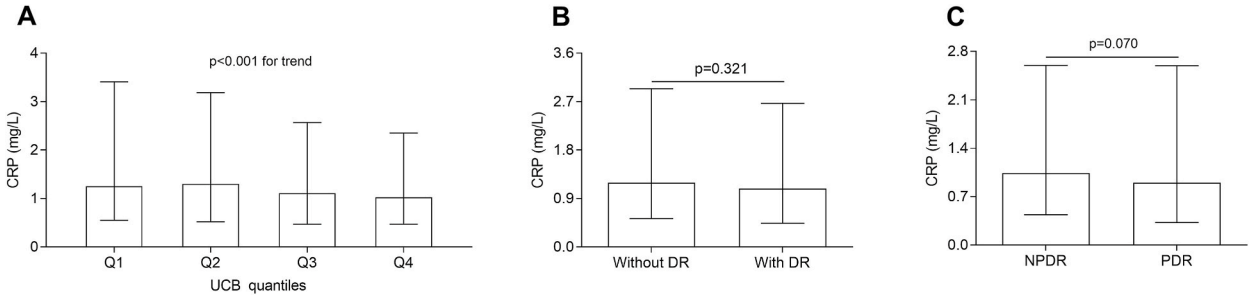

Fig. 4. Comparison of CRP levels in different groups
(A) Comparison of serum CRP levels stratified by UCB quartiles after controlling for gender, age, and DD ($p < 0.001$ for trend). (B) Comparison of serum CRP levels between the patients with and without DR after controlling for gender, age, and DD ($p = 0.0321$). (C) Comparison of serum CRP levels between the patients with NPDR and those with PDR after adjusting for gender, age, and DD ($p = 0.070$).

3.6. Comparison of CRP levels in different groups

Fig. 4 demonstrates the comparison of serum CRP levels in different groups. As shown in Fig. 4A, the levels of CRP significantly decreased across the UCB quartiles ($P < 0.001$). However, there was no significant difference in the CRP levels between the patients with and without DR, and between the patients with NPDR and those with PDR ($P = 0.321$ and $P = 0.070$, respectively, Fig. 4B and C).

3.7. Comparisons of DR prevalence stratified by sex, age and DD

Fig. S1 shows the comparisons of DR prevalence stratified by sex, age and DD. The total prevalence of DR in all subjects was 32.3 %, which had no significant difference between man (34.3 %) and women (30.5 %) ($P = 0.428$) (Fig. S1A). Meanwhile, the prevalence of DR significantly increased with age, although there was a slight decrease in the patients aged 70 years or older ($P < 0.001$ for trend) (Fig. S1B). We also observed a significantly increased prevalence of DR with prolonging DD ($P < 0.001$ for trend) (Fig. S1C).

3.8. Association of serum TB, UCB and CB levels with DR

Table 2 assesses the associations between serum TB, UCB and CB levels and DR presence. The obviously negative associations of serum TB and UCB levels with DR was observed after adjustment for age, gender, DD (model 1) (all $p < 0.001$ for trend). Moreover, decreased serum TB and UCB levels remained independently associated with increased odds of DR in T2DM, respectively, even after further controlling for other confounding variables (model 2–5) (all $p < 0.001$ for the trends in model 2, model 3, model 4, and model 5). Conversely, significant negative correlation between CB levels and DR prevalence were observed only in model 1 and model 2 ($p = 0.010$ in model 1 and $p = 0.022$ in model 2, respectively), while no significant correlations were found after further controlling for confounding factors (models 3–5) ($p = 0.095$, $p = 0.185$ and $p = 0.713$ for trends in model 3, model 4, and model 5, respectively).

3.9. Association of serum UCB quartiles with DR

Table 3 presents the association between serum UCB quartiles and DR. After controlling for age, gender, DD (model 1), serum UCB quartiles were independently and negatively associated with the presence of DR in T2DM patients ($p < 0.001$ for trend). After further adjustment for other confounding factors (model 2–5), serum UCB quartiles still retained a significantly negative association with DR (all $p < 0.001$ for trend). Compared with the subjects in first UCB quartile, the odds of DR decreased by 16.6 %, 21.5 %, and 31.5 % in the subjects in second, third, and fourth quartile, respectively.

4. Discussion

In this real-world study with a large sample of Chinese T2DM subjects, we found that increased serum TB and UCB concentrations within the normal range, rather than CB, were significantly associated with the decreased odds of DR. Furthermore, high-normal serum bilirubin was associated with lower severity of DR, which was indicated by the fact that the levels of three types of serum bilirubin were obviously increased in the patients with NPDR compared to those with PDR.

In recent years, diabetic complications have become a rapidly growing threat to public health worldwide. As a typical complication of diabetic microvascular diseases, DR has become the leading cause of preventable blindness in the working-age population [31], and its prevalence is increasing worldwide. A recent meta-analysis involving 59 populations speculated that the number of adults with DR worldwide was estimated at 103.12 million in 2020 and this number is expected to increase to 160.5 million by 2045 [32]. The DR prevalence in the present study was 32.3 % in 7617 T2DM patients, which was close to the prevalence of 28 % in a meta-analysis of 41 studies including 48,995 T2DM patients in Asia [33].

A significant negative correlation between serum TB and the occurrence of DR was revealed in our study. Consistently, several studies have also found that elevated TB might be a protective factor for DR in T2DM patients [34,35]. However, UCB and CB were not separated from TB in their investigations, and the findings of different studies were also controversial. For example, In a northern Chinese T2DM population, Zhang et al. observed that the prevalence of DR decreased with TB tertile, but the study did not limit the

Table 2
The association of serum UCB levels with DR.

	B statistic	OR	95 % CI	*P* values
Model 1	−0.179	0.836	0.787–0.889	<0.001
Model 2	−0.173	0.841	0.791–0.894	<0.001
Model 3	−0.176	0.838	0.788–0.892	<0.001
Model 4	−0.175	0.839	0.784–0.899	<0.001
Model 5	−0.189	0.828	0.763–0.899	<0.001

Model 1: adjusted for age, gender, DD.
Model 2: further adjusted for hypertension, obesity, alcohol drinking and smoking.
Model 3: further adjusted for the use of LLDs, ACAs, IIAs, Metformin, SU and AHA.
Model 4: additionally adjusted for SBP, DBP, WC, WHR and BMI.
Model 5: additionally adjusted for ALT, γ-GT, HDL, LDL, TG, TC, eGFR, Cr, SUA, UAE, FPG, 2h PPG, CRP, HbA1C, GA, HOMA2-IR, FCP and 2h PCP.

Table 3
The association of UCB quartiles with DR.

	ORs (95 % CI)				*P* values
	Q1	Q2	Q3	Q4	
Model 1	1	0.847(0.736–0.974)	0.733 (0.627–0.855)	0.700 (0.611–0.802)	<0.001
Model 2	1	0.859 (0.746–0.989)	0.738 (0.632–0.863)	0.710 (0.619–0.814)	<0.001
Model 3	1	0.855 (0.742–0.986)	0.743 (0.635–0.869)	0.704 (0.613–0.808)	<0.001
Model 4	1	0.843 (0.721–0.984)	0.742 (0.626–0.880)	0.716 (0.614–0.835)	<0.001
Model 5	1	0.834 (0.697–0.998)	0.785 (0.645–0.956)	0.685 (0.571–0.821)	<0.001

Model 1: adjusted for age, gender, DD.
Model 2: further adjusted for hypertension, obesity, alcohol drinking and smoking.
Model 3: further adjusted for the use of LLDs, ACAs, IIAs, Metformin, SU and AHA.
Model 4: additionally adjusted for SBP, DBP, WC, WHR and BMI.
Model 5: additionally adjusted for ALT, γ-GT, HDL, LDL, TG, TC, eGFR, Cr, SUA, UAE, FPG, 2h PPG, CRP, HbA1C, GA, HOMA2-IR, FCP and 2h PCP.

maximum concentration of TB [35]. Paradoxically, in a follow-up study of 5323 elderly males, a U-shaped relationship was found between serum bilirubin and DR rather than a simple linear relationship, with the lowest prevalence of DR in the third quintile of TB (12.60–13.80 μmol/L) [16]. They emphasized that the protective effect of bilirubin against DR was constrained by the concentration range of serum bilirubin [16]. Likewise, in the Hisayama Study, elevated TB quartiles in the physiological range were found to be accompanied by a decrease in DR prevalence [36]. Inconsistent with these findings, Zhu et al. found that TB was not associated with diabetic microvascular complications including CKD and DR in T2DM [37]. Therefore, the association of serum bilirubin and DR remained inconclusive, especially whether serum UCB and CB differentiated from TB within the normal range is associated with DR in T2DM patients.

Up to now, even though a few studies have distinguished different types of serum bilirubin especially UCB from TB, the association of serum UCB and CB with DR remained controversial. For example, an Indian study displayed that TB, CB and UCB levels were all negatively correlated with the occurrence and severity of DR in T2DM patients, but the range of concentrations of the three types of bilirubin was not restricted in this study [19]. Furthermore, Li et al. found a gender difference in the association between UCB levels and the odds of DR in T2DM patients, with a U-shaped relationship between UCB and DR in females and a negative association in males [38]. On the contrary, a multi-community study demonstrated that CB and TB rather than UCB were closely related to the prevalence of DR in Chinese adults with diabetes, even after controlling for other confounding factors [21]. Likewise, Karuppannasamy et al. observed higher levels of TB and CB in patients without DR compared to those with DR, while UCB was not significantly different between the two groups [20].

In contradiction to the above studies [19–21,38], our study observed an obvious negative correlation between serum TB and UCB, but not CB, and the occurrence of DR in T2DM subjects. In addition, we also found that serum TB and UCB, rather than CB, were significantly lower in the patients with DR compared to those without DR. Thus, we might conclude that serum TB and UCB levels presented a remarkable correlation with the odds of DR. Additionally, it is worth mentioning that the levels of serum bilirubin in the present study was within the normal physiological range despite the mild elevation.

In addition to the intimate association of serum bilirubin with the odds of DR, our study also revealed that serum bilirubin including TB, UCB and CB were all significantly higher in the patients with NPDR than in those with PDR, which indicated that bilirubin may be also significantly associated with the severity of DR. Similar to our findings, Kudo et al. found that serum bilirubin levels decreased as the severity of retinopathy increased [39]. Therefore, it is suggested that serum bilirubin not only was a simple indicator to evaluate the odds of DR, but also might be a reliable indicator of DR progression in clinical practice.

Presently, the pathogenesis of DR has been widely recognized to be related to inflammation and oxidative stress [12], and UCB has been characterized as an important endogenous antioxidant with protective properties against lipid peroxidation, free radicals, and especially against oxidative damage of LDL-C [4]. In a recent study, we demonstrated that UCB protects against chronic kidney disease in Type 2 diabetes, which is also a diabetic microangiopathy and closely associated with oxidative stress and inflammatory damage to the vascular endothelium [26]. Thus, we speculated that the protective roles of UCB in DR maybe mediated by the antioxidant stress effects of UCB, which was indicated by the fact that CRP levels tended to decrease and HDL-C increase across the UCB quartiles. HDL-C exerts an antioxidant stress effect by converting lipid hydroperoxides to lipid hydroxides [40], which thus indirectly confirmed the antioxidant stress effect of UCB in DR. Meanwhile, the anti-inflammatory effect of mildly elevated UCB has been confirmed by many studies including our recent clinical studies [27,28]. For example, in some chronic inflammatory diseases such as non-alcoholic fatty liver disease and atherosclerosis, elevated UCB showed a negative association with the occurrence and progression of these diseases [5,41]. Our recent studies also showed that high-normal UCB was closely associated with the decreased risk of chronic kidney disease, lower limb and carotid atherosclerosis through its anti-inflammation effect in T2DM subjects [27,28]. Therefore, the protective roles of UCB in DR may be partially attributed to its anti-inflammatory effect.

5. Limitation

Some limitations of the present study should be taken into consideration. Firstly, our study is a cross-sectional study and thus the close relationship between serum bilirubin and DR cannot be inferred as a causal relationship In addition, we did not measure the

parameters of oxidative stress and investigate their correlations with the occurrence and development of DR. Nevertheless, the antioxidant stress effect of UCB was assessed by HDL, an indirect indicator for oxidative stress. Finally, our study included only hospitalized T2DM patients, so it is uncertain whether these findings are applicable to other populations.

6. Conclusion

In summary, the present study demonstrated that high-normal serum TB and UCB levels were independently and negatively correlated with the presence of DR in Chinese T2DM patients, while all three types of serum bilirubin were negatively associated with the severity of DR. In clinical practice, serum bilirubin, especially UCB, may be a simple and readily available indicator to assess the risk of the development and progression of DR in type 2 diabetes.

Ethics approval and consent to participate

The involving human participants gave their written informed consents to join into this study, and these participants were reviewed and approved by Ethics Committee of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Consent for publication

Not applicable.

Data availability statement

The data associated with our study has not been deposited into a publicly available repository. In addition, the data that support the findings of this study are available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Man-Rong Xu: Writing – original draft, Investigation, Data curation. **Jun-Wei Wang:** Investigation, Formal analysis. **Yi-Lin Ma:** Investigation, Formal analysis. **Yu-Jie Wang:** Writing – review & editing, Data curation. **Meng-Han Li:** Writing – review & editing. **Jun-Xi Lu:** Writing – review & editing, Conceptualization. **Lian-Xi Li:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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