Serum bilirubin levels and risk of type 2 diabetes: results from two independent cohorts in middle-aged and elderly Chinese

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Serum bilirubin is a potent endogenous antioxidant and has been identified as cardiovascular risk in cohort studies, while the relation to type 2 diabetes (T2D) in the elderly remains unclear. We investigated both cross-sectional and prospective associations between serum bilirubin levels and T2D risk in the Dongfeng-Tongji (DFTJ) cohort, and replicated the prospective findings in a nested case-control study (509 cases and 509 controls) within the Singapore Chinese Health Study (SCHS). In the cross-sectional analysis of DFTJ cohort (15,575 participants with 2,532 diabetes cases), serum bilirubin levels (total, direct and indirect) increased in new on-set diabetes and decreased with the diabetic duration. In the longitudinal analysis of DFTJ cohort (772 incident diabetes cases during 4.5 years of follow-up among 12,530 diabetes-free participants at baseline), positive association was found between direct bilirubin and T2D risk comparing extreme quartiles, similar results were observed in the nested case-control study within SCHS. Total and indirect bilirubin levels were not significantly associated with T2D in either cohort. In conclusion, our findings do not support the protective association between serum bilirubin levels and incident T2D in the middle-aged and elderly adults; instead, direct bilirubin levels were associated with increased risk of T2D.

Diabetes has become a serious public health concern worldwide, affecting more than 415 million people in 2015, and about one fourth of them were Chinese. More than 90% of diabetes cases are type 2 diabetes (T2D)1. Oxidative stress and inflammation have been implicated in the underlying pathogenesis2–4. Bilirubin, a potent antioxidant5,6, is one of the end products of heme catabolism in the systemic circulation, and shown to decrease the risk of cardiovascular disease in prospective studies7–9. Some cross-sectional studies have reported that bilirubin was negatively related to diabetic risk factors such as hypertension and metabolic syndrome10–12. Experimental studies in animal models suggested that bilirubin could protect beta cells from oxidative injury13,14 and enhance insulin sensitivity by decreasing oxidative stress and inflammation15–17.

However, in current cross-sectional studies18–21 and longitudinal studies22–25, the relation between bilirubin and dysglycemia remains controversial. Such discrepancies might be due to age differences. Serum bilirubin levels decreased with age in young adults26, and increased with age in middle-aged and elderly adults18,27. Moreover,

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previous studies mainly focused on serum total bilirubin (TBil)\textsuperscript{18–20,22–25}, which is the sum of direct bilirubin (DBil) and indirect bilirubin (IBil). As traditional index of liver disease, TBil, DBil, and IBil have different clinical implications\textsuperscript{28}. When TBil is in normal range, higher DBil may indicate hepatocellular injury\textsuperscript{29}. Several studies had reported that DBil had more significant relationship to metabolic syndrome\textsuperscript{21,30} and stroke\textsuperscript{31} than TBil and IBil. Therefore, it is important to distinguish the temporal associations between different measures of bilirubin (TBil, DBil or IBil) and diabetic risk.

Therefore, we conducted the current analysis using data from the Dongfeng-Tongji (DFTJ) cohort, a prospective cohort study in a middle-aged and elderly Chinese population. We first examined the cross-sectional relation between serum bilirubin levels (TBil, DBil, and IBil) and prevalent diabetes, and then evaluated the prospective associations of bilirubin levels with incident diabetes during the 4.5 years of follow-up period. To validate our longitudinal results, we further replicated the analysis using data from a nested case-control study within the Singapore Chinese Health Study (SCHS).

**Results**

The median (interquartile range) of serum TBil, DBil, and IBil levels was 13.3 (10.3–16.9), 3.7 (3.0–4.6), 9.5 (7.1–12.4) $\mu$mol/L, respectively. Baseline data according to the quartiles of TBil are presented in Table 1.

In the DFTJ cohort, a total of 772 T2D cases were identified during 4.5 year (56,723 person-years) of follow-up, corresponding to an incidence rate of 13.6 per 1000 person-years. As shown in the Table 2, compared with those in the lowest quartile of DBil levels, the HRs (95% CIs) were 1.22 (0.98–1.52), 1.39 (1.12–1.72), and 1.29 (1.03–1.61) for Q2–Q4 in the full adjusted model ($P_{\text{trend}} = 0.03$). No significant relationship was observed for TBil ($P_{\text{trend}} = 0.27$) or IBil ($P_{\text{trend}} = 0.70$). In sensitive analysis, the associations changed to null when we excluded the participants with impaired fasting glucose (IFG, those with relatively higher bilirubin levels and more likely to develop diabetes) at baseline (Supplementary Table S1).

We further validated the relation of bilirubin levels and risk of T2D in the SCHS (Table 3). In this cohort, the mean duration between blood donation and diagnosis of T2D was 4.0 (SD 1.7) years. DBil levels were positively associated with the risk of T2D, and the OR (95% CI) across tertiles was 1.00 (reference), 1.68 (1.14–2.47), and

### Table 1. Baseline characteristics of the Dongfeng-Tongji cohort according to serum total bilirubin quartiles. Data are means (SD), percentage (%), or median (interquartile range); $P$-value was calculated after adjustment for age, sex except for itself. FPG, fasting plasma glucose; IFG, impaired fasting glucose; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

| Characteristics                          | Q1        | Q2        | Q3        | Q4        | $P$ for trend |
|-----------------------------------------|-----------|-----------|-----------|-----------|--------------|
| Total bilirubin ($\mu$mol/L)            | 2.8–10.3  | 10.3–13.3 | 13.3–16.9 | 16.9–34.2 | —            |
| N                                       | 3102      | 3168      | 3087      | 3173      | —            |
| Total bilirubin ($\mu$mol/L)            | 8.6 (7.9) | 11.6 (11.1) | 14.8 (14.0) | 19.2 (18.3) | —            |
| Direct bilirubin ($\mu$mol/L)           | 2.8 (2.5) | 3.4 (3.1) | 4.1 (3.5) | 5.1 (4.3) | —            |
| Indirect bilirubin ($\mu$mol/L)         | 5.8 (4.7) | 8.2 (7.7) | 10.7 (9.9) | 14.3 (13.2) | —            |
| Age, years                              | 60.9 (7.8) | 61.9 (7.8) | 62.6 (7.6) | 63 (7.4) | 0.019        |
| Sex, men (%)                            | 28.8      | 36.8      | 46.8      | 57.6      | <0.001       |
| High school or above, (%)               | 36.7      | 35.4      | 34.8      | 32.9      | <0.001       |
| Smoker, (%)                             | 21.7      | 25.2      | 29.6      | 35.1      | <0.001       |
| Drinker, (%)                            | 19.4      | 23.6      | 28.3      | 34.4      | 0.004        |
| Physical activity, (%)                  | 87.7      | 89.4      | 90.3      | 88.9      | 0.032        |
| FPG (mmol/L)                            | 5.5 (0.6) | 5.5 (0.6) | 5.5 (0.6) | 5.5 (0.6) | 0.085        |
| IFG (%)                                  | 43.4      | 43.2      | 45        | 47.3      | 0.18         |
| ALP (U/L)                               | 85 (69,102) | 86 (72,102) | 85 (70,101) | 83 (69,98) | 0.001        |
| ALT (U/L)                               | 19 (15,25) | 19 (15,25) | 19 (15,25) | 20 (15,26) | 0.45         |
| AST (U/L)                               | 22 (19,26) | 22 (19,26) | 23 (19,26) | 23 (20,27) | <0.001       |
| Total cholesterol (mmol/L)              | 5.2 (0.9) | 5.2 (0.9) | 5.2 (0.9) | 5.1 (0.9) | 0.89         |
| Triglyceride (mmol/L)                   | 1.2 (0.8) | 1.2 (0.8) | 1.1 (0.8) | 1.1 (0.8) | 0.057        |
| High density lipoprotein (mmol/L)       | 1.4 (0.4) | 1.5 (0.4) | 1.5 (0.4) | 1.5 (0.4) | <0.001       |
| Low density lipoprotein (mmol/L)        | 3 (0.8)   | 3 (0.8)   | 3 (0.9)   | 3 (0.8)   | 0.021        |
| Systolic pressure (mmHg)                | 126.2 (18.3) | 127.3 (18.4) | 127.4 (17.9) | 128.3 (18.3) | 0.19         |
| Diastolic pressure (mmHg)               | 76.8 (10.5) | 77.3 (10.9) | 77.4 (10.8) | 77.8 (10.8) | 0.019        |
| Overweight/obesity, (%)                 | 48.7      | 50.7      | 50.4      | 50.7      | 0.61         |
| Central obesity, (%)                    | 44.4      | 41.4      | 39.3      | 37.6      | 0.67         |
| Family history of diabetes, (%)         | 5.4       | 3.5       | 3.5       | 3.4       | 0.26         |
| Hypertension, (%)                       | 43.0      | 45.2      | 46       | 48.3      | 0.057        |
relationship was observed for TBil ($P_{\text{trend}} = 0.02$) in the multivariate model. Similar to the DFTJ cohort, no significant relationship was observed for TBil ($P_{\text{trend}} = 0.74$) or IBil ($P_{\text{trend}} = 0.86$) with diabetes risk.

We further investigated the interaction between bilirubin levels and other covariates (sex, BMI, physical activity, drinking status, smoking status, and hypertension) on T2D risk in the DFTJ cohort. Although significant interactions were observed between smoking and bilirubin in the DFTJ cohort (Supplementary Figure S1), the interactions were not replicated in the SCHS (Supplementary Table S2).

We additionally examined the associations of bilirubin levels with T2D risk in the cross-sectional design based on the baseline data of Dongfeng-Tongji cohort at 2008 ($n = 15,575$). As Table 4 showed, the serum TBil, DBil, and IBil levels were inversely associated with the risk of T2D ($P_{\text{trend}} < 0.05$) after adjustment for the traditional risk factors (model 1). Further adjustment for liver function and serum lipids diminished the associations to null. In addition, compared with the individuals with normal fasting glucose ($n = 7,207$), the IFG individuals ($n = 5,836$) had higher levels of bilirubin, and the new-onset diabetics ($\leq 1$ year, $n = 1,056, 41.2\%$ of the diabetics) had the highest bilirubin levels (Fig. 1). In the next three diabetic groups ($n = 454, 444$ and $578$, respectively), the bilirubin levels decreased with longer diabetic duration. Similarly, we found that the bilirubin levels were positively related to risk of T2D with 1-year duration but negatively with risk of T2D with more than 1-year duration (Supplementary Table S3).

### Discussion

In the present study, we found that elevated serum DBil concentrations were associated with an increased risk of developing T2D independent of traditional diabetes risk factors in two independent cohort studies of middle-aged and elderly Chinese adults. In contrast, no significant associations were found with total and indirect bilirubin levels. In addition, the serum bilirubin (TBil, DBil and IBil) levels were related to glucose metabolic status, and they increased in those with impaired fasting glucose and new-onset T2D, but decreased with the prolonged duration of diabetes.

Several cross-sectional studies have reported an inverse association between serum TBil and T2D ($P_{\text{trend}} < 0.05$) after adjustment for the traditional risk factors (model 1). Further adjustment for liver function and serum lipids diminished the associations to null. In addition, compared with the individuals with normal fasting glucose ($n = 7,207$), the IFG individuals ($n = 5,836$) had higher levels of bilirubin, and the new-onset diabetics ($\leq 1$ year, $n = 1,056, 41.2\%$ of the diabetics) had the highest bilirubin levels (Fig. 1). In the next three diabetic groups ($n = 454, 444$ and $578$, respectively), the bilirubin levels decreased with longer diabetic duration. Similarly, we found that the bilirubin levels were positively related to risk of T2D with 1-year duration but negatively with risk of T2D with more than 1-year duration (Supplementary Table S3).

### Table 2. Associations of serum bilirubin levels and risk of type 2 diabetes incidence in Dongfeng-Tongji cohort (hazard ratio and 95% confidence interval).

| Quartiles of serum bilirubin (μmol/L) | P for trend |
|---------------------------------------|------------|
| Q1                                    | Q2         | Q3         | Q4         |
| Total bilirubin                        |            |            |            |
| Range                                 | 2.8–       | 10.3–      | 13.3–      | 16.9–      | 34.2          |
| Median                                | 8.6        | 11.6       | 14.8       | 19.2       |
| Cases/Person-years                     | 185/14062.82 | 170/14571.89 | 224/13962.32 | 193/14326.08 |
| Age-, sex- adjusted                   | 1.00 (Ref) | 0.91 (0.74–1.13) | 1.22 (1.00–1.49) | 1.03 (0.84–1.27) | 0.31 |
| Model 1                               | 1.00 (Ref) | 0.90 (0.73–1.11) | 1.21 (1.00–1.48) | 1.02 (0.83–1.26) | 0.34 |
| Model 2                               | 1.00 (Ref) | 0.92 (0.74–1.15) | 1.28 (1.05–1.57) | 1.04 (0.84–1.29) | 0.27 |
| Direct bilirubin                       |            |            |            |
| Range                                 | 0.8–       | 3.0–       | 3.7–       | 4.6–       | 19.4          |
| Median                                | 2.6        | 3.3        | 4.1        | 5.3        |
| Cases/Person-years                     | 160/13090.16 | 190/14075.84 | 218/14923.01 | 204/14634.1 |
| Age-, sex- adjusted                   | 1.00 (Ref) | 1.15 (0.93–1.42) | 1.28 (1.04–1.58) | 1.21 (0.98–1.50) | 0.08 |
| Model 1                               | 1.00 (Ref) | 1.15 (0.93–1.43) | 1.31 (1.06–1.61) | 1.23 (0.99–1.52) | 0.06 |
| Model 2                               | 1.00 (Ref) | 1.22 (0.98–1.52) | 1.39 (1.12–1.72) | 1.29 (1.03–1.61) | 0.03 |
| Indirect bilirubin                     |            |            |            |
| Range                                 | 1.1–       | 7.1–       | 9.5–       | 12.4–      | 27.7          |
| Median                                | 5.7        | 8.1        | 10.7       | 14.3       |
| Cases/Person-years                     | 189/13865.8 | 170/14009.85 | 219/14627.99 | 194/14219.47 |
| Age-, sex- adjusted                   | 1.00 (Ref) | 0.89 (0.73–1.10) | 1.10 (0.90–1.34) | 0.98 (0.80–1.20) | 0.70 |
| Model 1                               | 1.00 (Ref) | 0.88 (0.71–1.09) | 1.11 (0.91–1.35) | 0.96 (0.78–1.18) | 0.83 |
| Model 2                               | 1.00 (Ref) | 0.90 (0.73–1.11) | 1.17 (0.96–1.43) | 0.97 (0.79–1.20) | 0.70 |

In contrast, our study and the other two East Asian prospective studies $^{22,23}$ did not find significant associations between TBil and T2D. Age and race might two main factors contribute to the inconsistent findings. Serum bilirubin levels are increased in older people $^{18,27}$. The average age in our study was relatively older. In addition, serum bilirubin concentrations in East Asia populations $^{22,23}$ are higher than those in whites $^{24}$. In the Dutch population, 95% of the individuals had TBil concentrations lower than 10 $\mu$mol/L $^{24}$, while in the present DFTJ cohort study, 75% of the individuals had TBil concentrations above 10 $\mu$mol/L. Therefore, the results in this European cohort study could not be directly generalized to East Asian population. In the Korean study which reported
result in a decreased expression of HO-139. Meanwhile, reactive oxygen species generated by hyperglycemia in
with overt diabetes, HO system might adapt to the long-term oxidative stress induced by hyperglycemia and
might be a biomarker of oxidative stress and inflammation in pre- and new-onset diabetes. While in individuals
suggested that plasma HO-1 levels are positively associated with T2D risk37,38, lending support to the present
and markers of inflammation and oxidative stress in the present population. However, previous studies have
played an important role in the bilirubin effects on T2D development, but we did not measure the levels of HO,
and IBil at the same time. More importantly, we found serum DBil was positively associated with incident T2D in
multiple covariates including liver function; and evaluation of the bilirubin-diabetes association using TBil, DBil
and indirect bilirubin (IBil) in the prospective cohort design and validation in another independent cohort; adjustment for
Strengths and limitations. The strengths of the present study include both cross-sectional and longitudinal
analyses based on the prospective cohort design and validation in another independent cohort; adjustment for
multiple covariates including liver function; and evaluation of the bilirubin-diabetes association using TBil, DBil
and IBil at the same time. More importantly, we found serum DBil was positively associated with incident T2D in
two independent cohorts, indicating the robustness of the present results.

There are also several limitations of the present study. Firstly, although we speculate that the HO system
played an important role in the bilirubin effects on T2D development, but we did not measure the levels of HO,
and markers of inflammation and oxidative stress in the present population. However, previous studies have
suggested that plasma HO-1 levels are positively associated with T2D risk37,38, lending support to the present
findings. Secondly, the follow-up periods in DFTJ and SCHS cohorts were relatively short, and statistical power
might be limited because of the small number of incident cases. Nonetheless, the effects of three type of bilirubin

| Bilirubin type       | Tertiles of serum bilirubin levels (μmol/L) | P for trend |
|---------------------|---------------------------------------------|------------|
|                     | T1  | T2  | T3   |          |
| Total bilirubin     | 4–  | 9–  | 11–44|          |
|                     | 7   | 9   | 13   |          |
| Age adjusted model  | 1.00 (Ref) | 1.03 (0.75–1.42) | 0.91 (0.67–1.24) | 0.46 |
| Model 1             | 1.00 (Ref) | 1.09 (0.78–1.54) | 0.88 (0.63–1.24) | 0.34 |
| Model 2             | 1.00 (Ref) | 1.02 (0.68–1.54) | 0.94 (0.62–1.43) | 0.74 |
| Direct bilirubin    | 1–  | 2–  | 3–8  |          |
|                     | 1   | 2   | 3    |          |
| Cases/controls      | 177/209 | 214/180 | 118/120 |          |
| Age adjusted model  | 1.00 (Ref) | 1.46 (1.09–1.97) | 1.22 (0.85–1.74) | 0.16 |
| Model 1             | 1.00 (Ref) | 1.41 (1.03–1.93) | 1.27 (0.87–1.86) | 0.14 |
| Model 2             | 1.00 (Ref) | 1.68 (1.14–2.47) | 1.63 (1.03–2.58) | 0.02 |
| Indirect bilirubin  | 3–  | 8–  | 9–36 |          |
|                     | 6   | 8   | 11   |          |
| Cases/controls      | 237/234 | 88/79  | 184/196 |          |
| Age adjusted model  | 1.00 (Ref) | 1.10 (0.77–1.58) | 0.93 (0.70–1.23) | 0.58 |
| Model 1             | 1.00 (Ref) | 1.15 (0.78–1.70) | 0.91 (0.67–1.24) | 0.52 |
| Model 2             | 1.00 (Ref) | 1.09 (0.68–1.72) | 0.97 (0.67–1.40) | 0.86 |

Table 3. Associations of serum bilirubin levels and risk of type 2 diabetes in Singapore Chinese Health Study (Odds ratio and 95% confidence interval). Model 1: adjusted for age, BMI, education level, smoking status, drinking status, physical activity; Model 2: additionally adjusted for alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, triglyceride, high density lipoprotein, history of hypertension and fasting status.
were similar in the two independent populations suggesting low probability of chance findings. Thirdly, we only measured serum bilirubin once at baseline, and the concentrations may not represent long-term exposure status. Finally, the current study was conducted in middle-aged and elderly Chinese population, and further studies with different ethnic and age populations are required to confirm our findings.

**Conclusions**

In summary, we found serum DBil concentrations were positively associated with the risk of incident T2D in middle-aged and elderly adults. Additional large and long-term prospective studies in different ethnic groups are warranted to establish the role of serum bilirubin in the T2D development.

**Methods**

**Study population.** The DFTJ cohort is an ongoing dynamic prospective cohort including 27,009 retirees who come from the Dongfeng Motor Corporation with an average age of 63.6 years at study inception in 2008\(^41\). Each participant completed a semi-structured questionnaire including the socio-demographic, lifestyle, health status, and medical history of diabetes, and history of hypertension; Model 2: additionally adjusted for alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase; Model 3: additionally adjusted for triglyceride and high density lipoprotein.

**Table 4.** Associations of serum bilirubin levels and risk of type 2 diabetes in the cross-sectional analysis of the Dongfeng-Tongji Cohort (\(n = 15,575\)). Model 1: adjusted for age, sex, body mass index, waist circumference, education level, smoking status, drinking status, physical activity, family history of diabetes, and history of hypertension; Model 2: additionally adjusted for alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase; Model 3: additionally adjusted for triglyceride and high density lipoprotein.

| Quartiles of serum bilirubin (μmol/L) | Total bilirubin | Direct bilirubin | Indirect bilirubin |
|---------------------------------------|----------------|------------------|-------------------|
| **Range**                             | 2.8– | 0.8– | 1.1– |
| **Median**                            | 8.5  | 2.6  | 5.7  |
| Cases/n                               | 673/3805 | 593/3571 | 678/3874 |
| Age-, sex-adjusted                    | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 1                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 2                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 3                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Age-, sex-adjusted                    | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 1                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 2                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 3                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Direct bilirubin                      | 0.8– | 1.1– | 1.1– |
| Median                                | 2.6  | 5.7  | 5.7  |
| Cases/n                               | 673/3805 | 678/3874 | 678/3874 |
| Model 1                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 2                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Model 3                               | 1.00 (Ref) | 1.00 (Ref) | 1.00 (Ref) |
| Indirect bilirubin                    | 1.1– | 1.1– | 1.1– |
| Median                                | 5.7  | 8.1  | 10.7 |
| Cases/n                               | 678/3874 | 619/3846 | 586/3881 |

were similar in the two independent populations suggesting low probability of chance findings. Finally, the current study was conducted in middle-aged and elderly Chinese population, and further studies with different ethnic and age populations are required to confirm our findings.

**Conclusions**

In summary, we found serum DBil concentrations were positively associated with the risk of incident T2D in middle-aged and elderly adults. Additional large and long-term prospective studies in different ethnic groups are warranted to establish the role of serum bilirubin in the T2D development.
Ethics statement. The DFTJ cohort study was approved by the Medical Ethics Committee of the School of Public Health, Tongji Medical College, and the Dongfeng General Hospital in the Dongfeng Motor Corporation. The SCHS cohort was approved by the Institutional Review Board of the National University of Singapore. The methods were carried out in accordance with the relevant guidelines. All subjects enrolled gave written informed consent for participation.

Laboratory measurements. In the DFTJ cohort, peripheral venous blood samples were collected after overnight fasting. Plasma glucose levels were measured with Aeroset automatic analyzer (by glucose oxidase method; Abbott Laboratories. Abbott Park, Illinois, USA). The serum bilirubin, lipids, hepatic function, and renal function were measured by the ARCHITECTCi8200 automatic analyzer (ABBOTT Laboratories. Abbott Park, Illinois, USA). In the SCHS, morning random blood samples were collected, and plasma lipids, hepatic enzymes, and bilirubin were measured via colorimetric method on a chemistry analyzer (AU5800 Analyzer, Beckman Coulter, Brea, CA).

T2D definition. In the DFTJ cohort, T2D was defined as having fasting plasma glucose (FPG) ≥ 7.0 mmol/L, according to the WHO criteria[45], or having self-reported doctor-diagnosed diabetes or taking antidiabetic medications. In the SCHS, history of physician-diagnosed diabetes was asked at each follow-up interview by the question: “Have you been told by a doctor that you have diabetes?” If the answer was “yes”, participants were further asked for the age at which they were first diagnosed. In a validation study of the SCHS cohort participants, 97% of the self-reported diabetes cases were confirmed to be valid, suggesting a very high positive predictive value[46].

Statistical analysis. In the DFTJ cohort, baseline characteristics data were compared across quartiles of TBil. Categorical variables were expressed in percentages and continuous variables in mean (SD) or median (interquartile range). Covariate distributions across baseline TBil quartiles were compared using logistic regression models for categorical variables and ANOVA for continuous variables.

Follow-up time was calculated from baseline to the date of diagnosis of T2D, death, or the follow-up interview, whichever came first. Cox proportional hazards models were used to examine hazard ratio (HR) and confidence intervals (CIs) of T2D for each bilirubin quartile compared with the lowest quartile, with adjustment for age
(continuous), sex, BMI (<24 and ≥24 kg/m²), central obesity (binary variable defined as waist circumference ≥85 cm in men or ≥80 cm in women), education (below secondary school, secondary school or higher), smoking status (ever and never smoker), drinking status (ever and never drinker), physical activity (yes, no), family history of diabetes (yes, no), baseline history of hypertension (yes, no). Liver function measures (ALT, AST, and ALP), triglyceride, and high density lipoprotein (HDL) were treated as continuous variables and included in the final model. To test the linear trend across bilirubin quartiles, we assigned the median value to each quartile, and treated it as continuous variable in the model. Stratified analyses were performed according to sex, BMI category, smoking, drinking, and history of hypertension. We also calculated the HRs associated with per 1 SD increase in bilirubin. Likelihood ratio tests were conducted to examine interactions.

In the cross-sectional analysis of Dongfeng-Tongji cohort, logistical regression models were used to examine Odds ratios (OR) and 95% CIs of T2D for three types of bilirubin. To investigate the change of bilirubin levels among different glucose metabolic groups (individuals with normal fasting glucose, impaired fasting glucose, diabetes with different duration), we used generalized linear models to calculate least squares means of bilirubin levels among different groups. According to diabetes duration, T2D cases were classified into four groups: duration ≤1 year; 1 year < duration ≤5 years; 5 years < duration ≤10 years; and duration > 10 years.

In the nested case-control study from SCHS, participants were classified into three categories according to the levels of bilirubin because of relative small sample size and accuracy of the data (values were rounded to integral numbers from the lab). Conditional Logistic regression models were used to estimate ORs and 95% CIs. Covariates in model included age, BMI (<24 and ≥24 kg/m²), education (below secondary school, secondary school or higher), smoking status (ever and never smokers), drinking status (ever and never drinkers), physical activity levels (<0.5, ≥0.5 hours/week), continuous values of ALP, ALT, AST, triglyceride, and high density lipoprotein, baseline history of hypertension, and fasting status. All statistical analyses were performed with SAS version 9.4 (SAS Institute, Cary, North Carolina, USA). Two-sided P < 0.05 was considered as statistically significant.

References
1. International Diabetes Federation. IDF Diabetes Atlas, 7 ed., http://www.diabetesatlas.org/ (2015).
2. Evans, J. L., Goldfine, I. D., Maddux, B. A. & Grodsky, G. M. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? Diabetes 52, 1–8, doi: 10.2337/diabetes.52.1.1 (2003).
3. Houstis, N., Rosen, E. D. & Lander, E. S. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440, 944–948, doi: 10.1038/nature04634 (2006).
4. Lu, J., Rosenblatt-Velin, N., Parapnov, R. & Lauded, L. The role of oxidative stress during inflammatory processes. Biol. Chem. 395, 203–230, doi: 10.1515/bch-2013-0241 (2014).
5. Stocker, R., Glazer, A. N. & Ames, B. N. Antioxidant activity of albumin-bound bilirubin. Proc Natl Acad Sci USA 84, 5918–5922 (1987).
6. Wu, T. W., Fung, K. P., Wu, J., Yang, C. C. & Weisel, R. D. Antioxidation of human low density lipoprotein by unconjugated and conjugated bilirubins. Biochem. Pharmacol. 51, 859–862 (1996).
7. Djourou, L. et al. Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. Am. J. Cardiol. 87, 1196–1200, doi: 10.1001/0002-9149(1991)087<1196:TSBARD>2.3.CO;2 (1991).
8. Breimer, L. H., Wannamethee, G., Ebrahim, S. & Shaper, A. G. Serum bilirubin and risk of ischemic heart disease in middle-aged British men. Clin. Chem. 41, 1304–1308 (1995).
9. Troughton, J. A. et al. Bilirubin and coronary heart disease risk in the Prospective Epidemiological Study of Myocardial Infarction (PRIME). Eur. J Cardiov. Prev. Rehabil. 14, 79–84, doi: 10.1097/01.hjr.0000230097.81202.9f (2007).
10. Wang, L. & Bautista, L. E. Serum bilirubin and the risk of hypertension. Int. J. Epidemiol. 44, 142–152, doi: 10.1093/ije/dyu242 (2015).
11. Lin, L. Y. et al. Serum bilirubin is inversely associated with insulin resistance and metabolic syndrome among children and adolescents. Atherosclerosis 203, 563–568, doi: 10.1016/j.atherosclerosis.2008.07.021 (2009).
12. Wu, Y. et al. Low serum total bilirubin concentrations are associated with increased prevalence of metabolic syndrome in Chinese. J Diabetes 3, 217–224, doi: 10.1111/j.1753-0407.2011.00138.x (2011).
13. Li, M. et al. Interdiction of the diabetic state in NOD mice by sustained induction of heme oxygenase: possible role of carbon monoxide and bilirubin. Antioxid Redox Signal 9, 855–863, doi: 10.1089/ars.2007.1568 (2007).
14. Fu, Y. Y. et al. Hyperbilirubinemia reduces the streptozotocin-induced pancreatic damage through attenuating the oxidative stress in the Gunn rat. Tohoku J. Exp. Med. 222, 265–273 (2010).
15. Ndsang, J. E., Lane, N. & Jadhav, A. The heme oxygenase system abates hyperglycemia in Zucker diabetic fatty rats by potentiating insulin-sensitizing pathways. Endocrinology 150, 2098–2108, doi: 10.1210/en.2008-0239 (2009).
16. Liu, J. et al. Bilirubin Increases Insulin Sensitivity by Regulating Cholesterol Metabolism, Adipokines and PPARGamma Levels. Sci. Rep. 5, 9886, doi: 10.1038/srep09886 (2015).
17. Dong, H. et al. Bilirubin increases insulin sensitivity in leptin-receptor deficient and diet-induced obese mice through suppression of ER stress and chronic inflammation. Endocrinology 155, 818–828, doi: 10.1210/en.2013-1667 (2014).
18. Cherityah, P. et al. High Total Bilirubin as a Protective Factor for Diabetes Mellitus: An Analysis of NHANES Data From 1999–2006. J. Clin. Med. Res. 2, 201–206, doi: 10.4021/jocmr252w (2010).
19. Han, S. S. et al. High serum bilirubin is associated with the reduced risk of diabetes mellitus and diabetic nephropathy. Tohoku J. Exp. Med. 221, 133–140 (2010).
20. Ohnaka, K. et al. Inverse associations of serum bilirubin with high sensitivity C-reactive protein, glycated hemoglobin, and prevalence of type 2 diabetes in middle-aged and elderly Japanese men and women. Diabetes Res. Clin. Pract. 88, 103–110, doi: 10.1016/j.diabres.2009.12.022 (2010).
21. Jo, J., Yun, J. E., Lee, H., Kim, H. & Lee, S. H. Total, direct, and indirect serum bilirubin concentrations and metabolic syndrome among the Korean population. Endocrine 39, 182–189, doi: 10.1515/1-s2.040-001-9417-2 (2011).
22. Oda, E. & Aizawa, Y. Total bilirubin is inversely associated with metabolic syndrome but not a risk factor for metabolic syndrome in Japanese men and women. Acta Diabetol. 50, 417–422, doi: 10.1007/s00592-012-0447-5 (2013).
23. Lee, M. J. et al. Serum bilirubin as a predictor of incident metabolic syndrome: a 4-year retrospective longitudinal study of 6205 initially healthy Korean men. Diabetol. Metab. 40, 305–309, doi: 10.1111/diabetol.2014.006 (2014).
24. Abbasi, A. et al. Bilirubin as a potential causal factor in type 2 diabetes risk: a Mendelian randomization study. Diabetes 64, 1459–1469, doi: 10.2337/db14-0228 (2015).
25. Jung, C. H. et al. Higher serum bilirubin level as a protective factor for the development of diabetes in healthy Korean men: a 4 year retrospective longitudinal study. Metabolism 63, 87–93, doi: 10.1016/j.metabol.2013.09.011 (2014).
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Author Contributions
J.W., M.A.H., T.C.W., H.D.Y., WPK., A.P. and FB.H conceived and designed the study. J.W., Y.R.L., X.H., H.H., and F.W. analyzed the data and wrote the first draft of the paper. X.L.L. and K.Y. collected, cleaned and analyzed the data; J.Y., P.Y., X.M.Z., H.G., Y.J.W., W.H.C., S.W., Y.L. and J.M.Y. supervised the field activities and F.W. analyzed the data and wrote the first draft of the paper. X.L.L. and K.Y. collected, cleaned and analyzed the data; J.Y., P.Y., X.M.Z., H.G., Y.J.W., W.H.C., S.W., Y.L. and J.M.Y. supervised the field activities and F.W. analyzed the data and wrote the first draft of the paper.

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