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Original Article

Association between serum total bilirubin levels, bone mineral density, and prevalence of osteoporosis in Chinese patients with type 2 diabetes

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Abstract: Serum bilirubin, a potent antioxidant substance, has been associated with bone mineral density (BMD) and osteoporosis. However, previous studies have produced conflicting results. Herein, this study was to compare serum total bilirubin (TBIL) within normal physiologic levels in type 2 diabetes mellitus (T2DM) patients with osteoporosis, osteopenia and control subjects, and to assessed their association with BMD and osteoporosis. A total of 923 elderly patients with T2DM (342 patients with osteoporosis, 353 patients with osteopenia and 228 control subjects) undergoing BMD measurement were enrolled. Serum TBIL levels in T2DM patients with osteoporosis and osteopenia were significantly lower than those of normal BMD subjects (11.36±3.77 vs. 11.94±4.38 vs. 13.53±5.69 μmol/L, both $P < 0.01$). Serum TBIL levels were positively correlated with BMD at the lumbar spine and hip ($P < 0.01$ or $P < 0.05$). Univariate regression analysis revealed that the prevalence of osteoporosis was significantly associated with serum TBIL levels [odds ratio (OR) 0.901, 95% confidence interval (CI) 0.865-0.938, $P < 0.01$]. Also, multivariate regression analysis revealed that higher serum TBIL levels related independently and negatively to the prevalence of osteoporosis [adjusted OR 0.926, 95% CI 0.865-0.990, $P < 0.05$]. Moreover, BMD at various sites markedly increased, and the prevalence of osteoporosis markedly decreased along with increasing serum TBIL quartiles ($P < 0.01$ or $P < 0.05$). Higher TBIL levels were associated with higher BMD at the lumbar spine and hip and lower risk of developing osteoporosis in T2DM patients.

Keywords: Total bilirubin, oxidative stress, bone mineral density, osteoporosis, type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder featuring chronic hyperglycemia that can cause enhanced oxidative stress [1]. Osteoporosis is an important and common but frequently overlooked complication in individuals with T2DM. Osteoporosis is a complex, multi-factorial skeletal disease characterized by reduced bone mass and micro-architectural deterioration of bone tissue, consequently resulting in increased bone fragility and risk for fracture. As the population gets older and the incidence of T2DM get higher, morbidity, mortality and financial cost attributed to osteoporosis have increased in the last few years, osteoporosis has become increasingly prevalent and attracted more and more attention in public health. Although the mechanisms involved in the pathogenesis of osteoporosis has not been clearly identified yet, there is now increasing evidence indicating that oxidative stress is associated with reduced bone mineral density (BMD) and the development and progression of osteoporosis [2-6].

Bilirubin, once considered merely a toxic end-product of heme degradation, has now emerged as an important endogenous anti-inflammatory and antioxidant molecule under physiological conditions [7-9]. Accumulating evidence have suggested that bilirubin can efficiently scavenge of peroxyl radicals and reactive nitrogen species (RNS), such as peroxynitrite or its oxidative products [10]. Bilirubin suppresses oxidation more strongly than many other antioxi-

dants, including a-tocopherol, SOD and catalase [11]. As little as 10 nM of bilirubin is enough to protect cells against a 10000-fold higher concentration of hydrogen peroxide through rapid regeneration of bilirubin by biliverdin reductase [12]. Also, bilirubin has a potent inhibitory effect on the activity of NAD(P)H oxidase, whichis probably an important source of ROS production in response to stimuli [9]. Moreover, bilirubin can suppresses the oxidation of lipids and lipoproteins, inhibit the peroxidation of linoleic acid and phospholipids, and the scavenge hypochlorous acid, thus acting against plaque formation and subsequent atherosclerosis [8, 13-15]. Recent studies also have suggested its powerful cytoprotective, anti-mutagenic, platelet inhibitory and immunodulatory properties that protect against inflammation [16-18]. Given the properties of bilirubin as described above, and the role of inflammation and oxidative stress in the pathogenesis of osteoporosis, it seems plausible that serum total bilirubin (TBIL) serves as a protective factor against the development of osteoporosis through its multiple biological activities, especially antioxidative properties. In agreement with the hypothesis, several cross-sectional and longitudinal studies have demonstrateda positive association between serum TBIL levels and BMD values and z-score at the lumbar spine, femur neck and trochanter, and an inverse relationship between serum TBIL levels, the prevalence of osteoporosis, and bone loss rates at multiple proximal femur sites in postmenopausal women, elderly men and women, and healthy middle-agedmen [11, 19-21], suggesting of a potential protective role of serum TBIL against bone loss via potential antioxidant properties. However, several recent epidemiological analyses reached opposite conclusions, showing that higher serum TBIL levels were associated with lower BMD T scores of femur neck, and bone loss rates at the femoralneck in patients with underlying liver disease, such as noncholestatic liver cirrhosis (NCLC), and primary biliary cirrhosis (PBC) [22-24]. Furthermore, some studies showed no association between serum bilirubin levels, reduced BMD, and histologically assessed bone formation rate in patients with chronic liver disease and various liver disorders [25, 26].

Although a number of studies on the association between serum TBIL levels, BMD values and osteoporosis have been done, but the conclusions were inconsistent and controversial. Additionally, it is also uncertain whether serum TBIL within normal physiologic levels is a risk or protective factor for osteoporosis and the association between serum TBIL levels, BMD values and osteoporosis in T2DM patients without potential liver disease has not yet been investigated so far. Therefore, we conducted a large, cross-sectional study of 923 Chinese type 2 diabetic population without potential liver disease to compare the serum TBIL levels within normal physiologic levels in patients with osteoporosis, osteopenia and normal BMD, and to explore their potential association with BMD values at the lumbar spine and hip as well as the prevalence of osteoporosis.

Materials and methods

Subjects and study design

This large cross-sectional study initially consisted of 3514 patients with T2DM, who were consecutively admitted to the inpatient ward of our endocrinology department for screening of osteoporosis between August 2012 and September 2015. The diagnosis of T2DM was based on oral glucose tolerance tests and the 1999 World Health Organization (WHO) criteria. Inclusion criteria for the study were: (1) confirmed or newly diagnosed T2DM patients; (2) postmenopausal women aged 45-87 years who had not menstruated for at least 1 year or men between 50-86 old years; (3) estimated glomerular filtration rate (eGFR) ≥ 30 ml/min/1.73 m^2; (4) long-term residence (≥ 5 y) in China's Sichuan province. Exclusion criteria were: 1) Subjects who had known metabolic bone disease or fractures and taken any drugs that might influence bone metabolism for more than 6 months or within the previous 12 months, such as thiazolidinediones (rosiglitazone and pioglitazone), calcitonin, bisphosphonates, loop diuretics, high-dose thiazide diuretics, systemic glucocorticoids, immunosuppressant, and estrogens; 2) Subjects with having diseases known to effect bone metabolism and/or oxidant-antioxidant status like cancer, thyroid diseases, hyperparathyroidism, hypogonadism, Cushing syndrome, evidence of ongoing infection or inflammation, Gilbert syndrome (GS), liver disease (any liver enzyme or TBIL above reference range), advanced chronic kid-

ney disease (eGFR < 30 mL/min/1.73 m^2), hematologic disease, malignancy, congestive heart failure, asthma/chronic obstructive pulmonary disease, autoimmune diseases, using medications that may affect the oxidant/antioxidant system, including vitamins (A, C, and E) and minerals (zinc and selenium) during the previous 6 months; 3) Subjects with incomplete data and without available informations including menopausal status. Subjects with incomplete data were also excluded from the study. After the exclusions, a total of 923 participants (332 men and 591 women) were included in the final analysis.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the human research ethics committee of the Affiliated Hospital of Southwest Medical University in Sichuan province. The study was retrospectively conducted, and the informed consent requirement for the study was exempt due to restrained database access for analysis purposes only.

Subject's classification

The subjects were categorized into three subgroups according to the WHO's criteria of osteoporosis [27]: normal BMD (T-score ≥ -1.0 SD), osteopenia (-2.5 SD < T-score < -1.0 SD) and osteoporosis (T-score ≤ -2.5 SD). Meanwhile, based on quartiles of serum TBIL levels, the patients with normal BMD and osteoporosis were further divided into four groups including Q1 (TBIL < 9.1 μmol/L) (n = 142), Q2 (9.1 μmol/L ≤ TBIL ≤ 11.5 μmol/L) (n = 145), Q3 (11.5 μmol/L < TBIL ≤ 14.7 μmol/L) (n = 142), and Q4 (14.7 μmol/L < TBIL) quartile groups (n = 141).

Clinical and biochemical measurements

We used a structured interview questionnaire and reviewed medical records of all the participants to collect information regarding the demographic characteristics (gender, age), smoking and alcohol consumption, menopausal status, diabetes duration, use of medications, and history of comorbidities upon admission. Anthropometric measurements were performed in all study participants before breakfast. Body weight, height, body mass index (BMI), and blood pressure were measured with the use of standard methods, as described previously [28].

Blood samples were collected following overnight fasting and were subsequently analyzed at a central, certified laboratory in our hospital. Serum calcium concentration was measured using cresolphthalein complexone methods and corrected for serum albumin level. Serum uric acid (UA) concentration was measured by the direct enzymatic method, in which UA was oxidized by uricase coupled with peroxidase. Serum Cystatin C (CysC) levels were measured using immunity transmission turbidity method. Total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum creatinine (Scr), and albumin were analyzed using a 7060 full-automatic biochemical analyzer (Hitachi, Tokyo, Japan). Serum TBIL and direct bilirubin (DBIL) concentrations were measured by an enzymatic method with bilirubin oxidase. Serum indirect bilirubin (IBIL) = TBIL-DBIL. Fasting blood glucose (FBG) and glycated hemoglobin A1c (HbA1c) were measured by the glucose-oxidase method and high-performance liquid chromatography (HPLC) method, respectively. Fibrinogen was measured by the thrombin time titration method originally described by Clauss et al. Blood total white blood cell (WBC) count, neutrophil and lymphocyte counts were determined using an automated blood cell counter (Mindray BC-6800, Shenzhen, China), according to the manufacturer's instructions. Neutrophil to lymphocyte ratio (NLR) was calculated as the simple ratio between the absolute neutrophil and lymphocyte count, which were both obtained from the same blood sample drawn on admission. Urinary microalbumin and creatinine were measured from at least two fresh morning spot urine sample on two separate occasions within 6 months. Urinary microalbumin was measured with immunoturbidimetric tests. Urinary creatinine was measured enzymatically. The urinary microalbumin to creatinine ratio (ACR; mg/g creatinine) was calculated. Renal function was evaluated by estimated glomerular filtration rate (eGFR) using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations modified by a Japanese coefficient. eGFR (mL/min/1.73 m^2) [29] = 141 × min (Scr/k, 1)$^{\alpha}$ × max (Scr/k, 1)$^{-1.209}$ × 0.993^{age} × 1.018 (if female), where k is 0.7 for females and 0.9 for male, α is -0.329 for females and -0.411 for

males, min indicates the minimum of Scr/k or 1, and max indicates the maximum of Scr/k or 1.

Ankle-brachial index (ABI) measurements were obtained per standard protocol. After the participant rested supine for 5 minutes, SBP was measured in both arms with the appropriate-sized arm cuff. For each leg, SBP in each posterior tibial and dorsalis pedis artery was measured. All pressures were detected with a continuous-wave Doppler ultrasound probe. Leg-specific ABI was calculated by dividing the higher SBP in the posterior tibial or dorsalis pedis by the higher of the right or left brachial SBP.

The Areal BMD at the lumbar spine (L1-L4) and hip (femoral neck, ward's triangle, trochanter, and total hip) in all the participants was measured by dual energy X-ray absorptiometry (DEXA) using a GE Lunar Prodigy and was expressed as the number of grams of bone mineral per square centimeter (g/cm^2). All measurements were taken by the same well-trained and qualified operator using standardized protocols for participant positioning to ensure machine accuracy of greater than 98%. The BMD coefficients of variation were 0.84%, 1.96%, and 1.72% for the lumbar spine, femoral neck, and total hip, respectively.

Statistical methods

All data were first analyzed for normality of distribution using the Kolmogorov-Smirnov test of normality. Data are expressed as mean ± SD for continuous variables or percentages (%) for categorical variables, respectively, unless otherwise specified.

The differences in clinical and biochemical parameters among normal BMD, osteopenia, and osteoporosis groups were compared using chi-square (χ^2) tests for categorical variables, one-way analysis of variance (ANOVA) followed by Post hoc LSD for normally distributed continuous variables, and Kruskal-Wallis test followed by multiple pairwise comparisons with Bonferroni post hoc adjustment for non-parametric distributed covariates. Thereafter, spearman rank correlation (for categorical variables and not normally distributed continuous variables) and Pearson correlation (for normally distributed continuous variables) coefficients were employed to assess the relationship between serum TBIL concentration, clinical and biochemical parameters.

To further test the hypothesis that lower serum TBIL levels may be associated with the prevalence of osteoporosis, binary logistic regression analysis were performed. In univariate regression analyses, baseline characteristics that were associated with the prevalence of osteoporosis at $P < 0.1$ were identified as potential confounders. Consequently, these confounders were entered simultaneously into the multivariate regression models to identify significant independent related factors for osteoporosis. Odds ratios (OR) and 95% confidence intervals (CI) were estimated.

Lastly, to better understand the clinical implications of these results, we divided the patients with normal BMD and osteoporosis into four quartile groups according to serum TBIL levels. BMD values at various sites and osteoporosis incidence of the participants in each of the four serum TBIL quartile categories were subsequently compared using analysis of Kruskal-Wallis tests for continuous variables and χ^2 test for categorical variables. In case of a statistically significant difference in the Kruskal-Wallis test, Bonferroni post hoc adjustment was used for multiple pairwise comparisons.

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS) statistical software (version 20.0, Chicago, IL, USA). All reported *P* values were two-sided. A *P* value of < 0.05 was considered statistically significant.

Results

Baseline characteristic of the study population

A total of 923 T2DM patients (mean age, 62.24±8.95 years; male/female, 332/591; and mean diabetes duration, 7.92±6.47 years) were finally enrolled in this study. The baseline characteristics of all the participants in the present study are shown in **Table 1**. Compared with T2DM patients with normal BMD and osteopenia, osteoporosis patients were significantly older, were more likely to be female, and had lower BMI ($P < 0.01$ or $P < 0.05$). Compared with patients with normal BMD and osteopenia, those with osteoporosis had higher PP, HDL-C, NLR, and urinary ACR ($P < 0.01$ or $P < 0.05$). Patients with and osteopenia and osteoporosis

Table 1. Baseline characteristic of T2DM patients with normal BMD, osteopenia and osteoporosis ($\overline{x}$ ±s)

Item	Normal BMD (n = 228)	Osteopenia (n = 353)	Osteoporosis (n = 342)	*P* for trend
Male/Female	130/98	152/201**	50/292**,##	0.000
Diabetes duration (years)	8.35±5.49	9.08±6.25	9.34±6.49	0.053
Age (years)	58.94±9.40	61.75±8.26**	66.03±8.10**,##	0.000
BMI (kg/m^2)	24.87±3.30	24.16±3.77**	23.37±20.46**,#	0.000
SBP (mmHg)	131.27±20.32	132.65±20.55	134.73±22.15	0.198
DBP (mmHg)	71.67±11.82	71.65±12.10	70.06±11.99	0.149
PP (mmHg)	59.60±18.24	61.00±17.89	64.78±19.19	0.004
TC (mmol/L)	4.86±1.15	4.86±1.09	4.85±1.20	0.987
TG (mmol/L)	2.08±1.55	2.09±1.37	1.94±1.42#	0.047
HDL-C (mmol/L)	1.18±0.33	1.23±0.37	1.31±0.40**,#	0.001
LDL-C (mmol/L)	2.86±0.93	2.80±0.88	2.80±1.00	0.704
FBG (mmol/L)	10.69±4.62	10.96±5.62	9.93±4.93*,#	0.008
HbA1c (%)	9.37±2.47	9.52±2.57	8.98±2.59*,##	0.003
ALT (U/L)	21.44±8.97	18.68±8.47**	17.35±7.99**	0.000
AST (U/L)	19.46±5.50	18.87±6.15	18.98±6.19	0.133
DBIL (μmol/L)	4.62±1.90	4.13±1.55**	4.01±1.46**	0.001
IBIL (μmol/L)	8.90±4.23	7.81±3.23**	7.34±2.86*	0.000
ALP (U/L)	81.92±29.30	82.72±30.75	88.93±50.61	0.371
Corrected calcium (mg/dL)	9.15±0.48	9.26±0.58*	9.02±0.59*,##	0.000
Albumin (g/L)	42.49±4.14	41.17±4.28**	40.68±4.76**	0.000
Scr (μmol/L)	63.79±18.95	66.28±23.93	64.00±24.50	0.101
UA (μmol/L)	306.94±88.56	302.66±93.54	290.05±100.6*	0.077
CysC (mg/L)	0.87±0.27	1.05±0.83**	0.96±0.33*	0.011
eGFR (mL/min/1.73 m^2)	98.81±18.22	92.53±20.06**	87.73±20.46**,##	0.000
WBC count (*10^9/L)	6.47±2.01	6.80±2.19	6.61±2.44	0.081
Neutrophil count (*10^9/L)	4.28±1.76	4.47±2.02	4.53±2.29	0.700
Lymphocyte count (*10^9/L)	1.66±0.66	1.76±0.64	1.56±0.55**	0.000
NLR	3.03±2.28	2.93±2.03	3.44±2.77#	0.016
Fibrinogen (g/L)	3.41±1.12	3.49±1.12	3.52±1.11	0.649
Urinary ACR (mg/g)	101.88±26.96	140.04±29.40	276.67±54.87*	0.032
ABI	1.11±0.10	1.04±0.11	1.00±0.16**,#	0.000
BMD values (g/cm^2)				
L1	1.031±0.14	0.899±0.094**	0.748±0.113**,##	0.000
L2	1.126±0.158	0.967±0.104**	0.791±0.114**,##	0.000
L3	1.202±0.540	1.020±0.106**	0.839±0.118**,##	0.000
L4	1.160±0.160	1.027±0.130**	0.844±0.131**,##	0.000
L1-L4	1.130±0.184	0.978±0.094**	0.806±0.104**,##	0.000
Femoral neck	0.940±0.115	0.806±0.086**	0.690±0.096**,##	0.000
Ward's triangle	0.755±0.134	0.618±0.098**	0.484±0.098**,##	0.000
Trochanter	0.828±0.114	0.716±0.093**	0.587±0.090**,##	0.000
Total hip	0.841±0.113	0.713±0.083**	0.587±0.087**,##	0.000

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBG, fasting blood glucose; HbA1c, glycated hemoglobin A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBIL, direct bilirubin; IBIL, indirect bilirubin; ALP, alkaline phosphatase; Scr, serum creatinine; UA, uric acid; CysC, cystatin C; eGFR, estimated glomerular filtration rate; WBC, white blood cell; NLR, neutrophil to lymphocyte ratio; ACR, microalbumin to creatinine ratio; ABI, ankle-brachial index. Corrected calcium concentration (mg/dL) = serum calcium concentration (mg/dL) +0.8 × [4.0 (g/dL)-serum albumin concentration (g/dl)]. Values were given as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$ compared with normal BMD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with osteopenia group.

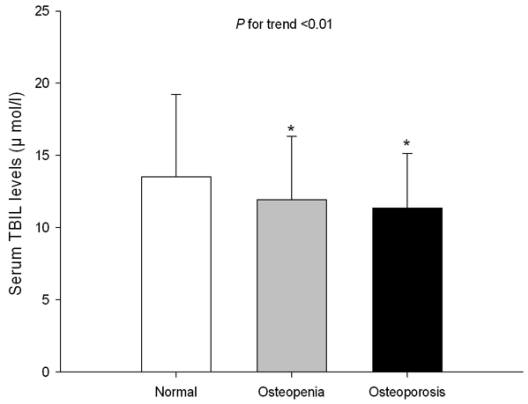

Figure 1. Comparison of serum TBIL levels in T2DM patients with normal BMD and osteopenia, and osteoporosis. vs. Normal BMD group, **P*<0.01.

displayed significantly elevated serum CysC levels compared to normal BMD patients ($P < 0.05$). However, BMD values at all skeletal sites, serum albumin, TG, FBG, HbA1c, ALT, DBIL, IBIL, corrected calcium concentration, eGFR, ABI, and lymphocyte count were significantly decreased ($P < 0.01$ or $P < 0.05$) in patients with osteoporosis, compared with those in normal BMD and osteopenia patients. There was a trend for serum UA concentration to decrease, whereas blood total WBC count and diabetes duration showed an increased tendency in patients with osteopenia and osteoporosis. The three groups did not differ significantly in terms of SBP, DBP, TC, LDL-C, AST, ALP, Scr, fibrinogen, and neutrophil count (all $P>0.05$).

Serum TBIL levels among T2DM patients with normal BMD, osteopenia, and osteoporosis

Data on serum TBIL levels in T2DM patients with normal BMD, osteopenia and osteoporosis groups are depicted in **Figure 1**. We observed that there was a progressive decline in serum TBIL levels from normal BMD subjects (13.53±5.69 μmol/L) to osteopenia (11.94±4.38 μmol/L) and osteoporosis (11.36±3.77 μmol/L) patients ($P < 0.01$).

Serum TBIL levels were significantly lower in osteoporosis subjects and osteopenia than in normal BMD subjects (both $P < 0.01$).

Association between serum TBIL levels, clinical characteristics and BMD values in all T2DM patients combined

Correlation analysis results between serum TBIL levels, clinical characteristics and BMD values are summarized in **Table 2**. Serum TBIL concentrations were positively correlated with BMD values at the lumbar spine, and hip, serum albumin, ALT, AST, DBIL, IBIL, TC, HDL-C, and eGFR ($P < 0.01$ or $P < 0.05$). A negative correlation was also observed between serum TBIL concentrations, age, diabetes duration, TG, CysC, blood total WBC count, lymphocyte count, fibrinogen, and urinary ACR ($P < 0.01$ or $P < 0.05$). Additionally, serum TBIL concentrations tended to be associated inversely with PP, and correlated positively with ALP, ABI, LDL-C and FBG. However, no significant relationship was observed between serum TBIL concentrations and other clinical and biochemical parameters (all $P>0.05$).

Binary logistic regression analyses of variables contributing to osteoporosis in T2DM patients with normal BMD and osteoporosis combined

The results of univariate and multivariate regression analysis of the possible correlates for osteoporosis are summarized in **Table 3**. Univariate regression analysis showed that the prevalence of osteoporosis was significantly associated with serum TBIL, gender, age, diabetes duration, PP, corrected calcium concentration, BMI, UA, CysC, urinary ACR, eGFR, HDL-C, ALT, serum albumin, lymphocyte count, and ABI ($P < 0.01$ or $P < 0.05$). Multivariate regression analysis were further used to examine the independent association of serum TBIL levels with the prevalence of osteoporosis after considering above all potential confounders. Higher serum TBIL levels still related independently and negatively to the prevalence of osteoporosis even after adjustment for above all potential confounders ($P < 0.05$). In the multivariable adjust model, increasing age and decreased eGFR were associated independently with the prevalence of osteoporosis, while BMI and ALT showed protective effects against the prevalence of osteoporosis ($P < 0.01$ or $P < 0.05$). Beta is the standardized coefficient and measures the influence of each variables on osteoporosis. OR is the odds ratio and refers to the risk of osteoporosis.

BMD values and the prevalence of osteoporosis across quartiles of serum TBIL levels in T2DM patients with normal BMD and osteoporosis combined

The BMD values at all sites of the lumbar spine and hip increased progressively along with

Table 2. Association between serum TBIL levels, clinical and biochemical parameters in all T2DM patients combined

Item	*r*	*P*		*r*	*P*
Diabetes duration	-0.123	0.000	Scr	-0.038	0.250
Age	-0.098	0.003	UA	-0.017	0.613
Gender	-0.177	0.000	CysC	-0.104	0.002
BMI	0.040	0.237	eGFR	0.133	0.000
SBP	-0.034	0.305	WBC count	-0.075	0.023
DBP	0.049	0.133	Neutrophil count	-0.034	0.314
PP	-0.054	0.099	Lymphocyte count	-0.072	0.032
TC	0.074	0.026	NLR	0.049	0.144
TG	-0.085	0.011	*Fibrinogen*	-0.096	0.040
HDL-C	0.122	0.000	Urinary ACR	-0.207	0.000
LDL-C	0.059	0.078	ABI	0.059	0.092
FBG	0.061	0.066	L1 BMD	0.098	0.003
HbA1c	-0.002	0.945	L2 BMD	0.120	0.000
ALT	0.158	0.000	L3 BMD	0.093	0.005
AST	0.100	0.000	L4 BMD	0.084	0.011
DBIL	0.789	0.000	L1-L4 BMD	0.104	0.002
IBIL	0.954	0.000	Femoral neck	0.142	0.000
ALP	0.058	0.076	Ward's triangle	0.152	0.000
Corrected calcium	-0.038	0.264	Trochanter	0.157	0.000
Albumin	0.247	0.000	Total hip	0.156	0.000

increasing serum TBIL quartiles ($P < 0.01$ or $P < 0.05$) (see **Table 4**). T2DM patients in the lowest TBIL quartile had significantly lower BMD values at all sites compared with those in the highest quartile ($P < 0.01$ or $P < 0.05$). Additionally, we found that the prevalence of osteoporosis decreased across decreases from Q1 to Q4 ($P < 0.01$), and the prevalence of osteoporosis in Q1 was 71.13% but this decreased to 45.39% in Q4 (see **Figure 2**).

Discussion

To the best of our knowledge, this is the first clinical study comparing serum TBIL levels in T2DM patients with osteoporosis, osteopenia, and normal BMD, including both men between 50-86 old years and postmenopausal women aged 45-87 years. We have found that serum TBIL levels in T2DM patients with osteoporosis and osteopenia were significantly lower than those in normal BMD subjects. Serum TBIL levels correlated positively with BMD values at the lumbar spine and hip. Additionally, univariate and multivariate regression analysis revealed that higher serum TBIL levels related independently and negatively to the prevalence of osteoporosis even after adjustment for all potential confounders. Finally, we have also demonstrated that the BMD values at all sites markedly increased, and the prevalence of osteoporosis significantly decreased along with increasing serum TBIL quartiles. Collectively, these data have demonstrated that serum TBIL levels within normal physiologic levels may play a crucial protective role in the development of osteoporosis, especially in T2DM patients without underlying liver disease.

Bilirubin, an end metabolic product of heme metabolism, has been known to play an important physiologic role as a strong antioxidant and cytoprotectant through efficient scavenging of peroxyl radicals and suppression of oxidation [12]. An increasing body of evidence has indicated that bilirubin is positively related to antioxidant enzyme activities such as SOD, catalase and glutathione peroxidase levels, and correlated inversely with markers of oxidative stress in jaundiced newborns [30]. Clinical evidence has shown that patients with GS, a congenital form of hyper-bilirubinemia, have an increased antioxidant capacity and improved resistance to lipid oxidation [31]. Our current results also showed a significantly positive association of serum TBIL levels with serum albumin, which was also decreased in T2DM patients with osteoporosis. Albumin may act as an indirect and sacrificial antioxidant and inhibits peroxidase free radical generation [32]. These results presented here further have confirmed that lower serum TBIL levels were associated with increased oxidativestress, while an increase in oxidative stress, lead to endothelial dysfunction, decreased bone blood flow and eventually to the development and progression of osteoporosis [20]. Therefore, it is reasonable to assume that moderate increase in serum TBIL concentrations within normal physiologic levels may improve BMD values and reduce the prevalence of osteoporosis through the ablation of oxidative stress in T2DM patients. In accordance with the hypothesis, a few studies [11, 19, 20] have

Table 3. Binary logistic regression analyses of variables contributing to osteoporosis in T2DM patients with normal BMD and osteoporosis combined

Variables	Univariate analysis			Multivariate analysis		
	B	OR (95% CI)	*P*-value	B	OR (95% CI)	*P*-value
Diabetes duration	0.034	1.035 (1.007-1.064)	0.013			
Age	0.093	1.097 (1.074-1.121)	0.000	0.061	1.063 (1.020-1.108)	0.004
gender	0.013	1.040 (1.014-1.066)	0.002			
BMI	-0.115	0.891 (0.848-0.936)	0.000	-0.090	0.914 (0.842-0.991)	0.030
SBP	0.008	1.008 (1.000-1.016)	0.060			
DBP	-0.011	0.989 (0.975-1.003)	0.116			
PP	0.015	1.015 (1.006-1.024)	0.002			
TC	-0.011	0.989 (0.856-1.142)	0.879			
TG	-0.064	0.938 (0.837-1.051)	0.268			
HDL-C	0.950	2.586 (1.581-4.228)	0.000			
LDL-C	-0.063	0.939 (0.790-1.117)	0.476			
FBG	-0.032	0.968 (0.935-1.002)	0.068			
HbA1c	-0.060	0.942 (0.882-1.006)	0.074			
ALT	-0.056	0.945 (0.926-0.965)	0.000	-0.044	0.957 (0.923-0.991)	0.014
TBIL	-0.105	0.901 (0.865-0.938)	0.000	-0.077	0.926 (0.865-0.990)	0.025
AST	-0.013	0.987 (0.959-1.015)	0.349			
ALP	0.005	1.005 (1.000-1.010)	0.064			
Corrected calcium	-0.440	0.644 (0.465-0.892)	0.008			
Albumin	-0.091	0.913 (0.877-0.951)	0.000			
Scr	0.000	1.000 (0.993-1.008)	0.915			
UA	-0.002	0.998 (0.996-1.000)	0.041			
CysC	0.993	2.698 (1.463-4.977)	0.001			
eGFR	-0.032	0.968 (0.958-0.978)	0.000	-0.023	0.977 (0.955-1.000)	0.046
WBC count	0.027	1.027 (0.952-1.107)	0.488			
Neutrophil count	0.060	1.061 (0.973-1.158)	0.178			
Lymphocyte count	-0.289	0.749 (0.563-0.998)	0.048			
NLR	0.067	1.070 (0.993-1.152)	0.076			
Fibrinogen	0.092	1.096 (0.877-1.370)	0.420			
Urinary ACR	0.001	1.001 (1.000-1.001)	0.025			
ABI	-3.137	0.043 (0.009-0.208)	0.000			

demonstrated that osteoporosis patients had significantly lower serum TBIL level compared with normalsubjects, osteopenia patients or non-osteoporosis patients in elderly men and menopausal women. Additionally, they found that serum TBIL levels were positively associated with BMD values and z-score at the lumbar spine and femur neck, and higher TBIL level was independently associated with a descending prevalence of osteoporosis before and after adjustment for several variables. Our study results are in consistent with these findings, suggesting of a potential protective role of serum TBIL against bone loss via potential antioxidant properties.

Recently, Beom et al [21] performed a 3-year longitudinalstudy of 917 healthy Korean men aged ≥ 40 years and observed that baseline BMD values at the femoral neck and trochanter tended to increase as serum TBIL quartile category rose. Moreover, the rates of bone loss at multiple proximal femur sites were significantly attenuated in a dose-response manner across increasing TBIL concentrations before and after adjustment for potential confounders. Those in the highest TBIL quartile category showed significantly less bone loss at all proximal femur sites compared to subjects in the lowest TBIL quartile category. Similarly, our study also has shown that BMD values at all

Table 4. Comparison of BMD values at the lumbar spine and hip across quartiles of serum TBIL in T2DM patients with normal BMD and osteoporosis combined

BMD values	TBIL quintiles				*P* for trend
	Q1	Q2	Q3	Q4	
L1	0.823±0.175**	0.874±0.197	0.855±0.189	0.893±0.179	0.007
L2	0.884±0.200**	0.934±0.221	0.915±0.213	0.970±0.204	0.004
L3	0.991±0.703*	0.986±0.219	0.956±0.208	1.005±0.206	0.033
L4	0.927±0.195*	0.982±0.214	0.962±0.207	1.009±0.220	0.014
L1-L4	0.906±0.249**	0.944±0.205	0.922±0.195	0.969±0.195	0.011
Femoral neck	0.751±0.161**	0.793±0.157	0.774±0.152**	0.842±0.160	0.000
Ward's triangle	0.555±0.181**	0.591±0.169	0.584±0.176*	0.640±0.165	0.000
Trochanter	0.646±0.151**	0.681±0.150	0.681±0.150	0.727±0.159	0.000
Total hip	0.651±0.159**	0.688±0.154	0.680±0.154*	0.736±0.156	0.000

vs. Q4, *$P < 0.05$, **$P < 0.01$.

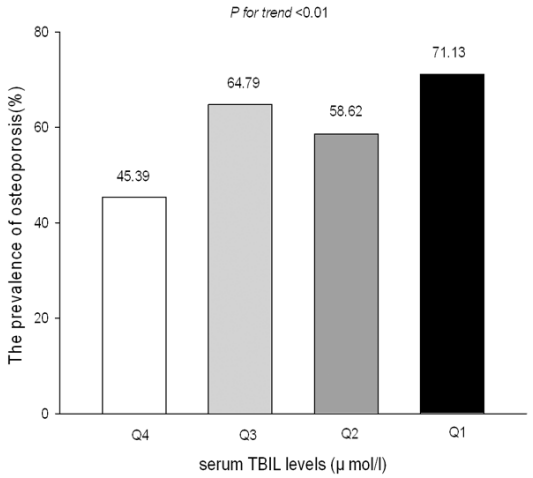

Figure 2. The prevalence of osteoporosis across quartiles of serum TBIL.

sites markedly increased, and the prevalence of osteoporosis significantly decreased along with increasing serum TBIL quartiles. Taken together, these previous and our present findings consistently demonstrated a positive relationship between serum TBIL levels and BMD, and an inverse relationship between serum TBIL levels and prevalence of osteoporosis, both cross-sectionally and longitudinally, is mediated to some great extent by its antioxidant activity. However, there have been several experiment and epidemiological studies, showing significant inverse or no association between TBIL and BMD values in patients with underlying liver disease [22-26]. In a cross-sectional study, Mehmet et al [22] reported a negative correlation between bilirubin and BMD T scores of femur neck in the NCLC patients. Moreover, Menon and his colleagues [23] performed a longitudinal study of 176 patients with PBC over 7 years of follow-up, and observed that a greater baseline serum bilirubin level was the only variable independently associated with bone loss rate over time, which is consistent with in vitro studies showing that bilirubin my adversely affect bone formation [33-35]. Similar resultshas been reported by Ormarsdóttir et al [24]. However, Smith et al [25] reported that serum bilirubin levels did not correlate with reduced BMD in a cohort of 86 consecutive patients with chronic liver disease referred forliver transplant evaluation, and chronic unconjugated hyperbilirubinemia does not lead to alterations in bone mineralization in Gunn rats. Subsequently, Diamond et al [26] demonstrated that serum bilirubin did not correlated weakly with histologically assessed bone formation rate in 80 patients with various liver disorders. These findings suggested that bilirubin is not a major contributing factor to hepatic osteodystrophy. Serum TBIL concentration is one of the most robust indicators of hepatic dysfunction, it is difficult to delineate whether TBIL is the direct causeof bone mineral loss or whether it merely reflects the severity of the underlying liver disease which per se causes osteoporosis. One of the proposed hypotheses explaining this paradox is related to a shift in the prooxidant/antioxidant properties of TBIL depending on its concentration as serum UA. Serum TBIL may become prooxidant under certain conditions, particularly when it is supersaturated inblood. Several previous studies have shown that TBIL levels at 13.6-25 μmol/L have been demonstrated to be protec-

tive against various pathologies, but bilirubin levels over 25 μmol/L are detrimental [20]. Ruiz-Gaspa and colleagues [34] also demonstrated that a high concentration of bilirubin (5.8 mg/dL) far exceeding the reference range in human, decreased the viability, differentiation, and mineralization of primary human osteoblasts, supporting the notion that the elevated bilirubin levels in advanced chronic liver diseases have deleterious consequences including disturbed bone formation related to osteoblast dysfunction and, thus, mayplay an important role in the pathogenesis of the osteoporosis associated with chronic jaundice. On the contrary, a low TBIL concentration (0.6 mg/dL) within normal physiologic levels in patients without liver diseasesnot only significantly increased osteoblast viability but also tended to increase osteoblast mineralization. This in vitro experiment implies that there may be a threshold regarding the role of TBIL in bone cells and is consistent with the results of our cross-sectional clinical study performed in T2DM patients without potential liver disease. In our study, we found that ALT levels were significantly decreased in patients with osteoporosis than in patients with normal BMD and osteopenia. Additionally, serum TBIL levels correlated positively with AST and ALT. More importantly, multivariate regression analysis revealed that ALT showed protective effects against the development of osteoporosis. These data have demonstrated that serum TBIL is one of reliable indicatorsof liver function and further supported the notion that normal liver function is independently protective factor for bone metabolism. Thus, these data may, in part, explain the higher prevalence of osteoporosis and greater bone mass loss rate in patients with underlying liver disease than in patients without underlying liver disease [22, 36, 37]. Further longitudinal studies are needed to determine if the association between hyperbilirubinemia, BMD valuesand the prevalence of osteoporosis in T2DM patients maydiffer from that in the physiologic range. Diabetic nephropathy (DN) could represent a possible mechanism linking serum TBIL with the development of osteoporosis. Many lines of evidence has indicated that individuals with DN and its related ESRD have a higher prevalence of osteoporosis and rates of hip bone loss as well as an increased risk for fractures [38, 39]. Our study demonstrated that osteoporosis patients had higher urinary ACR and serum CysC levels, and lower eGFR compared with patients with normal BMD and osteopenia. Moreover, eGFR was associated inversely with the development of osteoporosis. These data further provided additional support for the proposition that an inverse relationship exists between DN, impaired renal function, and osteoporosis. As anticipated, we also observed that serum TBIL levels in T2DM patients correlated positively with eGFR and negatively with serum CysC levels and urinary ACR, implying a critical role of serum TBIL in protection against the development and progression of DN and impaired renal function. Our findings are consistent with several previous cross-sectional and prospective studies.

Fukui et al [40] reported that patients without DN have higher bilirubin levels than those with DN. Moreover, TBIL concentration correlated negatively with albuminuria, and positively with eGFR in a hospital-based sample of 633 Japanese T2DM patients. Similarly, Huang and his colleagues [41] revealed that serum bilirubin level is associated with micro-albuminuria in patients with essential hypertension.

Likewise, Shin et al [42] performed a community-based cross-sectional study of 1363 Korea non-diabetic and diabetic adults, and observed that serum TBIL level was negatively correlated with 24 h urine protein, and positively with eGFR after adjustment for potential confounding factors. Recently, Kawamoto and his colleagues [43] conducted a hospital-based study of 1050 elderly Japanese persons, and found that serum bilirubin was significantly and independently associated with eGFR, and multivariate adjusted-odds ratio of hypobilirubinemia for stage 4 of CKD was 3.52. These data further indicatedthat serum bilirubin has a potential renoprotective effect because of antioxidant properties. Given the remarkable antioxidant and cytoprotective properties of bilirubin, and the role of oxidative stress in the pathogenesis of DN and osteoporosis, it is plausible that the relationship between serum TBIL levels, BMD values, and the prevalence of osteoporosis in T2DM patients is mediated to some extentby the association between serum TBIL levels, DN and its related impaired renal function.

Experimental and clinical studies have suggested that inflammation plays a key role in the development and progression of osteoporosis

and anti-inflammatory therapy can prevent osteoporosis. In the current study, we found that osteoporosis patients had higher NLR value and lowerlymphocyte count compared with those in normal BMD and osteopenia patients. Besides these, blood total WBC count showed an increased tendency in patients with osteopenia and osteoporosis compared with normal BMD patients (P = 0.081). Moreover, there were modest, but not statistically significant, correlations between NLR and the prevalence of osteoporosis (*P* = 0.076). These results further supported an important role of inflammation in the development and progression of osteoporosis. The present study further indicated that serum TBIL levels correlated negatively with blood total WBC, suggesting that high bilirubin levels may exert an anti-inflammatory effect, which may provide protection from osteoporosis. Indeed, abody of evidence have also demonstrated that serum bilirubin levels were inversely associated with hsCRP or C-reactive protein (CRP) in middle-aged and elderly Japanese men and women [44], children and adolescents [45]. Additionally, Vogel et al [46] suggested that bilirubinis able to suppress inflammatory responses by preventing the migration of leukocytes into target tissues through disruption of vascular cell adhesion molecule-1 (VCAM-1)-dependent cell signaling. Moreover, Zhu and his colleagues [47] found that administration of bilirubin can protect grafts from nonspecific inflammation-induced injury by inhibiting the production of inflammatory mediators and infiltration of Kupffer cells in a rat syngeneic intraportal islet transplantation model. Similar results have also been stated by Mazzone GL et al [48]. Recently, Alexandra and his co-workers [49] found that administration of exogenous bilirubin inhibited LPS-induced leukocyte-endothelial interaction and leukocyte accumulation in subjects with experimental endotoxemia. Taken all the above together, these previous and our present findings would further raise the possibility that bilirubin can serve as an endogenous regulator of host inflammatory responses, and accordingly exert an anti-inflammatory effect.

The ABI is initially a simple noninvasive measure of the severity of lower-extremity peripheral artery disease (PAD), and is now an indicator of atherosclerosis at other vascular sites. Fibrinogen, a biomarker of subclinical inflammation and cardiac risk, directly participates in the pathogenesis of atherosclerosis. Plasma fibrinogen levels were inversely correlated with BMD values in perimenopausal women [50], and anti-inflammatory drug tamoxifen can lead to an increase in BMD and reduction in fibrinogen in postmenopausal women [51]. As was expected, a significant reduction in ABI value was observed in patients with osteoporosis in our study, compared with those normal BMD and osteopenia patients. Furthermore, serum TBIL concentrations tended to be associated positively with ABI, and inversely and significantly correlated with fibrinogen, which was consistent with reports of Cho and his collaborators [52] showing that high bilirubin concentrations were significantly and independently associated with low levels of fibrinogen independent of conventional CVD risk factors in Korean subjects, and a low serum bilirubin concentration with various measures of subclinical atherosclerosis, implying a critical role of serum bilirubin in protection the progression of atherosclerosis. It is common knowledge that inflammation and related endothelial dysfunction are crucial in the development and progression of atherosclerosis as well as osteoporosis. Compelling evidence [7, 16, 53-55] have demonstrated that bilirubin at a physiological concentration suppressed activation of proinflammatory genes, ameliorated oxidative stress and vascular inflammation, restored endothelial nitric oxide synthase (eNOS) expression, increased nitric oxide (NO) bioavailability, improved endothelial function, prevented coronary microvascular dysfunction and coronary flow reserve impairment, and blocked vascular smooth muscular cell proliferation. Recently, Erdogan and colleagues [56] indicated that lower serum bilirubin levels are independently related to endothelial dysfunction and increased carotid IMT in healthy subjects. Collectively, these data further supported a protective effect of serum bilirubin on atherosclerosis through inhibition or alleviate of the effects of systemic inflammatory activity on endothelium. Our findings and those of previous studies also indicate that serum TBIL levels may be closely associated with osteoporosis and atherosclerosis and the beneficial effects of bilirubin on osteoporosis was at least partly attributed to its anti-inflammatory and related anti-atherosclerosis effect.

There are some limitations to our study. First, we could not determine the causal relationships between serum TBIL levels, BMD values, and the prevalence of osteoporosis due to the cross-sectional nature of the study with a single-center data. Second, we could not ascertain that subjects were representative of the general population in Luzhou city, as participants were recruited from the inpatient department and underwent strict screening; thus, there remains a possibility of selection bias. Third, the study population consisted of T2DM patients aged 45-87; therefore, these findings may not be generalizable to other populations, especially younger women or men. Fourth, although we considered the effects of multiple potential confounders on the associations between serum TBIL levels, BMD values, and the prevalence of osteoporosis, but the residual confounding still remained possible in our observational study due to uncontrolled or undetected variables such as bone turnover markers, phosphorus, PTH, 25-hydroxyvitamin D, vitamins C and E, SOD, and GPx. Finally, all biochemical parameters were performed once, a common practice in clinic studies, so as to avoid extra cost and burden to patients. Because single values of biochemical parameters are less accurate than using the means of several measures, we believed that our observations would underestimate the true association. Therefore, all biochemical parameters require multiple measurements. However, the use of standardized methods set in a single center and taking serum measurements in a fasted state likely improved reliability by reducing the influence of diet on bilirubin and other biochemical parameter levels. Nevertheless, our study has several strengths including relatively large sample size and BMD measurements done with DEXA, the current considered "gold standard". Furthermore, we intentionally applied strict exclusion criteria based on medical histories and routine laboratory findings and made careful adjustments for possible confounders to appropriately investigate the pure physiologic effects of serum TBIL on bone metabolism. Most importantly, our study is, to our knowledge, the first to evaluate the association between serum TBIL levels within normal physiologic levels, BMD values and the prevalence of osteoporosis in T2DM patients.

In conclusion, the present study showed that higher serum TBIL levels are significantly and positively associated with higher BMD values at the lumbar spine and hip, and lower prevalence of osteoporosis in T2DM patients, most likely by virtue of its anti-oxidative, anti-inflammatory and related anti-atherosclerosis and renoprotective properties. Further large and prospective studies are needed to establish the precise mechanism of action and to determine whether the positive association exists in the hyperbilirubinaemia range in T2DM patients.

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Disclosure of conflict of interest

None.

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References

[1] Baynes JW and Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes 1999; 48: 1-9.

[2] Maggio D, Barabani M, Pierandrei M, Polidori MC, Catani M, Mecocci P, Senin U, Pacifici R and Cherubini A. Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. J Clin Endocrinol Metab 2003; 88: 1523-1527.

[3] Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr Rev 2010; 31: 266-300.

[4] Mody N, Parhami F, Sarafian TA and Demer LL. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. Free Radic Biol Med 2001; 31: 509-519.

[5] Sánchez-Rodríguez MA, Ruiz-Ramos M, Correa-Muñoz E and Mendoza-Núñez VM. Oxidative stress as a risk factor for osteoporosis in elderly Mexicans as characterized by antioxidant enzymes. BMC Musculoskelet Disord 2007; 8: 124.

[6] Shouhed D, Kha HT, Richardson JA, Amantea CM, Hahn TJ and Parhami F. Osteogenic oxysterols inhibit the adverse effects of oxidative stress on osteogenic differentiation of marrow stromal cells. J Cell Biochem 2005; 95: 1276-1283.

[7] Kawamura K, Ishikawa K, Wada Y, Kimura S, Matsumoto H, Kohro T, Itabe H, Kodama T and Maruyama Y. Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction. Arterioscler Thromb Vasc Biol 2005; 25: 155-160.

[8] Stocker R and Peterhans E. Antioxidant properties of conjugated bilirubin and biliverdin: biologically relevant scavenging of hypochlorous acid. Free Radic Res Commun 1989; 6: 57-66.

[9] Fujii M, Inoguchi T, Sasaki S, Maeda Y, Zheng J, KobayashiK and Takayanagi R. Bilirubin and biliverdin protect rodents against diabetic nephropathy by down regulating NAD(P)H oxidase. Kidney Int 2010; 78: 905-919.

[10] Minetti M, Mallozzi C, Di Stasi AM and Pietraforte D. Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. Arch Biochem Biophys 1998; 352: 165-174.

[11] Kuyumcu ME, Yesil Y, Oztürk ZA, Cınar E, Kızılarslanoglu C, Halil M, Ulger Z, Yesil NK, Cankurtaran M and Arıoğul S. The association between homocysteine (HCY) and serum natural antioxidants in elderly bone mineral densitometry (BMD). Arch Gerontol Geriatr 2012; 55: 739-743.

[12] Baranano DE, Rao M, Ferris CD and Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A 2002; 99: 16093-16098.

[13] Sedlak TW, Saleh M, Higginson DS, Paul BD, Juluri KR and Snyder SH. Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc Natl Acad Sci U S A 2009; 106: 5171-5176.

[14] Stocker R, Yamamoto Y, McDonagh AF, Glazer AN and Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987; 235: 1043-1046.

[15] Greabu M, Olinescu R, Kummerow FA and Crocnan DO. The levels of bilirubin may be related to an inflammatory condition in patients with coronary heart disease. Acta Pol Pharm 2001; 58: 225-231.

[16] Kundur AR, Singh I and Bulmer AC. Bilirubin, platelet activation and heart disease: a missing link to cardiovascular protection in Gilbert's syndrome? Atherosclerosis 2015; 239: 73-84.

[17] Kirkby KA and Adin CA. Products of heme oxygenase and their potential therapeutic applications. Am J Physiol Renal Physiol 2006; 290: 563-571.

[18] Weinberger B, Archer FE, Kathiravan S, Hirsch DS, Kleinfeld AM, Vetrano AM and Hegyi T. Effects of bilirubin on neutrophil responses in newborn infants. Neonatology 2013; 103: 105-111.

[19] Cho JH, Kim MT, Lee HK, Hong IS and Jang HC. Factor analysis of biochemical markers associated with bone mineral density in adults. J Phys Ther Sci 2014; 26: 1225-1229.

[20] Bian LQ, Li RZ, Zhang ZY, Jin YJ, Kang HW, Fang ZZ, Park YS and Choi YH. Effects of total bilirubin on the prevalence of osteoporosis in postmenopausal women without potential liver disease. J Bone Miner Metab 2013; 31: 637-643.

[21] Kim BJ, Koh JM, Ahn SH, Lee SH, Kim EH, Bae SJ, Kim HK, Choe JW and Kim GS. High serum total bilirubin as a protective factor against hip bone loss in healthy middle-aged men. Calcif Tissue Int 2013; 92: 501-518.

[22] Turkeli M, Dursun H, Albayrak F, Okçu N, Uyanik MH, Uyanik A, Yıldırım R, Keleş M and Yılmaz O. Effects of cirrhosis on bone mineral density and bone metabolism. Eurasian J Med 2008; 40: 18-24.

[23] Menon KV, Angulo P, Weston S, Dickson ER and Lindor KD. Bone disease in primary biliary cirrhosis: independent indicators and rate of progression. J Hepatol 2001; 35: 316-323.

[24] Ormarsdóttir S, Ljunggren O, Mallmin H, Michaëlsson K and Lööf L. Increased rate of bone loss at the femoral neck in patients with chronic liver disease. Eur J Gastroenterol Hepatol 2002; 14: 43-48.

[25] Smith DL, Shire NJ, Watts NB, Schmitter T, Szabo G and Zucker SD. Hyperbilirubinemia is not a major contributing factor to altered bone mineral density in patients with chronic liver disease. J Clin Densitom 2006; 9: 105-113.

[26] Diamond TH, Stiel D, Lunzer M, McDowall D, Eckstein RP and Posen S. Hepatic osteodystrophy. Static and dynamic bone histomorphometry and serum bone Gla-protein in 80 patients with chronic liver disease. Gastroenterology 1989; 96: 213-221.

[27] Kanis JA, Melton LJ 3rd, Christiansen C, Johnston CC and Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res 1994; 9: 1137-1141.

[28] Yang W, Lu J, Weng J, Jia W, Ji L, Xiao J, Shan Z, Liu J, Tian H, Ji Q, Zhu D, Ge J, Lin L, Chen L, Guo X, Zhao Z, Li Q, Zhou Z, Shan G, He J; China National Diabetes and Metabolic Disorders Study Group. Prevalence of diabetes among

men and women in China. N Engl J Med 2010; 362: 1090-1101.
[29] Luo Y, Wang X, Wang Y, Wang C, Wang H, Wang D, Liu L, Jia Q, Liu G, Zhao X, Wang Y; CNSR Investigators. Association of glomerular filtration rate with outcomes of acute stroke in type 2 diabetic patients: results from the China national stroke registry. Diabetes Care 2014; 37: 173-179.
[30] Kumar A, Pant P, Basu S, Rao GR and Khanna HD. Oxidative stress in neonatal hyperbilirubinemia. J Trop Pediatr 2007; 53: 69-71.
[31] Inoguchi T, Sasaki S, Kobayashi K, Takayanagi R and Yamada T. Relationship between Gilbert syndrome and prevalence of vascular complications in patients with diabetes. JAMA 2007; 298: 1398-1400.
[32] Era S, Kuwata K, Imai H, Nakamura K, Hayashi T and Sogami M. Age-related change in redox state of human serum albumin. Biochim Biophys Acta 1995; 1247: 12-16.
[33] Janes CH, Dickson ER, Okazaki R, Bonde S, McDonagh AF and Riggs BL. Role of hyperbilirubinemia in the impairment of osteoblast proliferation associated with cholestatic jaundice. J Clin Invest 1995; 95: 2581-2586.
[34] Ruiz-Gaspà S, Martinez-Ferrer A, Guañabens N, Dubreuil M, Peris P, Enjuanes A, Martinez de Osaba MJ, Alvarez L, Monegal A, Combalia A and Parés A. Effects of bilirubin and sera from jaundiced patients on osteoblasts: contribution to the development of osteoporosis in liver diseases. Hepatology 2011; 54: 2104-2113.
[35] Dubreuil M, Ruiz-Gaspà S, Guañabens N, Peris P, Alvarez L, Monegal A, Combalia A and Parés A. Ursodeoxycholic acid increases differentiation and mineralization and neutralizes the damaging effects of bilirubin on osteoblastic cells. Liver Int 2013; 33: 1029-1038.
[36] Leslie WD, Bernstein CN and Leboff MS. AGA technical review on osteoporosis in hepatic disorders. Gastroenterology 2003; 125: 941-966.
[37] Diamond T, Stiel D, Lunzer M, Wilkinson M, Roche J and Posen S. Osteoporosis and skeletal fractures in chronic liver disease. Gut 1990; 31: 82-87.
[38] Alem AM, Sherrard DJ, Gillen DL, Weiss NS, Beresford SA, Heckbert SR, Wong C and Stehman-Breen C. Increased risk of hip fracture among patients with end-stage renal disease. Kidney Int 2000; 58: 396-399.
[39] Stehman-Breen CO, Sherrard D, Walker A, Sadler R, Alem A and Lindberg J. Racial differences in bone mineral density and bone loss among end-stage renal disease patients. Am J Kidney Dis 1999; 33: 941-946.
[40] Fukui M, Tanaka M, Shiraishi E, Harusato I, Hosoda H, Asano M, Hasegawa G and Nakamura N. Relationship between serum bilirubin and albuminuria in patients with type 2 diabetes. Kidney Int 2008; 74: 1197-1201.
[41] Huang SS, Huang PH, Chiang KH, Chen JW and Lin SJ. Association of serum bilirubin levels with albuminuria in patients with essential hypertension. Clin Biochem 2011; 44: 859-863.
[42] Shin HS, Jung YS and Rim H. Relationship of serum bilirubin concentration to kidney function and 24-hour urine protein in Korean adults. BMC Nephrol 2011; 12: 29.
[43] Kawamoto R, Ninomiya D, Hasegawa Y, Kasai Y, Kusunoki T, Ohtsuka N and Kumagi T. Association between serum bilirubin and estimated glomerular filtration rate among elderly persons. PLoS One 2014; 9: e115294.
[44] Ohnaka K, Kono S, Inoguchi T, Yin G, Morita M, Adachi M, Kawate H and Takayanagi R. 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 2010; 88: 103-110.
[45] Lin LY, Kuo HK, Hwang JJ, Lai LP, Chiang FT, Tseng CD and Lin JL. Serum bilirubin is inversely associated with insulin resistance and metabolic syndrome among children and adolescents. Atherosclerosis 2009; 203: 563-568.
[46] Vogel ME and Zucker SD. Bilirubin acts as an endogenous regulator of inflammation by disrupting adhesion molecule-mediated leukocyte migration. Inflamm Cell Signal 2016; 3: e1178.
[47] Zhu H, Wang J, Jiang H, Ma Y, Pan S, Reddy S and Sun X. Bilirubin protects grafts against nonspecific inflammation-induced injury in syngeneic intraportal islet transplantation. Exp Mol Med 2010; 42: 739-748.
[48] Mazzone GL, Rigato I, Ostrow JD, Bossi F, Bortoluzzi A, Sukowati CH, Tedesco F and Tiribelli C. Bilirubin inhibits the TNFalpha-related induction of three endothelial adhesion molecules. Biochem Biophys Res Commun 2009; 386: 338-344.
[49] Tutak E, Ozer AB, Demirel I and Bayar MK. The relationship between serum bilirubin level with interleukin-6, interleukin-10 and mortality scores in patients with sepsis. Niger J Clin Pract 2014; 17: 517-522.
[50] Chen JT and Kotani K. Inverse correlation between fibrinogen and bone mineral density in women: preliminary findings. J Formos Med Assoc 2016; 115: 54-56.
[51] Downs RW Jr, Moffett AM, Ghosh A, Cox DA, Dowsett SA and Harper K. Effects of arzoxifene on bone, lipid markers, and safety parameters in postmenopausal women with low bone mass. Osteoporos Int 2010; 21: 1215-1226.

[52] Cho HS, Lee SW, Kim ES, Shin J, Moon SD, Han JH and Cha BY. Serum bilirubin levels are inversely associated with PAI-1 and fibrinogen in Korean subjects. Atherosclerosis 2016; 244: 204-210.

[53] Dekker D, Dorresteijn MJ, Pijnenburg M, Heemskerk S, Rasing-Hoogveld A, Burger DM, Wagener FA and Smits P. The bilirubin-increasing drug atazanavir improves endothelial function in patients with type 2 diabete mellitus. Arterioscler Thromb Vasc Biol 2011; 31: 458-463.

[54] Gullu H, Erdogan D, Tok D, Topcu S, Caliskan M, Ulus T and Muderrisoglu H. High serum bilirubin concentrations preserve coronary flow reserve and coronary microvascular functions. Arterioscler Thromb Vasc Biol 2005; 25: 2289-2294.

[55] Yoshino S, Hamasaki S, Ishida S, Kataoka T, Yoshikawa A, Oketani N, Saihara K, Okui H, Shinsato T, Ichiki H, Kubozono T, Kuwahata S, Fujita S, Kanda D, Nakazaki M, Miyata M and Tei C. Relationship between bilirubin concentration, coronary endothelial function, and inflammatory stress in overweight patients. J Atheroscler Thromb 2011; 18: 403-412.

[56] Erdogan D, Gullu H, Yildirim E, Tok D, Kirbas I, Ciftci O, Baycan ST and Muderrisoglu H. Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women. Atherosclerosis 2006; 184: 431-437.