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ORIGINAL ARTICLE

Serum bilirubin levels are positively associated with glycemic variability in women with type 2 diabetes

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Keywords
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ABSTRACT

Aims/Introduction: Glycemic variability is known to induce oxidative stress. We investigated the relationships between glycemic variability and serum bilirubin levels, an endogenous anti-oxidant, in patients with diabetes.

Materials and Methods: A cross-sectional study was carried out with 77 patients with type 2 diabetes who had been recruited to two clinical studies from 2008 to 2014. There were no participants with diseases of the pancreas, liver, biliary tract and chronic renal insufficiency. Glycemic variation was calculated by a continuous glucose monitoring system, and correlation analyses were carried out to evaluate their association with bilirubin levels. Multiple linear regression was carried out to identify independent factors influencing bilirubin levels and glycemic variation.

Results: Among the participants, 42.3% were men. The mean (standard deviation) age was 61.5 years (10.4 years), body mass index was 24.2 kg/m^2 (2.8 kg/m^2), diabetes duration was 17.7 years (9.5 years), hemoglobin A_{1c} was 60.7 mmol/mol (7.1 mmol/mol; 7.7 [0.7]%) and bilirubin was 11.8 μmol/L (4.10 μmol/L). Serum bilirubin levels were not different according to age, body mass index and hemoglobin A_{1c}. However, the mean amplitude of glucose excursion was positively associated with bilirubin levels in women ($r = 0.588$, $P < 0.001$). After adjustment with duration of diabetes, serum albumin, liver enzymes, and mean glucose, the correlation between bilirubin and mean amplitude of glucose excursion remained significant ($r = 0.566$, $P < 0.001$). Multiple linear regression analyses showed that bilirubin was an independent determinant for the mean amplitude of glucose excursion in women. 1,5-Anhydroglucitol was also associated with bilirubin levels in women.

Conclusions: Bilirubin level within the physiological range might be an independent predictor for glycemic variability in women with type 2 diabetes.

INTRODUCTION

Because intermittent high glucose has more triggering effects on oxidative stress than chronic sustained hyperglycemia[1,2], and oxidative stress is suggested as one mechanism of complications of diabetes mellitus[3], glycemic fluctuation has been examined with regard to diabetes mellitus complications[4]. However, there is no simple marker to assess glycemic variability (GV), despite the current technical progress in glucose monitoring systems. A continuous glucose monitoring system (CGMS) is used to evaluate glycemic excursion for a short period, but the cost and inconvenience preclude general use in clinical practice. Therefore, a surrogate marker that can reflect GV is required.

Bilirubin is known as an endogenous anti-oxidant and anti-inflammatory molecule[5]. Heme oxygenase-1 (HO-1) is an oxidative stress-responsive enzyme, and degrades free heme to carbon monoxide, iron and biliverdin, which is converted to bilirubin[6]. It has become apparent that the HO-1 system can act protectively in a variety of models of disease[7]. There are a couple of studies on the relationships between serum HO-1 and diabetes: diabetes mellitus was found to be associated with high serum HO-1[8], and

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attenuation of oxidative stress was associated with decreasing HO-1 levels[9]. Therefore, we hypothesized that GV-induced oxidative stress in diabetes activates the HO-1 system and its product, bilirubin, in circulation. To examine it, we applied CGMS in patients with type 2 diabetes, calculated various indices of GV and analyzed relationships with serum bilirubin.

METHODS

Population

The participants were adults with type 2 diabetes who had been recruited to two different clinical studies using CGMS (Medtronic Minimed, Northridge, CA, USA) carried out at Seoul National University Hospital, Seoul, Korea, from November 2008 to November 2014. Group 1 was composed of 60 persons under various treatment regimens (38 insulin users and 22 insulin non-users)[10]. Group 2 was composed of 17 insulin users. Clinical characteristics of each group are presented in Table S1. We excluded participants with diseases of the pancreas, liver, biliary tract and chronic renal insufficiency, and Gilbert syndrome based on the presence of hyperbilirubinemia (serum bilirubin level >20.5 μmol/L) in the absence of hemolytic disease and/or hepatic dysfunction (Figure 1)[11].

Measurement

Medical history, concomitant drugs, bodyweight and height were investigated. After 12-h overnight fasting, venous blood was collected, and the following were measured: hemoglobin

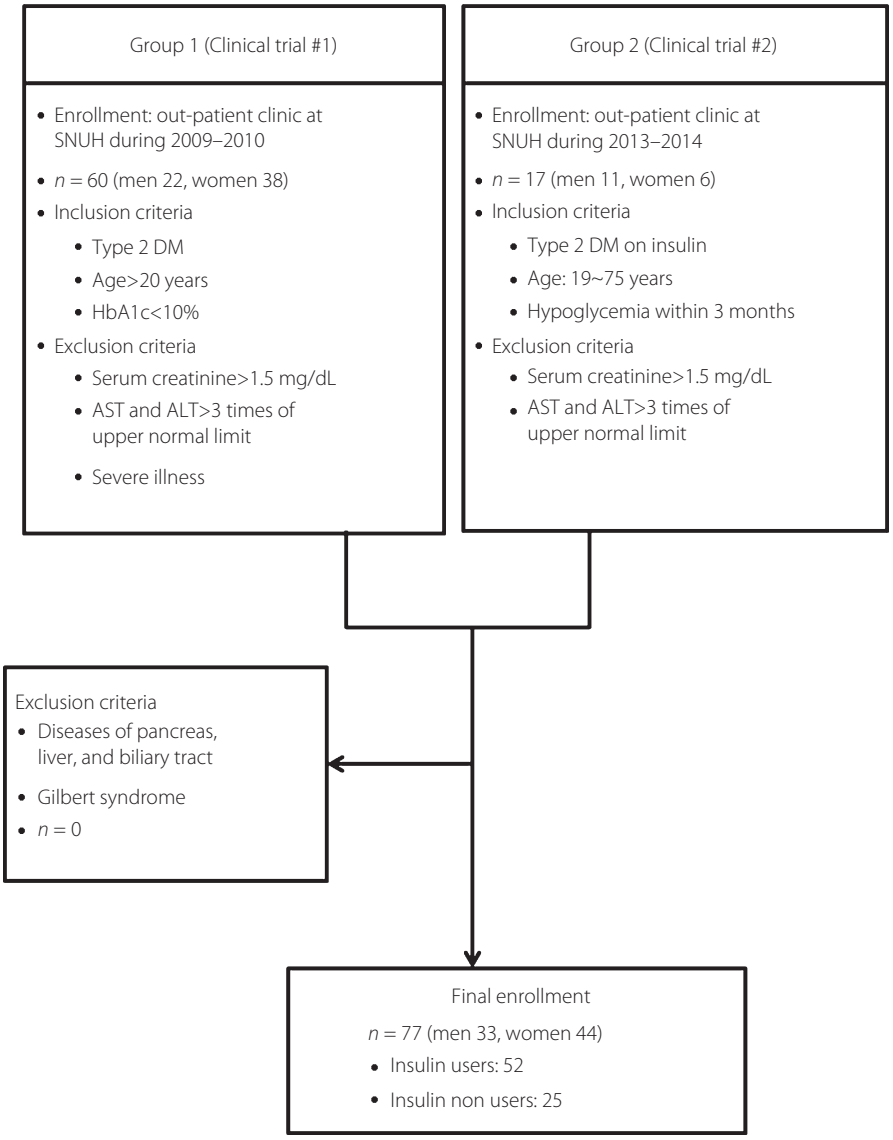

Figure 1 | A flow chart for enrollment of patients. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DM, diabetes mellitus; SNUH, Seoul National University Hospital.

(Hb), hemoglobin A_{1c} (HbA_{1c}; Sysmex XN-9000 with Bio-Rad Variant II Turbo; Bio-Rad Laboratories, Hercules, CA, USA), glucose, C-peptide, total cholesterol, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine levels (Toshiba-200FR auto-analyzer; Toshiba Medical Systems Corporation, Tokyo, Japan). Total serum bilirubin (Toshiba-200FR, HITACHI 7170 Auto Analyzer; Hitachi, Ltd., Tokyo, Japan) was measured using the azobilirubin method (Daiichi Pure Chemical Co. Ltd., Tokyo, Japan). Intra-assay coefficients of variance (CV) for bilirubin measurement was 1.9% and inter-assay CV was 0.6%. Spot urine albumin and creatinine (Toshiba-120FR auto-analyzer; Toshiba Medical Systems Corporation) were also measured. Estimated glomerular filtration rate was calculated using the Modification of Diet in Renal Disease Study equation method[12].

To assess GV, CGMS was applied for 3 days[10], and the first 48 h were used for the calculation of mean blood glucose (MBG) and the GV indices[4], such as standard deviation (SD), mean amplitude of glucose excursion (MAGE), continuous overall net glycemic action calculated with 6-h time intervals (CONGA-6), M100 (a measure of the stability of the glucose excursions in comparison with glucose value of 5.55 mmol/L [100 mg/dL]), J-index, mean post-meal maximum glucose (MPMG) and area under the curve for glucose above 9.99 mmol/L (180 mg/dL; area under the curve for glucose above 180 mg/dL). MAGE is the mean of the absolute difference of peak-to-nadir or nadir-to-peak direction, in the glycemic excursions more than one SD during the 48 h. CONGA-6 is the standard deviation of the differences between each glucose level and the corresponding glucose level measured 6 h earlier. M100 is a measure of the stability of the glucose excursions in comparison with glucose value of 5.55 mmol/L (100 mg/dL). J-index takes account of both the mean glucose level and variability of glycemia.

Statistical analysis

Continuous variables are expressed as mean ± SD, and categorical variables as the number and percentage. A normality test was carried out for all continuous variables. A log transformation was carried out for skewed data before further analyses. Student's *t*-test and chi square-test were used for comparisons between sex or groups. Analysis of variance (ANOVA) was applied for comparisons among the groups according to MAGE. The correlation coefficient was determined using Pearson's coefficient to determine the association between bilirubin levels and GV indices. Multiple linear regression using stepwise analyses were carried out to evaluate if MAGE was independently related with bilirubin levels. SPSS (version 18.0; SPSS, Chicago, IL, USA) was used for statistical analyses, and $P < 0.05$ was considered significant.

RESULTS

Clinical characteristics and laboratory data were compared between the two study groups, and the fasting C-peptide, albumin and MAGE were significantly different (shown in Table S1). However, according to general linear model analysis, all the relevance between bilirubin and C-peptide, between bilirubin and albumin, and between bilirubin and MAGE did not differ according to the group. Therefore, we combined the two groups to examine the relationships between bilirubin and GV. The clinical characteristics of the total 77 participants are shown in Table 1.

In the simple correlation analyses between bilirubin levels and the GV indices from CGMS, there were significant positive correlations between bilirubin and log(MAGE) ($r = 0.305$, $P = 0.007$), log(SD) ($r = 0.252$, $P = 0.027$), log(CONGA-6) ($r = 0.241$, $P = 0.034$), log(J-index) ($r = 0.286$, $P = 0.012$) and log(M100) ($r = 0.277$, $P = 0.015$). In the simple correlation analyses between bilirubin levels and GV-related variables, there were significant positive correlations between bilirubin and log (MBG) ($r = 0.262$, $P = 0.021$) and diabetes mellitus duration ($r = 0.266$, $P = 0.019$). Neither fasting glucose nor HbA1c was correlated with bilirubin levels. Hb, which is a main source of bilirubin, was significantly associated with bilirubin ($r = 0.300$, $P = 0.009$), but AST, ALT and albumin levels were not correlated with bilirubin. Because there were significant sex differences in important variables, such as bilirubin, GV indices, diabetes duration, C-peptide levels and Hb (Table 1), we carried out further analyses separately according to sex.

First, the participants were divided into three groups according to MAGE levels. Among the variables, body mass index was significantly different across the groups in the men participants ($F = 4.56$, $P = 0.019$), whereas bilirubin levels were significantly different across the groups in the female participants ($F = 4.499$, $P = 0.017$). Further correlation analyses showed that log(MAGE) was negatively correlated with body mass index in men ($r = -0.429$, $P = 0.013$), and positively correlated with bilirubin levels in women ($r = 0.588$, $P < 0.001$; Figure 2). Serum bilirubin was also positively associated with the duration of diabetes, serum albumin (which is an important transporter of unconjugated bilirubin in the circulation[6]), AST, ALT and log(MBG) in simple correlation analyses in women. Even after adjustment with these variables, the correlation between bilirubin and log(MAGE) remained significant, ($r = 0.566$, $P < 0.001$). In addition to MAGE, CONGA-6, MPMG, J-index and SD were also positively correlated with bilirubin levels in women (Table 2). In men, serum bilirubin levels were not correlated with log(MAGE) (Figure 2), regardless of adjustment with body mass index and other variables.

On the assumption that GV-induced oxidative stress contributed to serum bilirubin levels, multiple linear regression analyses were carried out to evaluate independent effects of MAGE on bilirubin levels in women, using log(MAGE), log (MBG), duration of diabetes mellitus, albumin, AST, ALT and Hb as independent variables. Among the independent variables, log(MAGE), ALT, AST and DM duration were significant determinants (Table 3). In the case of men, only Hb was a significant determinant for bilirubin levels ($\beta = 0.531$. $P = 0.019$), although the *F*-test was not significant ($P = 0.259$).

Table 1 | Clinical characteristics of the participants according to sex

	Total (n = 77)	Men (n = 33)	Women (n = 44)	P-value men vs women
Age (years)	61.5 ± 10.4	63.8 ± 10.2	59.8 ± 10.3	0.101
BMI (kg/m^2)	24.2 ± 2.8	23.9 ± 2.6	24.5 ± 2.9	0.321
DM duration (years)	24.2 ± 2.8	20.8 ± 10.0	15.4 ± 8.4	0.012
HbA_{1c} (mmol/mol)	60.7 ± 7.1	61.7 ± 8.0	60.7 ± 7.1	0.304
HbA_{1c} (%)	7.7 ± 0.7	7.8 ± 0.8	7.7 ± 0.7	
1.5-AG (μmol/L)	37.2 ± 17.7	37.1 ± 15.8	40.8 ± 17.7	0.020
C-peptide† (nmol/L)	0.53 ± 0.36	0.39 ± 0.37	0.62 ± 0.33	0.008
eGFR (mL/min/ 1.73 m^2)	68.1 ± 20.2	69.0 ± 23.1	67.5 ± 18.0	0.738
Urine albumin/creatinine (mg/mmol)	6.5 ± 19.8	3.7 ± 12.4	8.6 ± 23.8	0.282
Retinopathy (%) (none/NPDR/PDR)	50.0/37.1/12.9	40.0/43.3/16.7	57.5/32.5/10.0	0.336
CVD (%)‡	17.9	29.0	8.3	0.028
Insulin users (%)	71.4	75.8	68.2	0.466
OAD (%)	81.8	78.8	84.1	0.645
MTF/SU/others§	74.4/37.2/11.5	70.6/23.5/5.9	76.7/41.7/13.3	
Total bilirubin and the range (μmol/L)	11.80 ± 4.10 (1.71–23.95)	13.17 ± 4.28 (4.13–23.95)	10.95 ± 3.76 (1.71–20.52)	0.018
Hb (mmol/L)	8.3 ± 0.9	8.8 ± 0.8	7.9 ± 0.7	<0.001
Albumin (g/L)	43.0 ± 3.0	43.0 ± 3.0	43.0 ± 3.0	0.961
AST (IU/L)	25.9 ± 11.7	25.8 ± 13.1	26.0 ± 10.6	0.942
ALT (IU/L)	27.6 ± 17.5	27.1 ± 19.7	28.1 ± 15.8	0.800
MAGE† (mmol/L)	7.14 ± 2.75	8.06 ± 2.86	6.45 ± 2.48	0.010
SD† (mmol/L)	3.03 ± 1.08	3.26 ± 1.08	2.86 ± 1.05	0.086
CONGA-6† (mmol/L)	2.53 ± 0.90	2.74 ± 0.90	2.36 ± 0.87	0.045
M100†	23.0 ± 15.8	25.7 ± 15.9	20.9 ± 15.5	0.070
J-index†	50.0 ± 19.3	54.5 ± 19.3	46.7 ± 18.9	0.050
MPMG† (mmol/L)	13.11 ± 2.66	13.47 ± 2.39	12.84 ± 2.85	0.232
AUC-180† (mmol/L day)	1.12 ± 0.95	1.27 ± 0.96	1.00 ± 0.95	0.144
MBG† (mmol/L)	9.16 ± 1.72	9.51 ± 1.48	8.89 ± 1.85	0.077

Data are mean ± standard deviation for continuous variables. For frequency data, chi square-test was applied. †Log-transformed values were used for analysis. ‡Only available in 67 persons, 12 patients had cardiovascular disease. §Including α-glucosidase inhibitors, thiazolidinediones, nateglinides, a dipeptidyl peptidase-4 inhibitor and a glucagon-like peptide-1 (GLP-1) receptor agonist. 1,5-AG, 1,5-anhydroglucitol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC-180, area under the curve for glucose above 180 mg/dL; BMI, body mass index; CONGA-6, continuous overlapping net glycemic action calculated with 6-h time intervals; CVD, cardiovascular disease; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; M100, a measure of the stability of the glucose excursions in comparison with glucose value of 5.55 mmol/L (100 mg/dL); MAGE, mean amplitude of glycemic excursions; MBG, mean blood glucose; MTF, metformin; MPMG, mean post-meal maximum glucose; NPDR, non proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; SD, standard deviation; SU, sulfonylurea.

From a view of realistic significance in clinical practice, multiple linear regression analyses were carried out to identify independent factors determining GV, using log(MAGE) as a dependent variable, and bilirubin, log(C-peptide), duration of diabetes mellitus and log(MBG) as independent variables, because they would represent insulin insufficiency and mean glucose, which were reported to be related to GV[4]. Interestingly, the well-known factors, such as C-peptide and mean glucose, were significant determinants of MAGE in men as expected, whereas bilirubin was the only factor determining MAGE in women (Table 4). Similar results were also found if the dependent variable was replaced by log(SD), another GV index (data not shown).

1,5-Anhydroglucitol (1,5-AG) is a candidate biomarker for GV, in the case of moderately-controlled type 2 diabetes mellitus[4]. We explored the correlation between 1,5-AG and bilirubin, and observed that there was a significant correlation between them regardless of adjustment with log(MBG), which is known to affect 1,5-AG levels (Table 5). When we separated the participants by sex, the association between the biomarkers remained, although the statistical significance decreased in men.

DISCUSSION

In the present study, we found that in women, bilirubin was an independent factor for MAGE and SD, whereas insulin insufficiency and mean glucose significantly contributed to them in

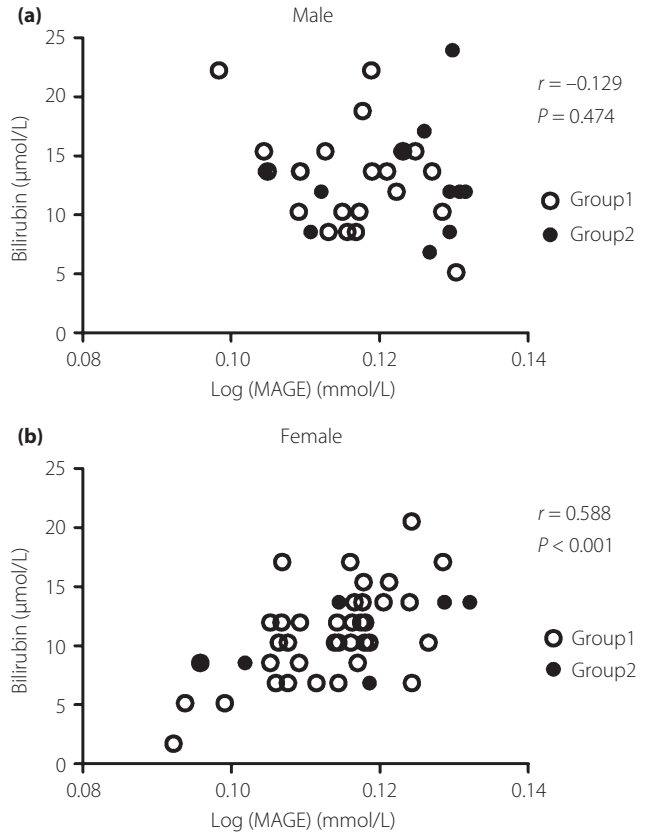

Figure 2 | Correlation analyses between mean amplitude of glucose excursion (MAGE) and bilirubin levels according to sex. There was no correlation between serum bilirubin levels and log(MAGE) in (a) men, whereas there was a significant correlation in (b) women.

Table 2 | Partial correlation analyses between bilirubin concentrations and glycemic variability indices in women

		r	*P*-value
Adjusted by DM duration, albumin, AST, ALT and log(MBG)	MAGE†	0.566	<0.001
	SD†	0.416	0.008
	CONGA-6†	0.420	0.008
	MPMG†	0.441	0.006
	J-index†	0.399	0.012
	M100†	0.297	0.067
	AUC-180†	0.293	0.079

†Log-transformed values were used for analysis. 1,5-AG, 1,5-anhydroglucitol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC-180, area under the curve for glucose above 180 mg/dL; CONGA-6, continuous overlapping net glycemic action calculated with 6-h time intervals; DM, diabetes mellitus; M100, a measure of the stability of the glucose excursions in comparison with glucose value of 5.55 mmol/L (100 mg/dL); MAGE, mean amplitude of glycemic excursions; MBG, mean blood glucose; MPMG, mean post-meal maximum glucose; SD, standard deviation.

Table 3 | Multiple linear regression analyses for bilirubin levels in women

Dependent variable	Bilirubin
Corrected r^2	0.480
F	6.414
P	<0.0001
β (*P*-value) of independent variables	
Log(MAGE)	0.578 (0.001)
AST	–0.011 (0.044)
ALT	0.011 (0.004)
DM duration	0.007 (0.031)
Log(MBG)	0.339 (0.292)
Albumin	0.094 (0.348)
Hb	0.004 (0.885)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DM, diabetes mellitus; Hb, hemoglobin; MAGE, mean amplitude of glycemic excursions; MBG, mean blood glucose.

Table 4 | Multiple linear regression analyses for prediction of mean amplitude of glycemic excursions

	Men (*n* = 33)	Women (*n* = 44)
Dependent variable	log(MAGE)	
Corrected r^2	0.345	0.309
F	5.074	5.817
P	0.004	0.001
β (*P*-value) of independent variables		
Bilirubin	–0.110 (0.256)	0.390 (0.002)
Log(MBG)	1.104 (0.008)	0.286 (0.297)
Log(C-peptide)	–0.119 (0.043)	–0.003 (0.958)
DM duration	0.002 (0.407)	0.002 (0.558)

DM, diabetes mellitus; MAGE, mean amplitude of glycemic excursions; MBG, mean blood glucose.

Table 5 | Correlation analyses between 1,5-anhydroglucitol and bilirubin levels

	Total participants (*n* = 77)		Men (*n* = 33)		Women (*n* = 44)	
	r	*P*	*r*	*P*	*r*	*P*
Simple correlation	–0.353	0.002	–0.231	0.195	–0.381	0.011
Adjusted by log(MBG)	–0.352	0.002	–0.333	0.063	–0.307	0.045

1,5-AG, 1,5-anhydroglucitol; MBG, mean blood glucose.

men. Another candidate biomarker for GV, 1,5-AG, was well correlated with bilirubin, especially in women.

Bilirubin is produced from heme through HO-1[6], and is a well-known anti-oxidant, as was described in the Introduction[5]. HO-1 gene expression in the muscle of patients with type 2 diabetes was reported to be defective[13], but the serum levels were found to be increased[8,9]. In particular, relationships

between HO-1 activity and glucose variability have not been evaluated. If glucose variability including both hypoglycemia and hyperglycemia enhances oxidative stress by diabetes mellitus, the reactive increase of HO-1 activity and its metabolites, such as bilirubin, is possible.

In addition, biliverdin reductase has been shown to have a role in glucose metabolism[14]. Biliverdin reductase (BVR) is not solely an enzyme converting biliverdin into bilirubin, but also a dual-specificity kinase (Ser/Thr and Tyr), not related to its reductase activity. Kinase BVR was found to be involved in the regulation of multiple steps of insulin signaling. Therefore, it might play a role in the pathogenesis of diabetes. GV-induced oxidative stress influences insulin signaling, too[15], and therefore there can be interactions between GV and BVR activity. Furthermore, in the nucleus, as a basic-leucine-zipper deoxyribonucleic acid/chromatin-binding transcription factor, BVR can act as a transcription factor, and has been shown to modulate HO-1 expression, which would further affect bilirubin levels.

Not only the process of bilirubin production, but also that of bilirubin metabolism is related to oxidative stress. When unconjugated bilirubin produced from hemoproteins throughout the body is delivered into the hepatocytes, where it becomes conjugated forms, it is bound to various proteins including the glutathione-S-transferase superfamily, to prevent efflux back into the serum[6]. The glutathione-S-transferase superfamily is another anti-oxidant detoxification enzyme that is associated with diabetes[16]. Therefore, oxidative stress might be able to regulate bilirubin levels through various pathways, which we cannot infer specifically with the current data.

We observed the close correlation between MAGE and bilirubin levels only in the women, but it is difficult to explain. We could presume that sex differences in response to oxidative stress caused by GV underlie it. It is known that men have insufficient anti-oxidant defense mechanisms compared to women[17,18], and bilirubin might also be included in that kind of anti-oxidant. In that case, bilirubin cannot reflect GV efficiently. Interestingly, in a cohort of 628 healthy participants aged 18~22 years, bilirubin levels were independently correlated with thiobarbituric acid-reactive substances only in women[19], suggesting bilirubin was a poor index for oxidative stress in men.

Our study had several limitations. First, owing to the cross-sectional nature of the present study, we were not able to conclude a causal relationship. Second, the study population was relatively small to control various confounding factors for GV. However, comprehensive assessment of GV from CGMS in the participants combined from two different groups makes our observation between GV and bilirubin more reliable.

In conclusion, we found that serum bilirubin levels were positively associated with GV in women with type 2 diabetes mellitus, independently from albumin, liver enzymes and mean glucose. If it is consolidated by interventional studies in larger populations, bilirubin levels can be a simple marker for glycemic excursions in women.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1| Clinical characteristics of the participants according to the study group.