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Bilirubin and progression of nephropathy in type 2 diabetes: A post-hoc analysis of RENAAL with independent replication in IDNT

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Abstract

Background. Bilirubin, a potent endogenous antioxidant, was found to protect against development of diabetic nephropathy (DN) in rodents. In humans, cross-sectional studies found an inverse relation between bilirubin and DN. We prospectively investigated whether bilirubin is associated with progression of DN toward end-stage renal disease (ESRD).

Methods. We performed a post hoc analysis in the Reduction of Endpoints in non-insulin dependent diabetes mellitus (NIDDM) with the Angiotensin II Antagonist Losartan (RENAAL) trial with independent replication in the Irbesartan Diabetic Nephropathy Trial (IDNT). Subjects with type 2 diabetes and nephropathy with alanine aminotransferase, aspartate aminotransferase (AST), and bilirubin levels <1.5 times the upper limit of normal were included. The renal end point was defined as the composite of confirmed doubling of serum creatinine or ESRD.

Results. Bilirubin was inversely associated with the renal end point in RENAAL independent of age, gender, race, BMI, smoking, total cholesterol, diastolic blood pressure, HbA1c, treatment, estimated glomerular filtration rate, albumin-to-creatinine ratio, and AST. These results were confirmed in IDNT.

Conclusions. This study indicates an independent inverse association of bilirubin with progression of nephropathy in RENAAL and IDNT. These data suggest a protective effect of bilirubin against progression of nephropathy in type 2 diabetes. The well-established role of bilirubin as an antioxidant is a potential explanation for our findings.

Introduction

The incidence of type 2 diabetes and its complications are increasing worldwide. One of the major complications of type 2 diabetes is diabetic nephropathy (DN). Nephropathy develops in approximately 20–40% of patients with diabetes and is the single leading cause of end-stage renal disease (ESRD) around the world (1).

Bilirubin is a product of heme catabolism and is known to be a potent endogenous antioxidant (2). As such, bilirubin has consistently been associated with protection against development of cardiovascular disease (CVD) (3,4). A recent study in rodents suggested that bilirubin is also protective against progression of DN (5). This notion is supported by several cross-sectional studies in humans demonstrating that low levels of bilirubin are associated with DN (6–8).

To our knowledge, there are no prospective studies to date that investigated whether bilirubin levels are associated with progression of DN toward ESRD. Therefore, our primary objective was to prospectively investigate the association of bilirubin with progression of nephropathy in patients with type 2 diabetes. To this end, we performed a historical prospective study in the Reduction of Endpoints in non-insulin dependent diabetes with the Angiotensin II Antagonist Losartan (RENAAL) trial (9,10). Subsequently, independent replication was sought in the Irbesartan Diabetic Nephropathy Trial (IDNT) (11,12). In the RENAAL and IDNT studies, patients were treated with an angiotensin receptor blocker (ARB) (losartan in RENAAL and irbesartan in IDNT). Several studies have shown that ARBs reduce hemoglobin levels (13–15). Because bilirubin is a product of heme catabolism, the use of ARBs could consequently reduce bilirubin levels. Therefore, our secondary aim was to investigate the effect of ARB treatment on serum concentrations of hemoglobin and bilirubin.

Patients and methods

Study Design and Population

The present study was conducted in patients with type 2 diabetes and nephropathy participating in the RENAAL and IDNT studies. The design, rationale, and study outcomes for these trials have been published elsewhere (9–12). Both trials investigated the efficacy of an ARB (losartan in RENAAL and irbesartan in IDNT) on renal outcomes in patients with type 2 diabetes, nephropathy, and proteinuria. Inclusion criteria for both trials were similar, with minor differences in details. Patients with type 2 diabetes, hypertension, and nephropathy aged 30–70 years were eligible for inclusion in both trials. Serum creatinine levels ranged from 1.0 to 3.0 mg/dL. All subjects were required to have proteinuria defined as a urinary albumin-to-creatinine ratio (ACR) ≥300 mg/g or

a 24-h urinary protein excretion >500 mg/day in RENAAL and ≥900 mg in IDNT. Major exclusion criteria for participation in both trials were type 1 diabetes, nondiabetic renal disease, and screening values of liver enzymes (alanine aminotransferase [ALT], aspartate aminotransferase [AST]) or total bilirubin >1.5 times the upper limit of normal. The inclusion and exclusion criteria of the RENAAL and IDNT studies are summarized in Supplementary Table S1 [adapted from Packham et al. (16)]. Subjects with missing data for baseline measurements of total bilirubin were excluded from the analyses (RENAAL n = 15 [1.0%], IDNT n = 8 [0.5%]).

Measurements and Clinical End Points

Laboratory and physical assessment data were collected every 6 months during follow-up for subjects participating in RENAAL and IDNT and included blood pressure measurements, glycated hemoglobin (HbA1c), lipid profile, hemoglobin, total bilirubin, serum albumin, ALT, AST, and serum creatinine. For both trials, all biochemical measurements were conducted in a central laboratory according to standardized conditions. Estimated glomerular filtration rate (GFR) was calculated using the Modification of Diet in Renal Disease (MDRD) equation (17). The primary end point for the current study was the composite of a confirmed doubling of serum creatinine (DSCR) or ESRD (defined as the need for long-term dialysis or renal transplantation). All end points were adjudicated by an independent committee using rigorous guidelines and definitions.

Statistical Analyses

Statistical analyses were performed using SPSS version 18.0 for Windows (IBM Corporation, Chicago, IL) and Stata 11 (StataCorp LP, College Station, TX). Results are presented as mean ± SD for variables with a normal distribution and as median (interquartile range) for variables with a nonnormal distribution. Nominal data are presented as the total number of patients with percentages. A two-sided $P < 0.05$ was considered statistically significant.

To assess which baseline variables were associated with baseline total bilirubin, the study populations were subdivided into tertiles of baseline total bilirubin concentration, and patient characteristics were presented accordingly. *P*-values for trend across tertiles of baseline total bilirubin were assessed using linear regression analyses. Variables with a skewed distribution were log-transformed to fulfill criteria for linear regression analyses. Multivariable linear regression analyses were used to investigate which clinical parameters at baseline were independently associated with bilirubin at baseline.

The course of clinical parameters over time are presented according to tertiles of baseline bilirubin. We investigated whether the change in total bilirubin concentration over time differed among tertiles in subjects with complete bilirubin measurements at baseline and 12, 24, and 36 months using one-way ANOVA.

To investigate the association of total bilirubin with progression of nephropathy, we used Cox proportional hazard regression analyses with time-varying covariates, which takes the change of clinical parameters over time into account. Logarithmic transformation (base 2) of bilirubin levels was applied in order to present the hazard ratios (HRs) derived from Cox regression analyses per doubling of bilirubin levels. Multivariable analyses were conducted using a Cox regression model, including the potential confounding factors of age, sex, baseline eGFR, baseline log ACR, race, smoking at baseline, history of CVD at baseline, baseline BMI, total cholesterol, diastolic blood pressure, HbA1c, treatment assignment, and log AST.

In sensitivity analyses, we repeated the Cox regression analyses in the subgroups receiving an ARB (i.e., losartan in RENAAL, irbesartan in IDNT) or placebo in both trials. In further sensitivity analyses, we investigated whether the change in bilirubin values during the course of the trials was associated with renal progression. To investigate the effect of treatment with an ARB on serum concentrations of hemoglobin and total bilirubin, we used the independent sample t test to compare hemoglobin and bilirubin concentrations between treatment groups in both trials.

Results

Patient Characteristics

In RENAAL, bilirubin concentrations were available for 1,498 (99.0%) patients. Mean baseline bilirubin level was 0.57 ± 0.19 mg/dL. Baseline patient characteristics according to tertiles of baseline bilirubin levels are presented in Table 1A and 1B. Prevalence of male sex, age, history of CVD, hemoglobin, serum albumin, liver enzymes, and eGFR increased with increasing bilirubin levels, whereas the prevalence in use of diuretics, use of insulin, BMI, HbA1c, cholesterol, triglycerides, and urinary ACR decreased with increasing bilirubin levels.

Table 1A. Baseline patient characteristics of the RENAAL study population presented as tertiles of bilirubin concentrations.

	RENAAL				
	All subjects	Tertile 1	Tertile 2	Tertile 3	*P*-value
N	1,498	374	776	348	-
Total bilirubin (mg/dL)	0.57 ± 0.19	0.1-0.4	0.5-0.6	0.7-2.1	-
Age (years)	60.1 ± 7.4	57.5 ± 7.7	60.8 ± 7.2	61.4 ± 7.0	<0.001
Male gender (n, %)	946 (63.2)	187 (50.0)	490 (63.1)	269 (77.3)	<0.001
History of CVD (n, %)	443 (29.2)	88 (23.5)	249 (32.1)	106 (30.5)	0.04
Race					
White (n, %)	723 (48.3)	119 (31.8)	416 (53.6)	188 (54.0)	0.06
Black (n, %)	228 (15.2)	92 (24.6)	112 (14.4)	24 (6.9)	
Hispanic (n, %)	276 (18.4)	109 (29.1)	113 (14.6)	54 (15.5)	
Asian (n, %)	252 (16.8)	49 (13.1)	125 (16.1)	78 (22.4)	
Other (n, %)	19 (1.3)	5 (1.3)	10 (1.3)	4 (1.1)	
Smoking status					
Smoker (n, %)	270 (18.0)	80 (21.4)	134 (17.3)	56 (16.1)	0.06
Body composition					
BMI (kg/m^2)	29.7 ± 6.3	30.2 ± 7.1	29.9 ± 6.1	28.7 ± 5.6	0.002
Blood pressure					
Systolic blood pressure (mmHg)	153 ± 19	152 ± 19	153 ± 20	151 ± 19	0.5
Diastolic blood pressure (mmHg)	82 ± 10	82 ± 10	82 ± 10	83 ± 11	0.1
Use of ACEi/ARB (n, %)	769 (51.3)	192 (51.3)	415 (53.5)	162 (46.6)	0.2
Use of diuretics (n, %)	870 (58.1)	251 (67.1)	440 (56.7)	179 (51.4)	<0.001
Glucose homeostasis					
Diabetes duration ≥ 5 years (n, %)	1,351 (90.2)	342 (91.4)	705 (90.9)	304 (87.4)	0.05
HbA1c (%)	8.4 ± 1.6	8.8 ± 1.6	8.5 ± 1.6	8.2 ± 1.6	<0.001
HbA1c (mmol/mol)	69 ± 18	73 ± 18	69 ± 18	66 ± 18	<0.001
Use of insulin (n, %)	901 (60.1)	252 (67.4)	471 (67.4)	178 (51.1)	<0.001
Laboratory measurements					
Hemoglobin (g/dL)	12.5 ± 1.8	11.6 ± 1.5	12.5 ± 1.7	13.5 ± 1.8	<0.001
Serum albumin (g/dL)	3.8 ± 0.4	3.5 ± 0.4	3.8 ± 0.4	4.0 ± 0.3	<0.001
Lipids					
Total cholesterol (mg/dL)	228 ± 56	244 ± 61	226 ± 54	216 ± 48	<0.001
HDL cholesterol (mg/dL)	45 ± 15	48 ± 17	44 ± 14	44 ± 14	0.001
LDL cholesterol (mg/dL)	142 ± 46	152 ± 53	141 ± 44	134 ± 39	<0.001
Triglycerides (mg/dL)	172 [122-245]	181 [133-270]	172 [120-244]	160 [111-228]	<0.001
Liver function					
ALT (U/L)	15 [12-21]	14 [11-19]	15 [12-21]	16 [13-24]	<0.001
AST (U/L)	16 [13-20]	15 [12-19]	16 [13-20]	17 [14-23]	<0.001
Renal function					
ACR (mg/g)	1247 [560-2559]	1917 [882-3730]	1193 [544-2334]	855 [433-1749]	<0.001
Serum creatinine (mg/dL)	1.9 ± 0.5	1.9 ± 0.5	1.9 ± 0.5	1.8 ± 0.4	<0.001
eGFR, MDRD (mL/min/1.73m^2)	39.8 ± 12.4	38.2 ± 12.7	39.7 ± 12.5	41.8 ± 11.5	<0.001

ACEi, angio-converting enzyme inhibitor; ACR, urinary albumin-to-creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein.

Table 1B. Baseline patient characteristics of the IDNT study population presented as tertiles of bilirubin concentrations.

	IDNT				
	All subjects	Tertile 1	Tertile 2	Tertile 3	*P*-value
N	1,707	588	413	706	-
Total bilirubin (mg/dL)	0.54 ± 0.21	0.1–0.4	0.5	0.6–2.0	-
Age (years)	58.9 ± 7.8	57.4 ± 8.4	58.7 ± 7.7	60.3 ± 7.0	<0.001
Male gender (n, %)	1,134 (66.4)	297 (50.5)	270 (65.4)	567 (80.3)	<0.001
History of CVD (n, %)	481 (28.2)	168 (28.6)	101 (24.5)	212 (30.0)	0.5
Race					
White (n, %)	1,238 (72.5)	323 (54.9)	323 (78.2)	592 (83.9)	<0.001
Black (n, %)	224 (13.1)	138 (23.5)	39 (9.4)	47 (6.7)	
Hispanic (n, %)	83 (4.9)	45 (7.7)	18 (4.4)	20 (2.8)	
Asian (n, %)	85 (5.0)	55 (9.4)	13 (3.1)	17 (2.4)	
Other (n, %)	77 (4.5)	27 (4.6)	20 (4.8)	30 (4.2)	
Smoking status					
Smoker (n, %)	299 (17.5)	119 (20.2)	76 (18.4)	104 (14.7)	0.009
Body composition					
BMI (kg/m^2)	30.8 ± 5.8	32.2 ± 6.8	30.3 ± 5.2	30.0 ± 4.9	<0.001
Blood pressure					
Systolic blood pressure (mmHg)	159 ± 20	160 ± 21	159 ± 19	159 ± 19	0.3
Diastolic blood pressure (mmHg)	87 ± 11	86 ± 11	87 ± 10	88 ± 11	<0.001
Use of ACEi/ARB (n, %)	797 (46.7)	306 (52.0)	175 (42.4)	316 (44.8)	0.01
Use of diuretics (n, %)	802 (47.0)	320 (54.4)	198 (47.9)	284 (40.2)	<0.001
Glucose homeostasis					
Diabetes duration ≥ 5 years (n, %)	1,533 (89.8)	537 (91.3)	374 (90.6)	622 (88.1)	0.06
HbA1c (%)	8.1 ± 1.7	8.1 ± 1.8	8.3 ± 1.8	8.1 ± 1.7	0.9
HbA1c (mmol/mol)	65 ± 19	65 ± 20	67 ± 20	65 ± 19	0.9
Use of insulin (n, %)	985 (57.7)	367 (62.4)	232 (56.2)	386 (54.7)	0.006
Laboratory measurements					
Hemoglobin (g/dL)	12.9 ± 1.9	12.0 ± 1.8	12.9 ± 1.8	13.8 ± 1.7	<0.001
Serum albumin (g/dL)	3.8 ± 0.4	3.6 ± 0.5	3.9 ± 0.4	4.0 ± 0.3	<0.001
Lipids					
Total cholesterol (mg/dL)	228 ± 58	239 ± 64	229 ± 58	218 ± 51	<0.001
HDL cholesterol (mg/dL)	42 ± 14	43 ± 15	44 ± 15	41 ± 13	0.1
LDL cholesterol (mg/dL)	142 ± 46	150 ± 50	144 ± 48	136 ± 41	<0.001
Triglycerides (mg/dL)	177 [119–270]	185 [127–276]	178 [116–270]	169 [115–266]	0.06
Liver function					
ALT (U/L)	18 [13–25]	17 [12–24]	18 [13–25]	19 [14–26]	0.001
AST (U/L)	18 [14–23]	17 [14–22]	18 [14–23]	18 [15–24]	0.001
Renal function					
ACR (mg/g)	1500 [780-2759]	2130 [1163-3692]	1429 [781-2609]	1106 [604-2015]	<0.001
Serum creatinine (mg/dL)	1.7 ± 0.6	1.8 ± 0.6	1.7 ± 0.6	1.6 ± 0.5	<0.001
eGFR, MDRD (mL/min/1.73m^2)	47.4 ± 17.5	43.3 ± 16.7	45.9 ± 18.2	51.6 ± 17.0	<0.001

ACEi, angio-converting enzyme inhibitor; ACR, urinary albumin-to-creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein.

Multivariable linear regression analyses showed that baseline bilirubin levels were independently associated with age, smoking, BMI, HbA1c, hemoglobin, serum albumin, log AST, and total cholesterol (Table 2A and 2B).

In IDNT, bilirubin concentrations were available for 1,707 patients (99.5%). The mean baseline bilirubin level in IDNT was similar to that in RENAAL (0.54 ± 0.21 mg/dL). In general, associations and trends of bilirubin with baseline characteristics were similar to those observed in RENAAL. In multivariable linear regression analyses, all variables that were independently associated with bilirubin in RENAAL (except AST) were also independently associated with bilirubin in IDNT. Sex, race, diastolic blood pressure, duration of diabetes (≥5 years), and eGFR were also significantly associated with bilirubin levels in IDNT.

Clinical Parameters Over Time

The course of clinical parameters over time is shown in Supplementary Table S2. In RENAAL, the change in total bilirubin concentration only differed among tertiles of baseline bilirubin at 12 months. After 24 and 36 months, there were no significant differences in change in bilirubin concentrations among tertiles of bilirubin (Supplementary Table S3). These results were confirmed in IDNT (Supplementary Table S3).

Progression of Nephropathy

After a mean follow-up period of 3.4 years, 471 (31%) subjects had reached the renal end point of DSCR or ESRD in RENAAL. Univariable time-varying Cox regression analysis showed that total bilirubin was significantly associated with progression of nephropathy in RENAAL (HR 0.54 [95% CI 0.45–0.65], $P < 0.001$) (Table 3, model 1). These associations remained significant after adjustment for potential confounding factors, which were age, sex, race, eGFR, log ACR, BMI, smoking status, history of CVD, total cholesterol, diastolic blood pressure, HbA1c, treatment, and log AST (HR 0.67 [0.55–0.83], $P < 0.001$). The risk for the renal end point according to total bilirubin concentrations in the RENAAL trial is shown in Figure 1A.

In IDNT, 381 (22%) patients reached the renal end point after a mean follow-up period of 2.6 years. The results of time-varying Cox proportional hazard regression analyses were similar to those of RENAAL (HR 0.64 [0.55–0.76], $P < 0.001$) for the final multivariable model (Table 3). The graph indicating the risk for the renal end point according to total bilirubin concentrations in IDNT is similar to that for RENAAL (Figure 1B).

In sensitivity analyses, we investigated whether total bilirubin was associated with progression of nephropathy irrespective of ARB or placebo assignment. In RENAAL, total bilirubin was significantly and inversely associated with progression of nephropathy

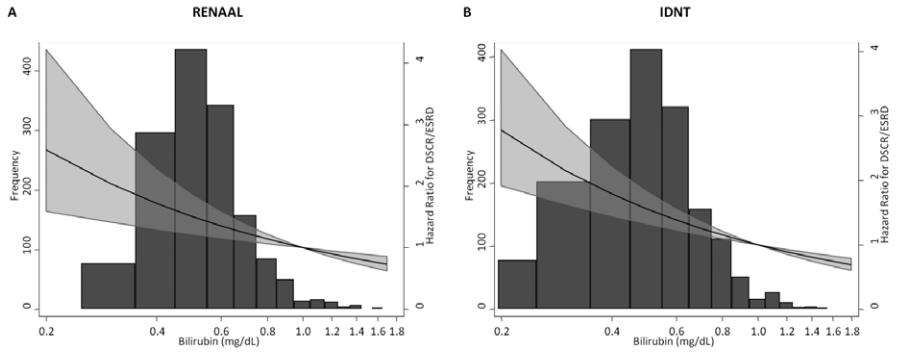

Figure 1. Histogram of baseline bilirubin concentrations in the RENAAL (A) and IDNT (B) studies. The line in the graph represents the risk for DSCR or ESRD. The grey area represents the 95% CI of the HR.

for subjects receiving losartan (HR 0.66 [0.48–0.89], $P = 0.008$) and those receiving placebo (HR 0.70 [0.52–0.94], $P = 0.02$). These results were confirmed in IDNT for subjects receiving irbesartan (HR 0.59 [0.43–0.81], $P = 0.001$) and those receiving placebo (HR 0.61 [0.46–0.80], $P < 0.001$).

In further sensitivity analyses, the change in total bilirubin during the course of the trial was not associated with the renal end point (HR 1.07 [0.97–1.19], $P = 0.2$, per 0.1 mg/dL), whereas total bilirubin remained significantly associated with the renal end point in RENAAL (HR 0.59 [0.44–0.79], $P < 0.001$). These results were confirmed in IDNT for change in total bilirubin (HR 1.05 [0.97–1.13], $P = 0.2$) and for total bilirubin (HR 0.62 [0.52–0.74], $P < 0.001$). The results remained essentially unchanged when stratified for treatment.

Table 2A. Univariable and multivariable associations of log_2-transformed bilirubin concentrations with clinical parameters in RENAAL.

	RENAAL					
	Univariable			Multivariable		
	Beta	**SE**	***P*-value**	**Beta**	**SE**	***P*-value**
Age (years)	0.01	0.001	<0.001	0.005	0.001	<0.001
Gender	-0.17	0.02	<0.001			
Race	-0.02	0.008	0.04			
History of CVD	0.06	0.03	0.02			
Smoking status						
Smoking	-0.06	0.03	0.05	-0.05	0.03	0.05
Body composition						
BMI (kg/m^2)	-0.006	0.002	0.002	-0.004	0.002	0.02
Blood pressure						
Systolic blood pressure (10 mmHg)	-0.005	0.006	0.4			
Diastolic blood pressure (10 mmHg)	0.01	0.01	0.3			
Use of ACEi/ARB	-0.04	0.02	0.08			
Use of diuretics	-0.10	0.02	<0.001			
Glucose homeostasis						
Diabetes duration ≥ 5 years	-0.07	0.04	0.09			
HbA1c (%)	-0.03	0.007	<0.001	-0.02	0.006	<0.001
Use of insulin	-0.09	0.02	<0.001			
Laboratory measurements						
Hemoglobin (g/dL)	0.09	0.006	<0.001	0.06	0.006	<0.001
Serum albumin (g/dL)	0.45	0.03	<0.001	0.28	0.03	<0.001
Lipids						
Total cholesterol (10 mg/dL)	-0.02	0.002	<0.001	-0.008	0.002	<0.001
HDL cholesterol (10 mg/dL)	-0.03	0.007	<0.001			
LDL cholesterol (10 mg/dL)	-0.02	0.003	<0.001			
Log triglycerides (log mg/dL)	-0.20	0.04	<0.001			
Liver function						
Log ALT (log U/L)	0.34	0.05	<0.001			
Log AST (log U/L)	0.52	0.07	<0.001	0.37	0.06	<0.001
Renal function						
Log ACR (log mg/g)	-0.26	0.03	<0.001			
Serum creatinine (mg/dL)	-0.09	0.02	<0.001			
eGFR, MDRD ($mL/min/1.73m^2$)	0.004	0.001	<0.001			

ACEi, angio-converting enzyme inhibitor; ACR, urinary albumin-to-creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; F, females; HDL, high-density lipoprotein; LDL, low-density lipoprotein; M, males.

Variables tested in multivariable analyses: age, gender, race, smoking, history of CVD, BMI, diastolic blood pressure, use of ACEi/ARB, use of diuretics, hemoglobin, serum albumin, total cholesterol, duration diabetes, HbA1c, use of insulin, log AST, log ACR, eGFR.

Table 2B. Univariable and multivariable associations of log_2-transformed bilirubin concentrations with clinical parameters in IDNT.

	IDNT					
	Univariable			Multivariable		
	Beta	**SE**	***P*-value**	**Beta**	**SE**	***P*-value**
Age (years)	0.01	0.002	<0.001	0.007	0.002	<0.001
Gender	-0.33	0.03	<0.001	-0.10	0.03	0.001
Race	-0.05	0.008	<0.001	-0.04	0.007	<0.001
History of CVD	0.01	0.03	0.6			
Smoking status						
Smoking	-0.11	0.04	0.002	-0.17	0.04	<0.001
Body composition						
BMI (kg/m^2)	-0.02	0.002	<0.001	-0.01	0.002	<0.001
Blood pressure						
Systolic blood pressure (10 mmHg)	-0.01	0.007	0.2			
Diastolic blood pressure (10 mmHg)	0.06	0.01	<0.001	0.04	0.01	<0.001
Use of ACEi/ARB	-0.06	0.03	0.03			
Use of diuretics	-0.12	0.03	<0.001			
Glucose homeostasis						
Diabetes duration ≥ 5 years	-0.10	0.05	0.04	-0.08	0.04	0.08
HbA1c (%)	0.003	0.008	0.7	0.02	0.008	0.06
Use of insulin	-0.09	0.03	0.002			
Laboratory measurements						
Hemoglobin (g/dL)	0.12	0.007	<0.001	0.08	0.009	<0.001
Serum albumin (g/dL)	0.50	0.03	<0.001	0.26	0.03	<0.001
Lipids						
Total cholesterol (10 mg/dL)	-0.02	0.002	<0.001	-0.01	0.002	<0.001
HDL cholesterol (10 mg/dL)	-0.03	0.01	0.02			
LDL cholesterol (10 mg/dL)	-0.02	0.003	<0.001			
Log triglycerides (log mg/dL)	-0.09	0.06	0.09			
Liver function						
Log ALT (log U/L)	0.22	0.06	<0.001			
Log AST (log U/L)	0.30	0.08	<0.001			
Renal function						
Log ACR (log mg/g)	-0.43	0.04	<0.001			
Serum creatinine (mg/dL)	-0.17	0.02	<0.001			
eGFR, MDRD ($mL/min/1.73m^2$)	0.006	0.001	<0.001	0.002	0.001	0.6

ACEi, angio-converting enzyme inhibitor; ACR, urinary albumin-to-creatinine ratio; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; F, females; HDL, high-density lipoprotein; LDL, low-density lipoprotein; M, males.

Variables tested in multivariable analyses: age, gender, race, smoking, history of CVD, BMI, diastolic blood pressure, use of ACEi/ARB, use of diuretics, hemoglobin, serum albumin, total cholesterol, duration diabetes, HbA1c, use of insulin, log AST, log ACR, eGFR.

Table 3. Associations of log_2-transformed bilirubin concentrations with the composite renal endpoint of DSCR or ESRD in univariable (model 1) and multivariable models adjusted for potential confounding factors.

Model	RENAAL		IDNT		RENAAL & IDNT	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
1.	0.54 (0.45–0.65)	<0.001	0.48 (0.43–0.55)	<0.001	0.50 (0.45–0.55)	<0.001
2.	0.59 (0.49–0.72)	<0.001	0.51 (0.45–0.58)	<0.001	0.53 (0.48–0.59)	<0.001
3.	0.60 (0.50–0.73)	<0.001	0.55 (0.48–0.63)	<0.001	0.55 (0.49–0.61)	<0.001
4.	0.73 (0.60–0.89)	0.002	0.64 (0.55–0.74)	<0.001	0.65 (0.58–0.73)	<0.001
5.	0.67 (0.55–0.83)	<0.001	0.64 (0.55–0.76)	<0.001	0.67 (0.59–0.76)	<0.001

Model 1: crude.

Model 2: adjusted for age and sex.

Model 3: adjusted for age, sex, and baseline eGFR.

Model 4: adjusted for age, sex, baseline eGFR, and baseline log ACR.

Model 5: adjusted for age, sex, baseline eGFR, baseline log ACR, race, smoking, history of CVD, baseline BMI, total cholesterol, DBP, HbA1c, treatment assignment, and log AST.

ACR, urinary albumin-to-creatinine ratio; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate.

ARB Treatment Effect

Because treatment with ARBs influence serum concentrations of hemoglobin (13) and could consequently affect bilirubin levels, we investigated the effect of treatment with an ARB (losartan in RENAAL and irbesartan in IDNT) on serum concentrations of hemoglobin and bilirubin. Hemoglobin and bilirubin concentrations over time in the RENAAL trial are shown in Figure 2A and 2B. Hemoglobin levels slowly decreased over time in the placebo group, while an initial decrease followed by a stabilization of hemoglobin levels was seen in losartan-treated patients (Figure 2A). After 48 months of treatment, hemoglobin levels were no longer significantly different between treatment groups (Figure 2A).

Bilirubin levels were slightly, but not significantly, lower in the losartan group than in the placebo group (Figure 2B). Despite the initial fall in hemoglobin levels, there was no initial fall in bilirubin levels in the losartan group. Bilirubin values decreased over time in both treatment groups, and no significant differences in bilirubin concentrations were observed between treatment groups after 12, 36, and 48 months.

Hemoglobin and bilirubin values of subjects using placebo and irbesartan in the IDNT trial are shown in Figure 2C and 2D. In general, the pattern of changes in these

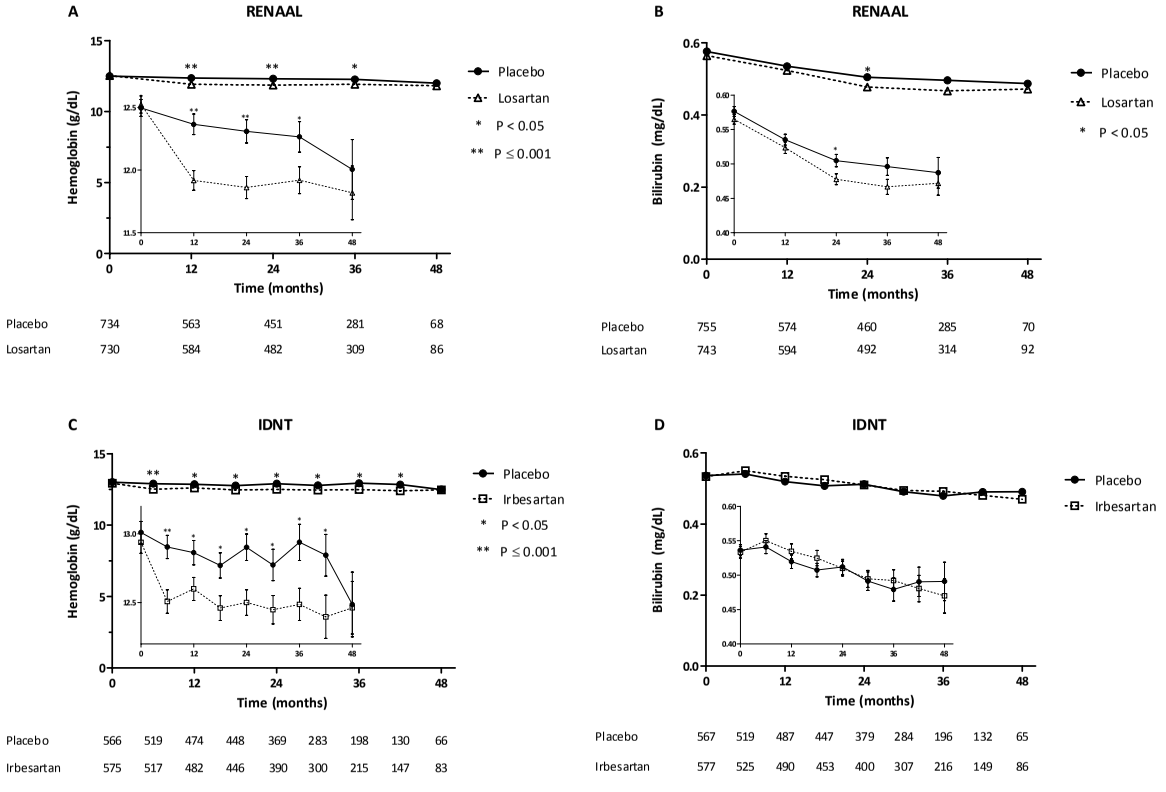

Figure 2. Hemoglobin (A, C) and total bilirubin (B, D) concentrations (mean ± SEM) of subjects using losartan or placebo in the RENAAL trial or irbesartan or placebo in the trial. The insets show a more-detailed version of each graph. The charts under each graph indicate the number of subjects with available measurements.

markers over time in IDNT was similar to the RENAAL trial. Although hemoglobin levels significantly decreased after initiation of treatment with irbesartan (Figure 2C), as in RENAAL, no significant differences in bilirubin concentrations were observed between treatment groups (Figure 2D).

Discussion

In this historical prospective analysis of the RENAAL trial, we found an independent inverse association of bilirubin levels with progression of nephropathy in patients with type 2 diabetes. This finding was independently replicated in IDNT. Furthermore, we showed that treatment with the ARBs losartan or irbesartan did not result in a decrease in bilirubin concentrations, despite an initial decrease in hemoglobin levels.

One of the major pathophysiologic mechanisms that has been identified in the development and progression of DN is oxidative stress, described as increased levels of reactive oxygen species (18–20). Bilirubin is known to be a potent endogenous antioxidant (2), and a recent study in rodents found a protective effect of bilirubin against DN through inhibition of oxidative stress by downregulation of renal NADPH oxidase (5).

In humans, several cross-sectional studies have provided additional evidence for a protective effect of bilirubin on DN. Inoguchi et al. (6) showed a lower prevalence of vascular complications as well as reduced markers of oxidative stress and inflammation in patients with Gilbert syndrome (a congenital hyperbilirubinemia) and diabetes. Fukui et al. (8) reported a negative correlation of bilirubin with log ACR and a positive correlation with eGFR. In addition, it was shown that bilirubin levels were higher in patients without DN than in those with DN (7). However, these studies were cross-sectional in design, precluding investigation of the prospective association of bilirubin with renal impairment. To our knowledge, the current prospective study is the first to indicate an inverse association of bilirubin and progression of nephropathy toward ESRD in type 2 diabetes.

In animal models, antioxidants have been shown to be effective in treating DN (21,22). In combination with the current human data showing an independent association between bilirubin and renal outcome, we speculate that treatments intended to slightly raise bilirubin levels might have a beneficial effect on progression of nephropathy in patients with type 2 diabetes and low bilirubin levels.

A moderate increase in bilirubin levels could be attained through induction of heme oxygenase-1 (HO-1), the enzyme that catalyzes the rate-limiting step in heme degradation. HO-1 splits heme into carbon monoxide (CO) and biliverdin, which is subsequently reduced to bilirubin (2,23). The HO-1 system and heme degradation products CO, biliverdin, and bilirubin have repeatedly been shown to have renoprotective properties (2,23). Therefore, the renoprotective effects of bilirubin in this study are possibly, and at least partly, mediated by induction of HO-1 and by-products of heme degradation

(i.e., CO, biliverdin). A study in rats showed that induction of HO-1 reduces renal oxidative stress and protects against diabetes-related renal injury (24). HO-1 is a highly inducible enzyme, which can be induced by many drugs routinely used in clinical medicine (i.e., nonsteroidal anti-inflammatory drugs [NSAIDs], peroxisome proliferator-activated receptor α agonists) (4). However, given the adverse effects of NSAIDs, long-term use of NSAIDs is not recommended in patients with advanced renal function impairment. Natural HO-1 inducers include curcuma and polyphenols (i.e., resveratrol) (4,25). Partial inhibition of conjugation of bilirubin by uridine diphosphate-glucuronyltransferase, an enzyme encoded by the UGT1A1 gene, reduces bilirubin excretion and is known to result in mild increases in bilirubin concentrations (4,23,26). Pharmaceuticals capable of a partial inhibition of UGT1A1 are probenecid and atazanavir (4).

A number of studies have reported that the use of ACE inhibitors and ARBs decrease hemoglobin levels (13–15), which can be enhanced by the use of diuretics (15). Because bilirubin is a product of heme catabolism, changes in hemoglobin levels could subsequently influence bilirubin concentrations. Although the use of losartan and irbesartan slightly, but significantly, decreased hemoglobin levels compared with placebo, it did not affect bilirubin levels in either trial. Several studies reported that the use of ACE inhibitors and ARBs reduce erythropoietin and, consequently, hemoglobin levels by blocking the effects of angiotensin II on erythropoiesis (27,28). Because the enzymatic degradation of hemoglobin by HO-1 is known to be the rate-limiting step in the formation of bilirubin (23,29) and not the synthesis of hemoglobin, it is conceivable that small changes in the synthesis of hemoglobin do not affect the formation and levels of bilirubin.

This study has several limitations. First, patients with liver enzymes (ALT, AST) and bilirubin levels >1.5 times the ULN were excluded from participation in both trials, which resulted in a relatively narrow range of bilirubin concentrations (i.e., 0.1–2.1 mg/dL, with a mean value of 0.57 mg/dL in RENAAL and 0.54 mg/dL in IDNT). In earlier cross-sectional studies on the association of bilirubin with DN, bilirubin levels were higher, with values of 1.4 (1.3–1.6) mg/dL in subjects with Gilbert syndrome in the study of Inoguchi et al. (6), and 0.71 ± 0.21 mg/dL in subjects with type 2 diabetes in the study of Fukui et al. (8). In a prospective study on development and progression of albuminuria in patients with type 2 diabetes of Mashitani et al. (30), mean bilirubin levels were 0.63 ± 0.28 mg/dL. Within the relatively small range of bilirubin levels in the current study, we could not identify a non-linear component in the association of bilirubin with the renal end point. A larger range of bilirubin concentrations in future studies might allow for identification of a cutoff value of bilirubin above which the association with progression of renal function might flatten, which could help to identify an optimal target concentration for bilirubin in intervention trials. Second, in both RENAAL and IDNT, only total bilirubin was measured. Direct (conjugated) bilirubin was not measured separately because serum bilirubin consists for >95% of indirect (unconjugated) bilirubin (26), and subjects with total bilirubin levels >1.5 times the ULN were excluded from participation in both trials. Therefore, examining differences between un-

conjugated and conjugated bilirubin levels was not possible. Furthermore, given the observational nature of this study and the inability to focus on the HO-1 system and its by-products in more detail, it is impossible to draw a definitive conclusion about the causality of bilirubin and progression of DN. Mendelian randomization has been proposed as a method that enables estimation of causal relationships in observational studies (31,32). This method uses genotype to estimate causal relationships between a gene product and physiological outcomes (32). Because there is a strong relation between UGT1A1 (genotype) and bilirubin levels (phenotype) (32), mendelian randomization can be used to establish a possible causal relation between bilirubin and DN. The strengths of this study are the large sample size, the large number of renal events, and the independent replication of the current findings in another large cohort of >1,700 subjects.

In conclusion, the results show an independent inverse association of bilirubin levels with progression of nephropathy in patients with type 2 diabetes, suggesting that measurement of bilirubin may identify subjects at risk for renal disease progression. In addition, the study suggests a protective effect of bilirubin against progression of DN, thereby potentially implying its role as an antioxidant.

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Supplementary Table S1. Inclusion and exclusion criteria for the RENAAL and IDNT trials.

	RENAAL	IDNT
Inclusion criteria		
Age range (years)	31 – 70	30 – 70
Diagnosis of Type 2 Diabetes	Yes	Yes
Diabetic Nephropathy		
Proteinuria/albuminuria	ACR ≥ 300m/g or 24h urinary protein excretion > 500 mg	24h urinary protein excretion ≥ 900 mg
SCr	Women: SCr 1.3 – 3.0 mg/dL Men: SCr 1.5 – 3.0 mg/dL	Women: SCr 1.0–3.0 mg/dL Men: SCr 1.2–3.0 mg/dL
Hypertension	Hypertension and seated SBP/DBP ≤ 200/110 mmHg or normotension (SBP ≥ 100 mmHg)	Seated SBP >135 mmHg and/or seated DBP > 85 mmHg or receiving antihypertensive medication
Exclusion criteria		
Other diseases	History of non-diabetic renal disease or type 1 diabetes	Age onset type 2 diabetes < 20 years or diagnosis of type 1 diabetes
Concomitant therapies	Patients with absolute requirements for ACEI or ARB	Patients with absolute requirements for ACEI, calcium antagonists or ARB
Cardiovascular disease	• History of MI or CABG within past month of study entry • CVA or PTCA within past 6 months • TIA within past 12 months • History of HF	• Unstable AP, MI, CABG, PTCA, or CVA within past 3 months of study entry • TIA within past 6 months • History of HF (class III or IV NYHA)
Years of conduct of the trials	1996 – 2000	1996 – 2000

Abbreviations: ACR, urinary albumin-to-creatinine ratio; AP, Angina Pectoris; CABG, coronary artery bypass grafting; CVA, cerebrovascular accident; DBP, diastolic blood pressure; HF, heart failure; MI, myocardial infarction; NYHA, New York Heart Association; PTCA, percutaneous transluminal coronary angioplasty; SBP, systolic blood pressure; SCr, serum creatinine; TIA, transient ischemic attack.

Supplementary Table S2. Time course of main patient characteristics of all subjects in the RENAAL and IDNT trials and stratified by tertiles according to baseline bilirubin concentrations.

		RENAAL								IDNT							
	Time	All subjects		Tertile 1		Tertile 2		Tertile 3		All subjects		Tertile 1		Tertile 2		Tertile 3	
Variable	(Month)	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
Bilirubin	Baseline	1498	0.57	374	0.38	776	0.54	348	0.84	1707	0.54	588	0.34	413	0.50	706	0.72
(mg/dL)	12	1165	0.53	280	0.39	609	0.51	276	0.71	1450	0.52	479	0.38	360	0.48	611	0.66
	24	952	0.49	207	0.36	498	0.47	247	0.64	1157	0.50	350	0.36	282	0.47	525	0.61
	36	599	0.48	106	0.37	314	0.44	179	0.62	613	0.47	163	0.30	151	0.43	299	0.58
	48	162	0.48	20	0.39	83	0.44	59	0.56	218	0.47	63	0.31	53	0.44	102	0.59
SBP	Baseline	1498	153	374	152	776	153	348	151	1707	159	588	160	413	159	706	159
(mmHg)	12	1317	147	321	149	685	148	311	146	1486	143	491	143	370	142	625	144
	24	1087	144	237	146	577	144	273	142	1149	142	340	142	282	141	527	142
	36	615	142	101	142	331	142	183	140	616	141	161	142	150	140	305	141
	48	96	142	11	141	47	142	38	142	205	139	53	142	51	137	101	139
DBP	Baseline	1498	82	374	82	776	82	348	83	1707	87	588	86	413	87	706	88
(mmHg)	12	1317	79	321	80	685	79	311	80	1486	79	491	77	370	79	625	80
	24	1087	77	237	79	577	77	273	77	1148	78	339	77	282	78	527	79
	36	615	75	101	76	331	75	183	76	616	78	161	77	150	78	305	78
	48	96	74	11	73	47	74	38	74	204	76	53	76	51	76	100	75
HbA1C	Baseline	1490	8.5	371	8.8	773	8.5	346	8.2	1553	8.1	553	8.1	368	8.3	632	8.1
(%)	12	1160	8.6	276	8.9	608	8.6	276	8.3	1453	8.3	480	8.3	360	8.6	613	8.2
	24	936	8.6	202	9.0	490	8.6	244	8.2	1145	8.2	342	8.2	277	8.6	526	8.1
	36	584	8.4	104	8.8	305	8.5	175	8.2	619	8.2	165	8.1	152	8.4	302	8.1
	48	159	8.4	19	8.2	81	8.5	59	8.2	214	8.1	61	8.2	52	8.0	101	8.0

Supplementary Table S2. (continued)

		RENAAL								IDNT							
	Time	All subjects		Tertile 1		Tertile 2		Tertile 3		All subjects		Tertile 1		Tertile 2		Tertile 3	
Variable	(Month)	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
HbA1C	Baseline	1490	69	371	73	773	69	346	66	1553	65	553	65	368	68	632	65
(mmol/mol)	12	1160	70	276	73	608	70	276	67	1453	67	480	67	360	70	613	66
	24	936	70	202	75	490	71	244	66	1145	67	342	66	277	70	526	65
	36	584	69	104	72	305	69	175	66	619	66	165	65	152	69	302	65
	48	159	68	19	66	81	69	59	67	214	65	61	66	52	64	101	64
Cholesterol	Baseline	1498	228	374	244	776	226	348	216	1564	228	558	239	371	229	635	218
(mg/dL)	12	1165	221	280	233	609	219	276	212	1445	220	479	225	359	223	607	214
	24	952	210	207	221	498	209	247	203	1149	210	348	209	282	209	519	210
	36	600	202	107	215	314	202	179	194	613	203	163	206	150	206	300	200
	48	162	192	20	210	83	195	59	183	216	197	63	206	51	201	102	190
SCr	Baseline	1498	1.9	374	1.9	776	1.9	348	1.8	1698	1.7	584	1.8	412	1.7	702	1.6
(mg/dL)	12	1267	2.3	304	2.6	667	2.3	296	2.1	1453	2.0	480	2.4	361	2.0	612	1.8
	24	1064	2.5	226	2.8	569	2.6	269	2.3	1160	2.2	351	2.5	283	2.2	526	2.0
	36	607	2.7	99	3.1	330	2.7	178	2.6	614	2.3	163	2.7	150	2.3	301	2.1
	48	94	2.9	11	2.8	47	3.2	38	2.7	218	2.4	63	2.8	53	2.4	102	2.3

DBP, diastolic blood pressure; SBP, systolic blood pressure; SCr, serum creatinine.

Supplementary Table S3. Mean annual change in total bilirubin concentrations in subjects with complete bilirubin measurements at baseline, 12, 24, and 36 months in RENAAL (n = 553) and IDNT (n = 587).

		RENAAL				IDNT			
	Time (Months)	Tertile 1	Tertile 2	Tertile 3	*P*-value	Tertile 1	Tertile 2	Tertile 3	*P*-value
Δ Bilirubin (mg/dL)	0–12	0.061	-0.018	-0.126	<0.001	0.068	0.026	-0.026	<0.001
	12–24	-0.067	-0.055	-0.071	0.5	-0.039	-0.042	-0.063	0.3
	24–36	0.002	-0.035	-0.026	0.2	-0.070	-0.049	-0.062	0.6