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Plasma bilirubin and late graft failure in renal transplant recipients

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The authors declare no conflict of interest.

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Summary

Exogenous bilirubin has been shown to protect against oxidative stress in ischemia-reperfusion injury. Oxidative stress has been implicated in the pathophysiology of chronic transplant dysfunction leading to late graft failure after renal transplantation. We prospectively investigated whether high endogenous bilirubin is protective against development of late graft failure in renal transplant recipients (RTR). Baseline data were collected between August 2001 and July 2003 in nonicteric outpatient RTR with a functioning graft for >1 year. At baseline, bilirubin and liver enzymes were measured using routine assays on a Merck Mega analyzer. Graft failure was prospectively recorded until May 19 2009. During follow-up for 7.1[6.2–7.2] years, 55 RTR developed graft failure. We found that circulating levels of bilirubin are inversely associated with late graft failure in RTR (HR = 0.29 [95% CI: 0.16–0.52], $P < 0.001$). This association was independent of potential confounders, including creatinine clearance, urinary protein excretion, calcineurin inhibitors, and gender (HR = 0.31 [95% CI: 0.15–0.62] $P = 0.001$). Our findings are consistent with a protective effect of increased endogenous bilirubin against development of late graft failure in RTR. If our findings are confirmed by other studies, intervention with endogenous or exogenous bilirubin may be of interest for long-term preservation of renal function in RTR.

Introduction

Short-term outcome of renal transplantation has improved impressively during the last decades [1]. However, beyond 1 year after transplantation, outcome is still poor because many renal transplant recipients (RTR) experience a slow, but relentless decline in graft function [2–5]. This will ultimately lead to graft failure, necessitating renewal of dialysis or retransplantation [2–5]. Frequently, the slow decline in graft function is not the consequence of immunological rejection, but an entity that is called chronic transplant dysfunction [3]. The exact pathophysiology is not known, but oxidative stress has been implicated [6–9]. Endogenous bilirubin is an established cytoprotectant and antioxidant [10–12]. In experimental studies, it has been shown that exogenous bilirubin is protective against oxidative stress after ischemia-reperfusion injury in the kidney [13]. It is, however, not known whether endogenous levels of bilirubin influence long-term renal outcome in RTR. Because oxidative stress has been implicated a detrimental role in the process of chronic transplant dysfunction [6–9], we hypothesized that high circulating bilirubin concentrations protect against late graft failure in RTR. We prospectively investigated this hypothesis with a large single-center cohort of RTR.

Patients and methods

To investigate whether high circulating bilirubin concentrations protect against late graft failure, we collected data in nonicteric outpatient RTR with a functioning graft for >1 year. Baseline data were collected between August 2001 and July 2003 at a median of 6 years after transplantation. All 603 patients available for analysis signed writ-

ten informed consent. Approval for this study has been obtained by the Institutional Review Board (METc 2001/039). Detailed explanation of this study has been published before [14–16]. Creatinine clearance was assessed using the Jaffé method on a MEGA AU 510 (Merck Diagnostica, Darmstadt, Germany) and calculated using the Cockcroft–Gault formula. Concentration of total endogenous bilirubin was measured using the Bilirubin DPD method on a MEGA (Merck Diagnostica). In our laboratory, a bilirubin concentration lower than 17 μmol/l is considered normal.

Standard immunosuppressive therapy was given as described previously [14]. Delayed graft function was defined as oliguria for more than 6 days. Allograft rejections were biopsy proven. To assess change in renal function over time, difference between creatinine clearance at baseline and latest creatinine clearance up to 4 years beyond baseline was calculated. All participating subjects visited the outpatient clinic at least once a year and creatinine clearance was assessed at every visit. Graft failure was defined as the return to dialysis or retransplantation and was censored for death. Graft failure and mortality of all RTR were prospectively recorded until May 19, 2009. There was no loss to follow-up.

Statistical calculations were performed using SPSS (version 18.0, SPSS Inc. Chicago, IL). Total bilirubin concentration was divided into sex stratified tertiles. Normally distributed data are given as mean ± SD, whereas skewed distributed data are expressed as median (interquartile range), categorical distributed variables are given as number (percentage). Skewed variables were normalized using logarithmic transformation. Calculations have been made using ANOVA for normally distributed variables, Chi-square test for categorical distributed variables and a Kruskal–Wallis test in the case of a skewed distribution. Initial survival analyses were performed according to Kaplan–Meier using log-rank testing. To determine whether bilirubin concentrations were independently associated with outcome, we subsequently performed cumulative adjustment for potential confounders in multivariate Cox-regression analyses. A probability value of <0.05 was considered significant.

Results

In 603 RTR [age 51 ± 12 years, 55% men], median [IQR] baseline bilirubin concentration was 17 [14–21] μmol/l. Bilirubin concentration was significantly higher in men than in women ($P < 0.001$). In women, median bilirubin concentration was 16 [13–20] μmol/l. In men, median bilirubin concentration was 18 [15–23] μmol/l. In 318 (53%), RTR bilirubin concentration was above the range considered normal for the general population. However, none of the included RTR was icteric or suffered from severe liver disease. Median time between transplantation and baseline measurements was 6.0 [2.6–11.4] years. In Table 1, recipient and donor characteristics are shown according to sex stratified tertiles of bilirubin concentration. Bilirubin concentration was inversely associated with use of ACE inhibitors or angiotensin II antagonists, cerebrovascular accidents, HbA1c, serum creatinine, and urinary protein and sodium excretion. Bilirubin concentrations were positively associated with ASAT, ALAT, and use of calcineurin inhibitors.

Decline in serum creatinine clearance was least pronounced in RTR with highest bilirubin concentrations (Fig. 1, $P < 0.001$). In line with this observation, graft failure was less frequent in RTR with high bilirubin concentrations than in RTR with lower bilirubin concentrations, with frequencies of 16, 10, and 2% in the respective tertiles after a median follow-up of 6.9 [6.1–7.4] years (log-rank $P < 0.001$). A Kaplan–Meier analysis of graft failure is show in Fig. 2a. Estimated 5-year graft survival was 88% in the first, 92% in the second, and 98.5% in the third tertile of bilirubin distribution. Mortality rates were similar in all three tertiles (log-rank $P = 0.70$). A Kaplan–Meier analysis of mortality is shown in Fig. 2b. Estimated 5-year patient survival was 84% in the three tertiles of bilirubin distribution.

In Cox-regression analyses for bilirubin as a continuous variable, the inverse association of bilirubin concentration with late graft failure was confirmed, with a hazard ratio (HR) of 0.29 [95% CI: 0.16–0.52], $P < 0.001$ for the unadjusted analysis (Table 2). After cumulative adjustment in multivariate analyses, this association appeared independent of potential confounders, including creatinine clearance, urinary protein excretion, use of calcineurin inhibitors, and gender, with a HR of 0.31 [95% CI: 0.15–0.62], $P = 0.001$ for the final model.

Discussion

In this prospective study, we investigated whether endogenous bilirubin assessed in stable, outpatient RTR at more than 1 year after transplantation is associated with subsequent development of late graft failure. We found that relatively high circulating levels of bilirubin are independently associated with low risk of late graft failure in RTR.

It has been shown that oxidative stress is important in the pathophysiology of chronic transplant dysfunction [6–9]. Chronic transplant dysfunction is the leading cause of a slow, but progressive decline in renal allograft function [3]. It is thought that chronic transplant dysfunction is usually not the result of immunological rejection, but of a constant ''response to injury'' because of a combination of several immunological and nonimmunological insults [3,17]. In the first year after trans-

Table 1. Demographic characteristics according to sex stratified tertiles of bilirubin.

	Tertiles of serum bilirubin concentration			
	I	II	III	*P*
N	181	218	204	
Men, *n* (%)	101 (56)	116 (53)	113 (55)	
Serum bilirubin (μmol/l)	13 (6–15)	17 (16–20)	25 (21–62)	
Women, *n* (%)	80 (44)	102 (47)	91 (45)	
Serum bilirubin (μmol/l)	12 (7–13)	15 (14–18)	22 (19–47)	
Recipient demographics				
Age (yr)	49.8 ± 11.4	51.9 ± 12	52.3 ± 12.7	0.10
Body composition				
BMI (kg/m^2)	25.8 ± 4.1	25.8 ± 4.2	25.4 ± 4.6	0.51
Waist circumference (cm)	96.7 ± 13.5	97.6 ± 13.5	97.1 ± 14.2	0.78
Smoking status				
Nonsmoker, *n* (%)	47 (26)	77 (36)	91 (45)	0.08
Current smoker, n (%)	63 (35)	44 (20)	25 (12)	
Ex-smoker, *n* (%)	10 (39)	97 (45)	87 (43)	
Blood pressure				
Systolic (mmHg)	151.0 ± 22.1	156 ± 25.9	151.5 ± 19.4	0.06
Diastolic (mmHg)	89.6 ± 10.2	89.7 ± 9.9	90.4 ± 9.6	0.72
Use of ACE-I or AIIa, *n* (%)	78 (43)	78 (43)	50 (25)	0.001
Number of antihypertensives	1.9 ± 1.1	2.0 ± 1.2	1.8 ± 1.1	0.08
Lipids				
Total cholesterol (mmol/l)	5.6 ± 1.2	5.6 ± 1.1	5.6 ± 0.9	0.88
HDL (mmol/l)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.30
LDL (mmol/l)	3.5 ± 1.1	3.6 ± 1.0	3.5 ± 0.9	0.80
Triglycerides (mmol/l)	2.2 ± 1.3	2.2 ± 1.2	2.1 ± 1.2	0.74
Use of statin, *n* (%)	88 (49)	106 (49)	105 (52)	0.80
Diabetes Mellitus				
Insulin (μU/ml)	13.6 (8.6–16.3)	10.9 (7.8–16.9)	10.8 (7.7–14.8)	0.26
Glucose (mmol/l)	4.7 (4.2–5.1)	4.5 (4.1–5)	4.5 (4–4.9)	0.30
Diabetes after Tx, *n* (%)	31 (17)	38 (17)	37 (18)	0.96
Use of antidiabetic drugs, *n* (%)	26 (14)	29 (13)	24 (12)	0.75
CRP (mg/l)	2.7 (0.8–6.5)	2.1 (0.9–4.6)	1.8 (0.7–4.0)	0.15
Liver function				
ASAT (U/l)	21 (17–25)	22 (19–27)	24 (20–29)	<0.001
ALAT (U/l)	17 (13–23)	19 (14–25)	18 (15–25)	0.01
Alkaline phosphatase (U/l)	72 (57–90)	75 (58–98)	71 (56–94)	0.34
Gamma glutamyl transferase (U/l)	23 (16–36)	25 (17–40)	24 (18–41)	0.27
CMV status				
CMV seropositivity, *n* (%)	119 (66)	165 (76)	146 (72)	0.09
Donor demographics				
Age (yr)	38 (16)	37 (15)	36 (15)	0.51
Men, *n* (%)	98 (54)	122 (56)	107 (53)	0.80
Number of HLA mismatches,	2 (1–2.5)	2 (0–3)	2 (1–3)	0.79
Time between Tx and baseline (yr)	6.1 (3–12)	5.6 (3–12)	5.9 (3–11)	0.38
Dialysis duration prior to Tx (mo)	26 (14–47)	27 (15–49)	28 (11–51)	0.40
Renal allograft function				
Serum creatinine (μmol/l)	141 (112–178)	136 (113–165)	128 (112–149)	0.009
Creatinine clearance (ml/min)	60.1 ± 23.1	62.5 ± 22.9	63.4 ± 21.5	0.34
Urinary protein excretion (g/24 h)	0.3 (0.1–0.6)	0.2 (0.0–0.5)	0.2 (0.0–0.4)	<0.001
Transplantation type				
Postmortem donor, *n* (%)	149 (82)	189 (87)	183 (90)	0.11
Living donor, *n* (%)	32 (18)	29 (13)	21 (10)	0.11
Acute rejection, *n* (%)	92 (51)	86 (39)	93 (46)	0.07
Delayed graft function, *n* (%)	28 (16)	25 (12)	22 (11)	0.36

Table 1. continued

	Tertiles of serum bilirubin concentration			
	I	II	III	*P*
Immunosuppression				
Prednisolon dose (mg)	10 (7.5–10)	10 (7.5–10)	10 (7.5–10)	0.75
Use of calcineurin inhibitor, *n* (%)	122 (67)	177 (81)	174 (85)	<0.001
Use of ciclosporin, *n* (%)	83 (46)	147 (67)	158 (77)	<0.001
Use of tacrolimus, *n* (%)	39 (22)	30 (14)	16 (8)	0.001
Use of proliferation inhibitor, *n* (%)	143 (79)	160 (73)	142 (70)	0.11

BMI, body mass index; ACE-I, ACE inhibitor; AIIa, Ang II antagonist; CRP, C-reactive protein; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; CMV, cytomegalovirus; Tx, transplantation.
Normally distributed data are given as mean ± SD, skewed distributed data as median (interquartile range) and categorical distributed variables as number (percentage).

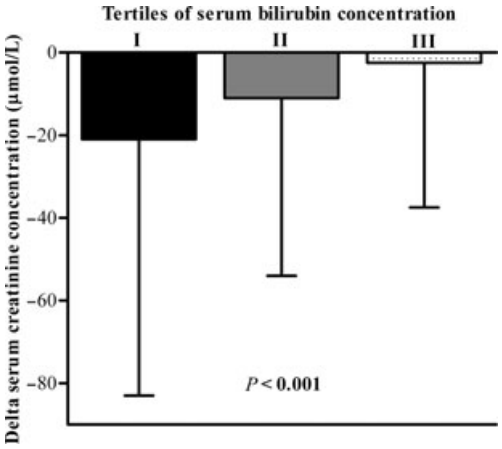

Figure 1 Changes in creatinine clearance (mean ± SEM) between baseline and follow-up according to sex stratified tertiles of serum bilirubin. Differences between groups were tested with Kruskal–Wallis test.

plantation, transplant dysfunction is predominantly caused by immunological factors [14], whereas after 1 year chronic transplant dysfunction is mainly attributed to nonimmunological risk factors, with many aspects of the pathophysiology shared with diabetic nephropathy [14]. Recent studies suggest a protective effect of relatively high levels of bilirubin against risks imposed by such nonimmunological risk factors. One is a study in which bilirubin was inversely associated with dyslipidemia in patients with metabolic syndrome [18]. The other study indicated that bilirubin may protect against progression of diabetic nephropathy [19], showing that bilirubin significantly inhibited reactive oxygen species production in human renal mesangial cells [19]. In line with this, diabetic hyperbilirubinemic rats did not develop renal mesangial expansion, a typical feature in diabetic nephropathy. Furthermore, Adin *et al.* [13] showed that exogenous bilirubin protected against kidney ischemia reperfusion injury.

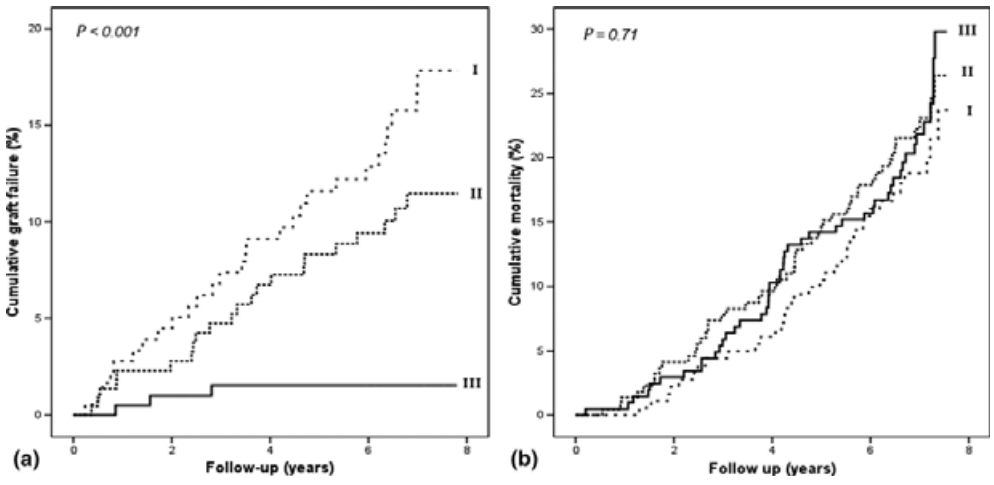

Figure 2 (a) Kaplan–Meier analysis for graft failure ($P < 0.001$) according to sex stratified tertiles of bilirubin concentration (I–III). (b) Kaplan–Meier analysis for survival ($P = 0.7$), according to sex stratified tertiles of bilirubin concentration (I–III).

Table 2. Cox regression analyses for late graft failure in RTR in sex stratified tertiles of bilirubin concentration.

	Bilirubin	
Model	HR [95% CI]*	*P*
Model 1	0.29 [0.16–0.52]	<0.001
Model 2	0.28 [0.15–0.51]	<0.001
Model 3	0.33 [0.18–0.61]	<0.001
Model 4	0.32 [0.17–0.61]	0.001
Model 5	0.35 [0.18–0.67]	0.001
Model 6	0.31 [0.15–0.62]	0.001

RTR, renal transplant recipients; HR, hazard ratio.
*Per 10 μmol/l change in bilirubin concentration.
Model 1: crude association for bilirubin.
Model 2: Model 1 + adjustments for age and gender.
Model 3: Model 2 + adjustments for creatinine clearance and urinary protein excretion.
Model 4: Model 3 + adjustments for use of calcineurin inhibitor, use of Alla or ACE-I, number of antihypertensives.
Model 5: Model 4 + adjustments for ASAT, ALAT.
Model 6: Model 5 + adjustments for CMV seropositivity, smoking status, systolic blood pressure, acute rejection.

Accumulating evidence has shown that higher bilirubin levels are associated with low prevalence of death and disease as high serum bilirubin levels were suggested to be protective against coronary artery disease [20], cancer mortality [21], and colorectal cancer [22]. But to our knowledge, we are the first to report that relatively high circulating levels of bilirubin are associated with low risk of late graft failure in RTR. In line with this association, we found that the decline in creatinine clearance was least pronounced in RTR with highest bilirubin concentrations.

Accumulating evidence implicates bilirubin as a powerful antioxidant [10,23,24]. Bilirubin has been shown to protect against kidney ischemia/reperfusion injury in rats [13]. It has also been shown that bilirubin might modulate immunological factors [23,25]. High concentrations of biliverdin, a precursor of bilirubin, downregulate several inflammatory pathways, hereby inducing tolerance to cardiac allografts in mice [25]. Protective effects in organ transplantation may include anti-inflammatory, antiapoptotic, and antiproliferative properties [23]. Thus, it may not only be nonimmunological antioxidative effects of bilirubin that explain our findings, but also effects that involve modulation of the immune system, with less pronounced chronic rejection in subjects with higher circulating concentrations of bilirubin.

This study has some limitations. Despite the fact that bilirubin has been implicated as protective against ischemia-reperfusion injury [13], we found no association of circulating bilirubin concentrations with occurrence of delayed graft function. It should, however, be realized that our study was designed to prospectively investigate the hypothesis that circulating endogenous bilirubin concentrations are associated with late graft failure and that assessment of bilirubin for our study was performed 1 year or more after transplantation. A prospective study of a potential association with occurrence of delayed graft function would have required assessment of pre- or perioperative concentrations of bilirubin. We found that bilirubin concentrations above what is considered normal for the general population are quite common late after transplantation. This may indicate preferential occurrence of delayed graft failure and early graft failure in RTR with constituently low bilirubin concentrations. In future studies it would therefore be interesting to investigate the potential association of bilirubin concentrations assessed in the pre- or perioperative phase with occurrence of delayed graft function and early graft failure. We did not record data on extended criteria donors, but such donor data, known to influence occurrence of delayed graft function of recipient characteristics would be important to be recorded to allow for adjustment as potential confounders in multivariate analyses in such studies. Other limitations are that this study was set up as a single-center study, so differences with other centers have not been assessed. Also, RTR patients were included at different time points after transplantation (2001–2003), which might have caused healthy survivor bias. Another limitation is that we have no repeated measurements of bilirubin concentrations. Most epidemiological studies use a single baseline measurement to predict outcomes, which adversely affects predictive properties of variables associated with outcomes. If intraindividual variability of predictive parameters is taken into account, this results in much stronger relations with outcomes [26,27]. A particular strength of this study is that there was no loss to follow-up.

Our study suggests a protective effect of endogenous bilirubin against development of graft failure in RTR, hereby implying its potential role as an antioxidant. If our findings are confirmed by other studies, and a mechanistic role supported, intervention with exogenous bilirubin may be of interest for long-term preservation of renal function in RTR. Given the results of our study, and particularly because we found relatively high bilirubin concentrations in RTR with preserved graft function until relatively late after transplantation, it would also be of interest to address in future studies whether pre- or perioperative bilirubin concentrations or bilirubin concentrations shortly after transplantation would be associated with delayed graft function or early graft loss.

Authorship

PED: analyzed the data and performed manuscript preparations. DMZ: helped with analyzing the data and

drafting the manuscript. JJHH: participated in subject care and manuscript revisions. GJN: participated in intellectual contributions and manuscript revisions. ROBG: participated in protocol development and manuscript revisions. SJLB: initiated the study, performed study planning, supervised data collection, and participated in subject care and manuscript revisions. All authors commented on drafts and all authors have seen and approved the final version.

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