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Circulating bilirubin and defense against kidney disease and cardiovascular mortality: mechanisms contributing to protection in clinical investigations

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BILIRUBIN IS PRODUCED IN THE reticuloendothelial system, as the end product of heme catabolism, derived from the oxidation of heme from hemeoproteins. Heme is degraded to form biliverdin and iron by heme oxygenase (HO) (68, 79, 182). Biliverdin is then reduced by biliverdin reductase to unconjugated bilirubin by macrophages of the spleen, bone marrow, and hepatic Kupffer cells (Fig. 1) (45, 182). Unconjugated bilirubin (UCB) possesses potent antioxidant (50, 156, 178), anti-inflammatory (80, 181, 197), complement inhibitory (5, 7), and possibly lipid-lowering properties (14, 20, 181). These properties are contrasted by the commonly accepted neurotoxic properties of bilirubin and support an emerging hypothesis that bilirubin is a physiologically important, potentially essential molecule in human physiology (164, 191). Furthermore, large epidemiological studies indicate that mildly elevated bilirubin concentrations may protect against all-cause mortality and cardiovascular disease (CVD) (31, 73, 75, 165, 177). These findings extend to protection from renal dysfunction/damage (25, 145) and also to protection from mortality in patients undergoing hemodialysis, which may be explained by bilirubin’s cardiovascular-protective effects (Fig. 2) (24).

Studying individuals with benign hyperbilirubinemia (Gilbert’s syndrome; GS) has greatly aided the exploration of bilirubin’s physiological protective effects. GS affects 3–18% of the population and is caused by a mutation in the gene promoter for bilirubin uridine glucuronyl transferase 1A1 (UGT1A1), which decreases hepatic bilirubin conjugation and, therefore, biliary excretion, increasing the concentration of circulating unconjugated pigment (44, 68). GS individuals are rarely jaundiced and are most clearly protected from CVD (89, 97, 98, 119). GS individuals can experience mild jaundice (particularly when dehydrated, suffering illness, stressed, etc.) and can report bilirubin concentrations of up to 100 μM (178). Indeed, having GS was recently shown to reduce the incidence of all-cause mortality by ~50% (72). Possible mechanisms of action that could explain protection from renal and CVD in GS are discussed under the following subheadings and are summarized in Fig. 2.

Although mildly elevated bilirubin is associated with reduced mortality rates, grossly elevated UCB is associated with the development of encephalopathy in infants. For example, a severe, chronic and nonhemolytic unconjugated hyperbilirubinemia (serum bilirubin levels of 15–38 mg/dl) is experienced in individuals with Crigler-Najjar syndrome (CNS). Patients with CNS type 1 have complete loss of UGT1A1 activity and can report bilirubin concentrations of up to 100 μM (178).
The pathological phenotype of jaundice develops in these patients due to excessive bilirubin accumulation and increases the risk of neurological dysfunction occurring, which is associated with neuronal apoptosis (83). Jaundice commonly occurs in neonates with hepatic immaturity, Rh incompatibility, or due to the replacement of fetal hemoglobin, delay in clearing meconium, and impaired conjugation of bilirubin during the transitional period after birth (151). Most importantly, bilirubin neurotoxicity can only occur in adults if an imbalance between bilirubin production and elimination occurs, such as in CNS type 1 (84, 151). In the presence of severe hyperbilirubinemia, limited options exist to chronically lower bilirubin concentrations (e.g., liver transplant). However, treatments including phototherapy and exchange transfusion both achieve rapid and clinically significant reductions in the short term, with the potential to prevent bilirubin encephalopathy in infants (2, 77).

Potential Protective Effects of Bilirubin

Antihypertensive effects. Bilirubin has demonstrated antihypertensive effects in animal models. For example, HO-1 knockout mice with superimposed uninephrectomy and DOCA administration developed systolic hypertension, whereas HO-1-competent mice remain normotensive (115). The DOCA-salt hypertensive model decreases circulating plasma renin and increases concentrations of aldosterone and thus induces excessive reabsorption of sodium and water from the proximal convoluted tubule, resulting in hypertension (168). Whether bilirubin per se was responsible for protection from hypertension was then explored using the same technique in Gunn rats (hyperbilirubinemic, UGT1A1 mutant) and littermate controls (115). Although both Gunn rats and littermates were subjected to uninephrectomy and DOCA administration, the Gunn rat model was selected for investigation because Gunn rats are used as a model of severe hyperbilirubinemia and mild, chronic hypertension (115). Bilirubin concentrations in Gunn rats were higher than those in littermate controls (14, 18, 178). Elevated bilirubin concentrations in Gunn rats attenuated the increase in systolic pressure caused by uninephrectomy and DOCA administration by ~50%. Hypertension-induced kidney damage and proteinuria in wild-type animals was almost completely attenuated in Gunn rats (115). Therefore, in this model of salt overload, protection from kidney damage appeared secondary to bilirubin’s hypotensive action. These effects might arise from bilirubin’s ability to neutralize reactive oxygen species (ROS), including the superoxide anion (116), which interacts with nitric oxide (NO) to form peroxynitrite, therefore inhibiting the ability of NO to induce vasodilation (127). After uninephrectomy and DOCA administration, Gunn rats produced significantly less ROS than controls. Therefore, bilirubin could increase NO bioavailability (see Bilirubin and endothelial dysfunction) by neutralizing superoxide and improving flow-mediated dilatation (FMD) in salt-overload models of hypertension.

Gunn rats are also resistant to angiotensin (ANG II)-induced hypertension (132, 150, 174, 175). Systemic blood pressure in Gunn rats was reduced by 50% compared with controls after chronic ANG II administration. Moreover, endothelial-dependent vasorelaxation in aortic rings was preserved in Gunn rats, suggesting that bilirubin’s superoxide-scavenging ability increased NO bioavailability (132). These findings are supported by animal data that indicate moderate hyperbilirubinemia induced by indinavir (a UGT1A1 inhibitor) administration attenuates ANG II-dependent hypertension (174). Indinavir administration or bilirubin infusion significantly lowered mean arterial pressure and increased plasma nitrate/nitrite concentrations after ANG II administration. Moderate hyperbilirubinemia also restores glomerular filtration rate (GFR) and renal blood flow in ANG II-dependent hypertensive mice (175). In addition, urinary protein excretion was significantly lower in bilirubin-treated ANG II-infused rats (93). Hence moderate hyperbilirubinemia lowers vascular resistance and improves renal hemodynamics, indicating bilirubin exerts both antihypertensive and...
renoprotective effects in ANG II-treated animal models (150). Interestingly, these studies are also complemented by evidence to suggest bilirubin accumulation in the myocardium in otherwise healthy aged animals is associated with a reduced rate of ventricular pressure development in ex vivo perfused Gunn rat hearts (Bakrania B, Du Toit E, Wagner KH, Headrick JP, Bulmer AC, unpublished observations). This effect was compensated for in vivo, as demonstrated by equivalent ventricular pressure development via Millar catheterization in Gunn and control animals. However, aortic pressure development remained depressed in Gunn rats, with reduced rates of pressure development and evidence of mild aortic luminal dilatation during systole and diastole. Together, these data suggest that bilirubin influences both vascular and cardiac function, which lead to reductions in absolute vascular pressures (in disease models) and rate of vascular pressure development (in healthy animals).

Human studies also demonstrate inverse relationships between serum bilirubin concentration and blood pressure. The Bogalusa Heart Study reported a positive correlation between bilirubin and radial artery pulse pressure (large- and small-artery compliance) (10). A marked delay in the development of increased carotid intima-media thickness (CIMT) is also reported in hyperbilirubinemic subjects (179). These results were supported by increased CIMT in individuals with lower serum bilirubin levels (40). These in vivo effects may be explained by the antioxidant properties of bilirubin and biliverdin, which inhibit NADPH oxidase-dependent proliferation and migration of vascular smooth muscle cells (VSMC) in vitro. Moraes et al. (112) also showed that the endogenous HO-derived by-products (bilirubin, biliverdin, and carbon monoxide) suppressed the prooxidant effect of heme and the proliferation of VSMC via modulation of NADPH oxidase activity. Moreover, higher serum bilirubin concentrations are also independently and positively correlated with improved FMD in overweight Japanese patients (197), and a negative relationship between bilirubin levels and incidence of hypertension is reported in a Korean population (26). In the latter study, subjects with lower bilirubin levels (<1.1 mg/dl) had higher mean arterial pressures and serum creatinine concentrations after adjustment for confounders. During a 15-yr follow-up, the cumulative incidence of hypertension was lower in subjects with higher bilirubin concentrations, especially in women (26). Despite these findings, evidence also exists indicating no relationship between bilirubin and blood pressure. For example, in a trial of age-, gender-, and body mass index (BMI)-matched GS and controls, no significant differences in systolic or diastolic pressures were noted (14). Additional studies also report that serum bilirubin concentration is not related to blood pressure (61). These data likely indicate a subtle effect of bilirubin on blood pressure that can only be detected in highly powered studies.

Antioxidant effects. Oxidative stress is progressively enhanced in patients with chronic kidney disease (CKD), with uremic patients on hemodialysis experiencing a marked increase in circulating oxidative damage (39, 71, 96), which contributes significantly to the pathogenesis and progression of CKD (99). Excessive free radical production results in lipid and protein oxidation (99), alteration of glomerular hemodynamics (186), damage to endothelial/tubular cells, and tubular cell hypertrophy (38, 67, 94). Biomarkers of oxidative stress, including oxidized low-density lipoprotein (oxLDL), protein carbonyls and reduced thiols, malondialdehyde (MDA), and plasma/urinary F2-isoprostanes, all represent sensitive and specific biomarkers for CKD progression and associated CVD mortality (37, 43, 90, 139).

Bilirubin is a potent antioxidant, protecting against the effects of free radical production and, therefore, oxidative damage (14, 18, 140, 153). Reduced glutathione is a thiol-containing antioxidant that provides intracellular protection against oxidative stress and subsequent protein/enzyme damage (21, 35). GS individuals also possess greater circulating reduced glutathione (GSH) concentrations compared with controls, the concentration of which was positively correlated with
UCB concentrations (14). This observation agrees with another report in GS individuals, who possessed an elevated serum reduced thiol concentration (64). Therefore, increased reduced thiol and GSH concentrations in GS indicate that elevated bilirubin protects from thiol oxidation in vivo (14). Vitek et al. (178) also reported significantly elevated total antioxidant status (TAS) in GS compared with patients with ischemic heart disease and controls. More recently, significantly increased circulating antioxidant status (Troxol equivalent antioxidant capacity and ferric-reducing ability plasma) was also reported in individuals with GS (18). Therefore, elevated bilirubin in vivo increases circulating antioxidant capacity and could protect against oxidation reactions in vivo throughout the life span.

Hyperbilirubinemic Gunn rats are also resistant to oxidative injury when exposed to hyperoxia (>95% O2) (36). Blood thiobarbituric acid-reactive substances, serum lipid hydroperoxide, and protein carbonyls were lowered in Gunn rats vs. controls after hyperoxic exposure, and these oxidized products were negatively correlated with serum bilirubin concentration. These data indicate that bilirubin is a functionally important antioxidant in the circulatory compartment of the neonatal Gunn rat, providing an important line of defense in the first few weeks of life. Oh et al. (121) recently demonstrated that administration of bilirubin (60 mg/kg ip) attenuates renal tubular injury after cyclosporine (CsA)-induced nephropathy in rats and in HK2 cells via protection from oxidative stress and apoptosis. In CsA-treated rat kidneys, kidney injury markers (urine kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin) are markedly reduced, and histopathological assessment showed significantly improved afferent arteriopathy, tubulointerstitial fibrosis, and tubular injury compared with the CsA-treated control rats (121). Bilirubin pretreatment inhibited oxidative stress by inactivation of NADPH oxidases (57, 121). These findings support the findings of Kwak et al. (92), who demonstrated that exogenous UCB inhibits superoxide-producing NADPH oxidase in membrane and cytosolic fractions derived from neutrophils and vascular endothelial cell culture (51). Fujii et al. (51) also showed that elevated bilirubin in Gunn rats downregulates NADPH oxidase activity in streptozotocin-induced diabetic nephropathy. Moreover, Oh et al. (121) reported a reduction in intracellular ROS production and inhibition of apoptosis via the upregulation of antiapoptotic protein (bcl-2) and downregulation of proapoptotic bax expression in bilirubin-treated CsA-induced rats.

Protection of LDL from oxidation is of particular relevance to CVD, because it is internalized by macrophages, stimulating a powerful inflammatory response, which might promote atherosclerotic lesion formation (154). Bilirubin neutralizes a number of physiological and synthetic radical species. Stocker and coworkers (152, 155) demonstrated the effectiveness of bilirubin ditaurate, a water-soluble form of bilirubin, in scavenging peroxyl radical [generated by 2,2'-azobis(2-amidinopropane) hydrochloride (AAHP) and 2,2'-azobis(2,4-dimethylvaleronitrile)]-induced oxidation of phosphatidylcholine. Furthermore, bilirubin prevents free radical damage to plasma lipids and inhibits AAHP-induced lipid peroxidation in human blood plasma (50). Mildly elevated bilirubin concentrations in GS reduce the susceptibility of plasma lipids to copper (Cu2+)-induced oxidation compared with control subjects (18, 194). These results are supported by data indicating exogenous UCB prevents the oxidation of human LDL, mediated by Cu2+ (188, 189). In addition, recent studies demonstrate significantly reduced oxLDL concentrations in GS compared with controls, which were negatively correlated to serum bilirubin concentrations (14, 100, 162, 180). Together, these findings suggest that the antioxidant capacity of bilirubin reduces the susceptibility of lipids and lipoproteins to oxidation in vivo and indicate that GS individuals may be protected against atherosclerosis and subsequently CVD by the oxidation modification hypothesis (154).

The effects of hemodialysis are complex and include the removal of circulating antioxidants, including uric acid and ascorbate, from the aqueous phase of plasma, leaving proteins vulnerable to oxidation (29). Bilirubin is retained in the circulation, due to its affinity for circulating albumin and therefore remains free to react with radicals generated in plasma (50). Although markers of oxidative stress were not measured, Chen et al. (24) showed that patients on hemodialysis with higher bilirubin (>0.99 mg/dl) had a significantly reduced incidence of cardiovascular events and all-cause mortality compared with patients with normal and low circulating bilirubin. In this situation, it is likely that bilirubin is one of the few low-molecular-weight antioxidants available to quench free radicals within the vasculature. Due to constant formation of bilirubin in vivo and reduced clearance, particularly in the presence of UGT1A1 deficiency, it is likely that bilirubin plays a vital role in protecting the vasculature and circulating blood components from oxidation, particularly in hemodialysis patients. Indeed, bilirubin concentrations are reduced in hemodialysis patients, likely indicating that bilirubin may represent a sacrificial antioxidant in these patients (190).

Protection from ischemia-reperfusion injury and graft rejection. Ischemia-reperfusion (I/R) injury is a consequence of restoration of blood supply after tissue ischemia (e.g. organ transplantation), which leads to immune system activation, endothelial cell apoptosis, inflammation, and deterioration of organ function (15, 124). Endogenously elevated bilirubin or exogenously administered UCB/biliverdin protects from I/R injury in the heart (28, 95, 113), intestine (22, 65, 114), lung (157), brain (87), liver (85, 192), and kidney (1, 86, 113) via antioxidant, anti-inflammatory, and antiapoptotic mechanisms (124). Biliverdin is a precursor of bilirubin and via reduction by biliverdin reductase, biliverdin can be recycled back to bilirubin providing a powerful redox cycle (Fig. 3) (143, 185). Mice treated with biliverdin before cardiac transplantation and daily for 13 days after transplantation survived longer than untreated controls (193). Biliverdin treatment also suppresses T cell-mediated immune responses by inhibiting leukocyte infiltration and proliferation of T cells in grafts. These findings suggest that biliverdin/bilirubin modulates the transcription of IL-2 through inhibition of nuclear factor of activated T cells (NFAT) and NF-κB (193). These results were supported by a recent study by Lee et al. (95), who demonstrated that IL-6, macrophage infiltration, T cell proliferation, lipid peroxidation (MDA), signal transduction of ERK1/2, and NF-κB activation (important cascade of proinflammatory cytokines) were inhibited in Gunn rats after heart transplantation compared with controls. Together, these studies indicate that elevated bilirubin prevents cardiac I/R injury and chronic allograft rejection, thereby improving long-term graft function and survival (95).
Interestingl, renal transplant recipients with higher bilirubin concentrations are protected from chronic transplant dysfunction and have improved survival compared with patients with lower bilirubin concentrations after 5 yr of follow-up. Bilirubin is independently associated with a lower risk of late graft failure, even after cumulative adjustment for confounders (34). Bilirubin modulates the immune system, resulting in inhibition of chronic rejection in individuals with high concentrations of bilirubin (34, 124). Therefore, both bilirubin and biliverdin inhibit acute T-cell-mediated immune responses and thus could contribute to prevention of transplant organ rejection (81). Organ rejection also can induce proliferation of VSMC causing allograft arteriosclerosis, organ damage, and eventually allograft loss (135). Bilirubin inhibits VSMC proliferation and neointima formation (see Bilirubin and vascular calcification) and thus may improve organ survival by improving organ perfusion, after transplantation in hyperbilirubinemic individuals (124). Exogenous bilirubin administration also reduces vascular resistance and tubular injury and increases creatinine clearance compared with control treatment in the isolated, perfused rat kidney (1). Despite generally favorable effects of bilirubin on transplant survival, one report indicates failure of exogenous bilirubin to protect from renal I/R. Treatment with bilirubin 1 h before ischemia did not protect the renal medulla or improve GFR; however, cortical proximal tubule histology was preserved (86).

**Bilirubin and endothelial function.** Endothelial cells serve as a reactive biological barrier between the blood and VSMC in blood vessels, functioning to regulate vascular tone and to maintain vascular homeostasis (32, 33). Endothelial dysfunction occurs due to an imbalance in endothelium-derived relaxing and contracting factors being released from endothelial/smooth muscle cells. Dysfunction can be caused by behavioral and pathological stresses, including smoking, hypercholesterolemia, hypertension, obesity, and oxidative stress (63, 107). Dysfunction of coronary or peripheral vascular endothelium can lead to impaired endothelium-dependent vaso dilation, which is an early predictor for the development, progression, and manifestation of CVD (Fig. 3) (32, 33). Elevated bilirubin concentrations are associated with decreased oxidative stress status (DNA and LDL oxidation) and augmented endothelium-dependent vasodilation in male GS subjects. Serum bilirubin concentrations were significantly positively correlated with flow-mediated vasodilation. Bilirubin reduces oxidative stress and augments endothelial function, probably due to its antioxidant capacity, although more specific measures of oxidative stress (e.g., F2-isoprostanes, protein chlorination/oxidation) are necessary to support these study conclusions (100). Despite this limitation, data indicating reduced oxidative stress in GS due to enhanced antioxidant status were recently reported in other studies (14, 18).

Oxidative stress is an important contributing factor in the pathogenesis of contrast-induced nephropathy (CIN) (75). The production of reactive oxygen and nitrogen species is associated with the amount of injected contrast media administered. For example, nitrotyrosine, a stable biomarker of peroxynitrite-mediated protein oxidation, is produced in proportion to the amount of contrast media infused into patients during cardiac angiography (46). Bilirubin directly scavenges peroxynitrite and inhibits the formation of nitrotyrosine ex vivo (108). Contrast media also has direct toxic effects on endothelial cells, reduces medullary blood flow, and causes renal artery vasoconstriction and renal ischemia (58, 69, 106, 131, 137). The risk of CIN is reduced in patients with greater bilirubin.
concentrations, the most likely explanation for which is that bilirubin suppresses/scavenges contrast-induced ROS production and prevents endothelial dysfunction (75).

In addition, the relationship between total bilirubin concentrations and endothelial function in overweight patients was recently reported. Flow-mediated dilation was significantly greater in overweight patients with the greatest bilirubin concentrations (>0.77 mg/dl), further suggesting that bilirubin protects against endothelial dysfunction (197). Elevated serum bilirubin concentrations are also associated with improved endothelium-dependent function of coronary arteries in patients without coronary heart disease (CHD) (196). Acetylcholine-mediated increases in coronary blood flow (CBF) and coronary artery diameter were positively correlated with serum bilirubin concentrations. Furthermore, hs-CRP and HDL were independent predictors correlating with serum bilirubin concentrations and therefore suggested that bilirubin may mediate these effects by reducing inflammation and increasing HDL availability (196). The reported beneficial effect of bilirubin on lipid status was recently reviewed, indicating bilirubin might have discrete effects on cholesterol metabolism (20). Zhu et al. (200) reported an association between pulsatile arterial function and serum bilirubin concentrations in patients with coronary artery disease (CAD). Furthermore, increased bilirubin was a determinant of decreased brachial-ankle pulse wave velocity in men. Further evidence has shown that increased arterial stiffness is associated with albuminuria, decreased GFR, and an elevated prevalence of CKD (16, 169, 195). Together, these observations indicate that serum bilirubin concentrations are negatively correlated with arterial stiffness and therefore may provide an explanation for the improvement in vascular function seen in patients with elevated bilirubin concentrations (195, 200).

Gullu et al. (61) investigated the relationship between bilirubin concentrations and coronary flow reserve (CFR) in young adults with no cardiovascular risk factors. CFR values were significantly greater in subjects with elevated bilirubin concentrations (>1 mg/dl). These data indicate that bilirubin can protect against coronary microvascular dysfunction and preserve CFR by improving endothelium-dependent vasodilation (61). Several studies demonstrate a negative relationship between endothelial function and ESRD in patients on hemodialysis (4) or peritoneal dialysis (110). However, it remains to be established whether elevated bilirubin concentrations, within the physiological range, can improve renal and endothelial function and thus reduce cardiovascular morbidity and mortality in patients with CKD.

Erdogan et al. (41) first investigated the possible effects of serum bilirubin on coronary collateral development in patients with CAD who experienced total coronary occlusion. This study indicated that serum total bilirubin concentrations were positively related to collateral development. NO plays a crucial role in the development of collateral vessels, and therefore bilirubin may be an important mediator increasing NO bioavailability, augmenting collateral development (41) and reducing amputation rates in type II diabetic patients (23). NO is considered the most important endothelium-derived relaxing substance (54), with endothelial dysfunction often characterized by a loss of NO bioavailability. NO inhibits platelet and leukocyte activation and therefore smooth muscle cell proliferation and migration within the vasculature (13, 32, 60). Many studies report a positive relationship between bilirubin, HO-1, and preservation of vascular bioactive NO (82, 104, 128). In addition, bilirubin attenuates the expression of endothelial adhesion molecules (E- and P-selectins) and prevents the adhesion and infiltration of leukocytes into the vessel wall (103, 147, 161, 171). Most recently, exogenous UCB was also shown to inhibit collagen-induced platelet activation, indicating that bilirubin might resist thrombus formation (91).

Endothelial cell injury in response to oxidative stress and inflammatory stimuli could contribute to cell growth and proliferation of VSMC. Abnormal proliferation of VSMC within the intima is an important event in the pathogenesis of atherosclerosis and vascular stenosis (101, 136, 142). Bilirubin inhibits neointima formation following carotid artery balloon injury in the rat (130). Bilirubin also inhibits the proliferation and migration of human aortic smooth muscle cells in a concentration-dependent manner, and this effect might be related to the expression of cell cycle-regulatory proteins (130). These conclusions agree with those of Ollinger et al. (123), who revealed that neointima formation was significantly suppressed in balloon injury-induced vascular damage in Gunn rats. These authors then investigated the effect of bilirubin on rat and mouse VSMCs in vitro, showing that UCB dose dependently reduced VSMC proliferation (123). Ollinger et al. (123, 125) argued that the antiproliferative action of bilirubin was not due to the induction of VSMC apoptosis or necrosis. Rather, bilirubin arrested cell cycle progression in VSMC by inhibiting phosphorylation of retinoblastoma tumor suppressor protein (Rb), JNK activation, and modulated MAPK signaling (123, 125). Imbalance of VSMC proliferation and apoptosis may lead to pulmonary vascular remodeling processes, which include thickening of the pulmonary artery wall and an accompanying increase in vascular resistance. A recent study by Song et al. (148) demonstrated that exogenous bilirubin inhibited pulmonary arterial smooth muscle cell apoptosis in a dose-dependent manner. From these data, it was suggested that bilirubin is a potential antiapoptotic compound protecting against hypoxia-induced pulmonary vascular function (148) and progression to CVD and mortality.

Endothelial progenitor cells (EPCs) derived from bone marrow play a crucial role in endothelial repair following injury. A strong correlation is reported between the number of circulating EPCs, endothelial function, and cumulative cardiovascular risk in men (70). It is hypothesized that endothelial injury in the absence of sufficient circulating EPCs is important in the pathogenesis of CVD (70). Interestingly, induction of HO-1 (using probucol) enhanced reendothelialization in a rabbit model of aortic balloon injury, which increased circulating EPCs and enhanced the maturation of bone marrow–derived progenitor cells. The end product of HO-1, bilirubin, may be involved in this action (187).

Bilirubin and vascular calcification. Vascular calcification is a common complication of CKD and is strongly correlated with cardiovascular mortality in patients with ESRD (9, 47). The pathogenesis of vascular calcification in CKD is complex and leads to endothelial dysfunction/damage and apoptosis of VSMCs. Smooth muscle cells are transformed into osteoblast-like cells capable of tissue mineralization in the presence of calcium phosphate deposition due to abnormal bone metabolism and impaired renal excretion of calcium and phosphate (30, 117, 146). Several contributing risk factors for vascular
calcification have been identified, including hypertension, diabetes, mineral metabolism abnormalities, hyperparathyroidism, and oxidative stress (56, 111). Vascular calcification manifests itself as medial and intimal calcification in arteries, leading to downstream organ dysfunction. In dialysis patients, vascular media calcification is responsible for calcific uremic arteriolopathy/calciphylaxis (144, 149) with the prevalence of vascular calcification approximating 50–80% in hemodialysis patients (47, 55). Calcification in coronary arteries reduces arterial elasticity, leading to stiffening and reduced compliance. Due to the loss of arterial elasticity, pulse wave velocity and pulse pressure are increased, leading to further impaired arterial distensibility, decreased coronary artery perfusion, and development of ventricular hypertrophy (30, 117, 118).

Previous studies have demonstrated an inverse relationship between bilirubin concentrations and vascular calcification. Tanaka et al. (159) investigated the relationship between serum bilirubin concentrations and coronary artery calcification (CAC) measured by noninvasive multislice computed tomography, which is a better predictor of severity of atherosclerotic disease than CIMT (48). Additional 0.058-mg/dl increments of serum bilirubin concentration were associated with decreased odds of patients obtaining a CAC score of ≥400 by 14% after adjustment of several risk factors (159). A supporting study showed an inverse relationship between total serum bilirubin and CAC score in healthy men. For example, an increase of 0.1 mg/dl bilirubin concentration was associated with reduced odds of patients having a CAC score of ≥100 by 29.2% (198). Furthermore, the CAC score was lowered in subjects with elevated bilirubin concentrations and was inversely associated with bilirubin concentrations (158). Vascular calcification is strongly associated with myocardial infarction, CAD (134), and all-cause mortality in ESRD patients (11). Hence, these data indicate that CKD patients with elevated bilirubin concentrations are protected from coronary artery vascular calcification and thus may explain the low prevalence of CVD events and mortality in these patients as reported by Chen et al. (24).

Systematic Review of Clinical Studies

The studies discussed above indicate potential mechanisms by which bilirubin may protect from hypertension, atherosclerosis, and development of CVD and kidney disease/dysfunction, mostly in animal models. Therefore, a systematic review of the literature was conducted to investigate whether a similar relationship existed between bilirubin and protection from kidney disease and CVD mortality in human clinical studies. Clinical studies were selected for inclusion in Supplemental Table S1 based upon a systematic search of the literature using the PubMed and Google Scholar databases that was completed on March 27, 2014 (all supplemental material for this article is accessible on the journal website). The PubMed search criteria identified studies with bilirubin or GS (in title/abstract) as independent variables and kidney injury or other related end points as dependent variables. The search was restricted to studies published between 1987 (when the landmark study to conclusively demonstrate bilirubin’s antioxidant potential was published) and 2014 (Fig. 4). A series of exclusion terms (i.e., malaria, human immunodeficiency virus, carcinoma, transplant, cirrhosis, anemia, cholestasis, hepatitis, liver failure, and hepatectomy) were added to focus the search on the relationship between bilirubin and kidney disease in the absence of confounding comorbidities. A total of 615 studies were identified and individually screened and excluded based upon the criteria in Fig. 4. A search of Google Scholar was conducted with similar inclusion and exclusion criteria, revealing three additional articles (identified with an asterisk in Supplemental Table S1).

**Clinical studies.** The aetiology and progression of most kidney diseases involve immunological and/or inflammatory processes followed by free radical production, oxidative stress,
cellular destruction, fibrosis, and repair (17, 49). Clinical studies have explored the relationship between circulating bilirubin concentrations and incidence of CKD and mortality in dialysis patients (24, 53) and healthy male population (Supplemental Table S1; entry 19) (141). A large cross-sectional study of Korean adults revealed that serum bilirubin is associated with improved renal function, after adjusting for age, gender, and confounders (Supplemental Table S1; entry 5) (66). Individuals with higher bilirubin levels have a reduced prevalence of diabetes in both men and women (men: 12.7–9%, women: 21.1–12.5%, respectively). In women, the risk of diabetic nephropathy was negatively associated with serum bilirubin levels. In addition, serum bilirubin was also negatively associated with insulin resistance [Homeostasis Model Assessment-Insulin Resistance (HOMA-IR)] and C-reactive protein (CRP) (62, 66). The results of this study support those of Park et al. (129), who also showed higher high-sensitivity CRP (hs-CRP) and HOMA-IR in type II diabetic patients with lower serum bilirubin concentrations (Supplemental Table S1; entry 13). Carotid intima-media thickness, plaque scores, insulin, serum creatinine, estimated GFR (eGFR), period of diabetes, and hypertension were significantly improved in patients with higher bilirubin concentrations, especially in women (129). These findings indicate that bilirubin might protect from renal damage, possibly by inducing anti-inflammatory mechanisms related to complement activation and cytokine release (7, 81). Temme et al. (165) was the first to publish a prospective study evaluating the association between serum bilirubin concentrations and all-cause, cardiovascular, and cancer mortality in a Belgian population over a period of 10 yr (Supplemental Table S1; entry 1). A longitudinal study by Aja et al. (3) further supported lower all-cause and cardiovascular mortality in men with higher bilirubin concentrations (Supplemental Table S1; entry 9). More recently, Vasović et al. (173) investigated the prediction of cardiovascular mortality in the disabled elderly (Supplemental Table S1; entry 15). This study demonstrated that multivariate predictors of cardiovascular mortality included albumin, BMI, total bilirubin, blood urea nitrogen, and hs-CRP, which combined, formed the inflammatory-malnutrition-renal involved score (IMRIS). Over a period of 32 mo, elderly subjects with a negative IMRIS had improved survival rates (~50%) compared with those with a positive IMRIS scores. The authors discussed that increasing the sample size of the cohort and follow-up period may strengthen the outcomes of this study and argued that total bilirubin should be a part of this new scoring system (173).

In chronic hemodialysis patients, preliminary studies suggest that circulating bilirubin concentrations predict long-term risk of cardiovascular events. Mildly elevated bilirubin concentrations protect from all-cause and cardiovascular mortality, which accounts for a large proportion of deaths in this cohort (Supplemental Table S1; entry 8) (24). Approximately 90% of patients with higher serum bilirubin levels (0.99 ± 0.25 mg/dl) survived after 12 yr of hemodialysis. Incremental (0.1 mg/dl) increases in serum bilirubin decreased the risk of a cardiovascular event and all-cause mortality by 9 and 10%, respectively (24). Oxidative stress is recognized as a CVD risk factor in hemodialysis (12, 59), which may be due to increased free radical production during hemodialysis via the direct contact of blood components with artificial biocompatible dialysis membranes (96). Although water-soluble antioxidants are dialyzed, unconjugated bilirubin, which is bound to albumin (126), is retained in the vascular compartment and may represent an important “sink” for free radical reduction in these individuals. Therefore, these observations indicate that serum bilirubin concentration is an important prognostic factor for CVD risk and all-cause mortality in end-stage renal disease (ESRD) patients. A recent population-based cohort study also reported that the mortality rates were 50% lower in GS subjects compared with those with normal bilirubin concentrations from CVD, diabetes, and chronic respiratory disease (Supplemental Table S1; entry 14) (72). However, an important limitation of this study included lack of data concerning nutrition, exercise habits, and ethnicity in primary care data (72).

Chin et al. (25) demonstrated that mildly elevated serum bilirubin levels were negatively associated with ESRD incidence in IgA nephropathy (Supplemental Table S1; entry 4). Improved eGFR, lowered serum creatinine, and reduced urinary protein concentration were associated with elevated bilirubin levels. ESRD incidence was 2.8% in the highest bilirubin quartile (>0.8 mg/dl) compared with 10.7% in the lowest quartile after 5 yr of follow-up (25). Oda et al. (120) showed a significant correlation between higher total bilirubin concentrations and improved eGFR in patients. Hypobilirubinemic patients (>1.24 mg/dl) have a higher incidence of ESRD after 1 yr of follow-up and suggested that total bilirubin might be an important risk factor for ESRD (Supplemental Table S1; entry 11) (120). Fukai et al. (53) also demonstrated lower serum bilirubin concentrations are associated with increased CVD risk in hemodialysis patients with type II diabetes (Supplemental Table S1; entry 6). An inverse association between serum bilirubin levels and log urinary albumin excretion was reported (Supplemental Table S1; entry 3) (52). Also, serum bilirubin concentrations independently predict urinary albumin excretion (micro- and macroalbuminuria) in hypertensive Taiwanese patients (Supplemental Table S1; entry 7) (74). A significant positive correlation between serum bilirubin and eGFR and a negative correlation with 24-h urinary protein excretion was reported in both diabetic and nondiabetic adults (Supplemental Table S1; entry 10) (145). Recent studies by Toya et al. (170) and Mashitani et al. (102) demonstrated that type II diabetic patients with elevated bilirubin concentrations have a reduced risk of progressing from urinary microalbuminuria to macroalbuminuria (Supplemental Table S1; entries 16 and 17) and have improved eGFR after 7 yr of follow-up. Furthermore, Okada et al. (122) confirmed that type II diabetic patients with lower bilirubin concentrations have a greater incidence of developing albuminuria (Supplemental Table S1; entry 18). In another cross-sectional diabetic study including GS and control subjects, an inverse relationship between bilirubin and the prevalence of vascular complications, oxidative stress, and inflammatory markers was reported (Supplemental Table S1; entry 2) (78). These studies suggest bilirubin is an important endogenous co-/antioxidant that might preserve glomerular and vascular function.

Interestingly, the renoprotective effects of bilirubin are also observed after contrast media administration during coronary diagnostic and interventional procedures. Contrast media administration has been associated with increased incidence of long-term morbidity, mortality, and renal impairment (138). For example, a negative association between serum bilirubin concentrations and future major adverse cardiovascular events
in patients undergoing coronary intervention is reported (Supplemental Table S1; entry 12) (75). This study indicated patients with higher bilirubin concentrations have significantly reduced incidence of CIN, CVD death, all-cause mortality, and the requirement for hemodialysis compared with patients with low bilirubin concentrations.

Choosing the most appropriate clinical bilirubin biomarker. Of the clinical studies identified in this systematic review, most report total bilirubin concentrations, which reflect the combined concentration of unconjugated and conjugated bilirubin. This is not optimal because measurements of total bilirubin alone do not allow authors to determine whether a patient’s elevated bilirubin is caused by hepatic pathology or a benign deficiency in excretory capacity (i.e., GS). Reporting of total bilirubin concentrations is acceptable if patients are screened for hepatic dysfunction/damage (i.e., using serum transaminases), indicators of hemolysis (reticulocyte counts), associated conditions (blood smears for spherocytosis), and excluded based upon appropriate criteria. Once this has been completed, either reporting total and/or unconjugated bilirubin would be acceptable. A meta-analysis has previously shown that in the presence of hepatopathy the association between total bilirubin and protection from cardiovascular disease is lost, emphasizing problematic use of total bilirubin measures in an investigation of the protective effects of bilirubin (119). An important problem in a small number of published studies is the report of direct bilirubin with renal function, metabolic syndrome, or cardiovascular protection (76, 141). The meaning of these associations is unclear and suggests that liver dysfunction (i.e., accumulation of direct reacting conjugated bilirubin in the blood) is associated with protection, which is clearly inaccurate. These findings are likely explained by a variable fraction of total bilirubin reacting in the diazo reaction (without a detergent/emulsifier) and therefore would not accurately reflect the relationship between bilirubin and protection in vivo. In conclusion, authors should investigate the relationship between total and/or unconjugated bilirubin and renal/cardiovascular protection, so as not to confuse readers and incorrectly link markers of hepatic dysfunction (direct bilirubin) to renal/cardiovascular protection.

Inconclusive/negative studies. Although the majority of clinical investigations indicate a potential beneficial effect of bilirubin on renal and CVD, there is a small body of literature suggesting potential negative associations between bilirubin and clinical end points. Targher et al. (163) reported that serum total bilirubin was negatively associated with eGFR and positively with albuminuria in 13,184 adults. The authors concluded that these associations indicate an association between serum bilirubin concentrations and nonalcoholic fatty liver disease (NAFLD). However, liver ultrasonography for diagnosing NAFLD was not performed, and medical history of existing CVD/CKD of the participants was not reported and excluded from the study (Supplemental Table S1; entry 20) (163). Negative findings of bilirubin on kidney function have mainly been observed in patients with comorbidities such as severe liver dysfunction, cholelithiasis (8, 172), cholestasis (133), infection with malaria (109, 160) and bacteria (166), and heart failure (88), which confound the effect of bilirubin on disease outcome and are thus excluded from Supplemental Table S1. For example, in a recent case Rafat et al. (133) reported that elevated bilirubin is associated with acute tubular necrosis in a kidney transplant patient with severe cholestasis and liver failure. Due to the obstruction of bile flow and accumulation of bile salts in hepatocytes, conjugated and unconjugated bilirubin concentrations were also increased in the circulation. Kidney function progressively deteriorated as both serum creatinine and bilirubin concentrations continued to increase for a period of 49 days after transplantation. Renal histology showed marked tubular damage with loss of brush border, tubular necrosis, and extensive bile pigment present in tubules (133). However, bilirubin is one (of many) organic anions normally excreted in bile, the excretion of which was clearly obstructed in this patient. Therefore, it is possible that any number of compounds (including bile acids) accumulated and crystalized with bilirubin, inducing renal tubular damage. Furthermore, Terg et al. (166) reported that cirrhotic patients with spontaneous bacterial peritonitis have a greater risk of renal failure and mortality. The results of this study showed that patients with serum bilirubin <4 mg/dl and creatinine <1 mg/dl had improved renal function and survival rate by ~20% compared with the high-risk group with elevated bilirubin concentration (166). However, patients with advanced liver disease (and elevated bilirubin) have a greater risk of hepatic failure and death due to sepsis, as a consequence of compromised bacterial clearance and not bilirubin per se. No regression analysis has been performed on potential predictors of renal failure during bacterial infection with concomitant liver dysfunction, due to the small number of patients within these studies.

Choi et al. (27) conducted a retrospective case-control study of 30 acute kidney injury (AKI) patients with hepatitis A. These data indicated that total bilirubin is an independent risk factor for AKI with hepatitis. This conclusion is supported by the studies of Misra et al. (109) and Tangpukdee et al. (160), who reported that increased total bilirubin is associated with acute renal failure among patients with severe falciparum malaria. Patients had a greater incidence of hemorrhheological disorder due to massive intravascular hemolysis upon sequestration of parasitized red blood cells in the kidney caused by acute viral hepatitis and parasitic infection (109). Thus the association between higher bilirubin concentrations and the development of AKI/renal failure in infected hyperbilirubinemia might be explained by increased destruction of red blood cells occurring in complicated infection, which thereby increases bilirubin concentrations in the circulation. These negative studies also indicated that patients with severe liver disease are more likely to develop hepatic encephalopathy and hepatorenal syndrome (166). Therefore, the assessment of renal function must be accompanied by an evaluation of liver function, including liver enzymes to exclude confounding influences of hepatic damage on pathology. Koyama et al. (88) reported a positive correlation between bilirubin concentrations and urine albumin excretion in patients with acutely decompensated heart failure. However, diagnosis of liver disease/function was not performed in this study, to exclude any confounding effect of concomitant liver damage/failure.

Summary

Clinical studies show that individuals with elevated bilirubin concentrations, in the absence of liver pathology, are at reduced risk of CKD, CVD, and related mortality. Mildly elevated bilirubin concentrations are consistently associated with
reduced vascular resistance (10, 52), improved eGFR (25, 145), renal tubular function, and slowing of the progression of kidney damage (1), in the absence of comorbidities including liver dysfunction and bacterial infection (42, 133, 167). Multiple mechanisms likely explain the protective effect of bilirubin (Fig. 3), including antioxidant and anti-inflammatory effects, suggesting that it could represent a biomarker and potential therapeutic target to reduce CKD/CVD risk (19).

Indeed, recent studies have identified numerous molecules that either induce HO activity or partially inhibit UGT1A1, which could be used therapeutically to induce the protective effects of bilirubin in individuals at increased risk of CVD/CKD (81, 105, 184, 199).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES
BILIRUBIN AND KIDNEY DISEASE


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BILIRUBIN AND KIDNEY DISEASE


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