Bilirubin and beyond: a review of lipid status in Gilbert's syndrome and its relevance to cardiovascular disease protection

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Abstract

Gilbert's syndrome (GS) is characterized by a benign, mildly elevated bilirubin concentration in the blood. Recent reports show clear protection from cardiovascular disease in this population. Protection of lipids, proteins and other macromolecules from oxidation by bilirubin represents the most commonly accepted mechanism contributing to protection in this group. However, a recent meta-analysis estimated that bilirubin only accounts for ~34% of the cardioprotective effects within analysed studies. To reveal the additional contributing variables we have explored circulating cholesterol and triacylglycerol concentrations, which appear to be decreased in hyperbilirubinemic individuals/animals, and are accompanied by lower body mass index in highly powered studies. These results suggest that bilirubin could be responsible for the development of a lean and hypolipidemic state in GS. Here we also discuss the possible contributing mechanisms that might reduce circulating cholesterol and triacylglycerol concentrations in individuals with syndromes affecting bilirubin metabolism/excretion, which we hope will stimulate future research in the area. In summary, this article is the first review of lipid status in animal and human studies of hyperbilirubinemia and explores possible mechanisms that could contribute to lowering circulating lipid parameters and further explain cardiovascular protection in Gilbert's syndrome.

List of Abbreviations

AhR: Aryl hydrocarbon receptor

Arnt: AhR nuclear translocator

BAIF: Bile acid independent flow

BDT: Bilirubin ditaurate

BMI: Body mass index

BW: Body weight

CN: Crigler-Najjar

GS: Gilbert's syndrome

GY: Groningen yellow

HDL: High-density lipoprotein

Hmox: Heme oxygenase

LCAT: Lecithin cholesterol acyltransferase

LDL: Low-density lipoprotein

MRP2: Multi-drug resistance protein 2

MS: Metabolic syndrome

OR: Odds ratio

PC: Phosphatidylcholine

PL: Phospholipid

SD: Sprague Dawley

TBIL: Total bilirubin

TC: Total plasma cholesterol

TAG: Triacylglycerols

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

UCB: Unconjugated bilirubin

UGT1A1: Uridine glucuronosyl transferase 1A1

WHR: Waist to hip ratio

1 Introduction

Within the clinical domain, bilirubin, the end product of heme catabolism, has long been associated with liver pathology and haemolytic conditions and is assumed to possess little or no biological function. Indeed, circulating concentrations of unconjugated bilirubin (UCB) approaching or exceeding the binding capacity of albumin are associated with neuronal toxicity in neonatal jaundice[1] and in children or adults with the rare condition of Crigler-Najjar syndrome (genetically diminished or absent uridine diphosphate glucuronyltransferase 1A1 function[2]). However, unconjugated bilirubin concentrations rarely approach these toxic levels and approximates 10 µM in the general population[3], which is 30-60 times below the reported toxic concentration of UCB. More than 20 years ago, a possible physiological role of UCB was suggested by Stocker and colleagues[4], based on the demonstration of its potent *in vitro* peroxyl radical scavenging activity. Numerous studies followed demonstrating UCB participated in many redox reactions, in the presence of various radicals and oxidising species[5]. Despite these supportive data, an understanding of the importance of UCB in the protection from radical induced molecule/cellular damage *in vivo*, has remained elusive. An exciting clinical report showed that individuals with endogenously elevated UCB were protected from coronary atherosclerosis[6], the effects of which are most graphically demonstrated in Gilbert's syndrome[7], which is prevalent in ~10% of the population[8]. Investigations in these persons, in addition to experiments in a rat model of hyperbilirubinemia (Gunn rat), showed protection from *ex vivo*[3,9] and *in vivo*[10] exposure to oxidative conditions. The field has evolved to show that circulating lipids, including cholesterol, in GS are less susceptible to *ex vivo* oxidation, compared to controls[3,11], possibly linking elevated UCB to prevention of atherosclerosis via prevention of sterol oxidation. These data are also supported by recent evidence showing reduced prevalence of CVD in hypercholesterolemic patients with serum bilirubin concentrations above 1mg/dL (>17.1 µM)[12] Interestingly, however, a recent publication shows that decreased oxidised LDL concentrations in GS are predominantly attributed to lower

circulating LDL concentrations rather than to bilirubin concentrations *per se*.[13] Indeed, when corrected for LDL content, LDL appears to be *more* oxidised in GS, indicating an antioxidant role for UCB in atheroprotection is debateable[13]. This observation has generated considerable interest, because previous reports of modified lipid status in hyperbilirubinemic subjects appear to have gone unnoticed, or have only been published very recently[14,15]. These data, however, present a very important mechanism *in vivo* that could contribute to the protection associated with elevated bilirubin concentrations against atherosclerosis and IHD, independent of bilirubin's well accepted antioxidant potential. This article briefly describes the heme catabolic and bilirubin excretion pathways and conditions affecting them. It then presents the association between circulating bilirubin concentrations and conditions affecting bilirubin excretion, in both rodents and man, with lipid status. The possible mechanisms in these conditions that might perturb lipid and cholesterol absorption, metabolism and excretion are presented, followed by discussion of the potential effects of such perturbation on atheroma and ischemic heart disease development. These observations are likely to generate a new avenue of research aimed at revealing how UCB, or conditions affecting its excretion, influences lipid metabolism *in vivo*.

1.1 Heme catabolism

Heme catabolism is a continuous process, resulting in approximately 300 mg of bilirubin generated and excreted each day in adults[16]. Heme oxygenases (*hmox*) are a family of enzymes, consisting of two isoforms. Hmox2 is constitutively expressed, particularly within brain and testis. Hmox1 is an inducible isoform and is only expressed under conditions of metabolic and oxidative stress [17]. Heme oxygenases are distributed within the endoplasmic reticulum, mitochondria, plasma membrane and nucleus[18], the activity of which represents the rate limiting step of heme catabolism, primarily accomplished within the cells of the reticulo-endothelial system (mostly resident splenic and hepatic macrophages[19]). Heme's iron atom is first abstracted forming protoporphyrin, which is subsequently oxidised and ring opened at the expense of NADPH oxidation[19]. This step liberates CO and biliverdin, which is rapidly reduced, in another energy consuming reaction, by biliverdin reductase to unconjugated bilirubin (UCB). Unconjugated bilirubin, which is lipophilic at physiological pH due to intermolecular hydrogen bonding of its propionate groups, then diffuses into the blood, where it is bound by at least one binding site on albumin[20]. Albumin then carries bilirubin to the liver, where it is actively and passively taken up by the hepatocyte[21,22]. Within the hepatocyte, UCB is bound to hepatic glutathione-s-transferase (ligandin) [21] and delivered to the endoplasmic reticulum where it is mono- and then di-conjugated with glucuronic acid by uridine diphosphate glucuronyltransferase 1A1 (UGT1A1[23]), which is discussed further in the sections below.

1.2 Bilirubin excretion

1.2.1 Liver

The liver is the primary organ responsible for bilirubin excretion and under physiological conditions facilitates bilirubin removal from the extravascular space and its excretion from the body. Multi-drug resistant protein 2 (MRP2) exports bilirubin glucuronides from the hepatocyte into the hepatic canaliculus, where it travels with the bile, into the duodenum via the biliary tract[21]. The

importance of the liver for bilirubin excretion is clearly evident as impeded bile flow causes an obstructive jaundice, indicated by elevated conjugated bilirubin in the blood. Other conditions including hepatitis also impair bilirubin conjugation and excretion resulting in elevated unconjugated and conjugated bilirubin concentrations in the blood[24].

1.2.2 Kidney

Interestingly, the kidney represents a secondary site for bilirubin excretion. Unconjugated bilirubin is strongly bound to plasma albumin and, therefore, very little is filtered at the glomerulus[25]. However, unconjugated bilirubin can be transported from the vascular compartment into proximal tubular cells by organic anion transport proteins (OATPs), followed by conjugation by UGT1A1 and export into the urine or back into the blood. Conjugated bilirubin can then be filtered at the glomerulus[26]. The kidney is very important for the excretion of conjugated bilirubin and oxidised bilirubin species including biopyrrins[27,28]. For example, in conditions of conjugated hyperbilirubinemia (e.g. obstructive jaundice) the kidney is responsible for the excretion of bilirubin, perhaps operating as a back-up system in the event of hepatic insufficiency[29].

1.2.3 Intestine

The importance of the intestine in re-absorbing and excreting bilirubin remains a popular and important area of research. The intestine is a target for the reduction of circulating unconjugated bilirubin concentrations in conditions including neonatal hyperbilirubinemia and Crigler-Najjar syndrome[30,31]. For example, in conditions of unconjugated hyperbilirubinemia, bilirubin excretion takes place via transepithelial transport across the intestinal wall into the intestinal lumen[30,32]. In hyperbilirubinemic Gunn rats, the transepithelial route has been estimated to account for at least 50% of total bilirubin turnover[33]. By increasing intestinal motility[30] and increasing fecal fat excretion[32,34], clinically significant reductions in circulating unconjugated bilirubin can be achieved. The intestine also reabsorbs appreciable amounts of both conjugated and unconjugated pigment by passive transport and/or paracellular diffusion[35,36], constituting the well-known

entero-hepatic circulation. These observations demonstrate the importance of clearing the infant meconium/producing stools for reducing unconjugated bilirubin concentrations in jaundiced babies and in using the commonly accepted fasting test to diagnose Gilbert's syndrome[37].

1.3 Congenital conditions of impaired bilirubin excretion

1.3.1 Gilbert's Syndrome

Gilbert's syndrome is a benign condition of mildly (~40-60%) impaired bilirubin glucuronidation. The number of TA repeats within the promotor region of the UGT1A1 gene ultimately influences the serum unconjugated bilirubin concentration, by reducing inducibility of the UGT1A1 gene and therefore hepatic bilirubin conjugation and excretion. Individuals with an increased number of TA repeats in the gene promoter for UGT1A1 (usually >7 in both alleles) are often diagnosed with Gilbert's syndrome, which is defined by an individual having an unconjugated bilirubin concentration > 1mg/dL (>17.1 µM)[38]. A number of polymorphisms in the promotor region of UGT1A1 exist, including the UGT1A1*28 (7/7 TA repeats in each allele) that clearly elevate unconjugated bilirubin concentrations[39]. In addition, Gilbert syndrome is also associated with impaired hepatic uptake of bilirubin (and bromosulfophthalein) into the hepatocyte, which is controlled by the activity of organic anion transporters[40,41]. Interestingly, this observation also explains the presentation of Rotor Syndrome, which is caused by a deficiency in conjugated bilirubin re-uptake into hepatocytes by organic anion transporting polypeptides OATP1B1 and OATP1B3[42]. Finally, circulating bilirubin concentrations in GS may also be influenced by the activity of hmox1, the rate limiting enzyme for bilirubin production. For example, apparently healthy adults with reduced GT repeats in the promoter region for hmox1 (increasing hmox1 inducibility) experience elevated bilirubin concentrations[43], which could interact with UGT1A1 polymorphisms to induce hyperbilirubinemia[44].

1.3.2 Crigler-Najjar Syndrome

Crigler-Najjar (CN) syndrome is divided into type 1 and 2 variants and is a rare, however, potentially fatal condition of impaired bilirubin glucuronidation. Crigler-Najjar syndrome type 1 and 2 are characterised by absent or strongly diminished glucuronidation capacity of the liver[40]. Unconjugated serum bilirubin concentrations can reach very high levels (up to 500 μM) and, if untreated, exceed the binding capacity of circulating albumin. This results in the net transfer of bilirubin from the extracellular compartment into the cellular compartment, particularly within the brain[45]. When the unbound (free) concentrations of unconjugated bilirubin exceed the exchange transfusion threshold of 75 nM, affected individuals can experience abnormalities in hearing and are more likely to suffer bilirubin encephalopathy[46]. The mechanism by which UCB in the CNS induces damage is not fully understood[47] but it is clear that bilirubin decreases the mitochondrial trans-membrane potential and induces ER stress, leading to apoptosis[48,49]. If not liver transplanted, Crigler-Najjar patients are life-long dependent upon phototherapy to reduce bilirubin concentrations[2], in addition to plasma exchange transfusion during periods of extremely high serum bilirubin levels[50]. Phenobarbital treatment induces hepatic glucuronidation capacity[51] and acutely lowers circulating bilirubin concentrations in Crigler-Najjar Syndrome type 2, but not type 1[52]. A more definitive treatment for CN disease has been liver transplantation, but transplantation in itself, together with lifelong immunesuppressive therapy, carries the risk of short- and long-term morbidity and even mortality[53]. Recent studies show considerable promise for orally administered polyethylene glycol[30] and orlistat[32], in combination with phototherapy, for short term reduction in circulating unconjugated hyperbilirubinemia, in animal and human subjects.

1.3.3 Dubin-Johnson Syndrome

Dubin-Johnson syndrome is characterized by genetically impaired transport of bilirubin glucuronides and other organic anions (e.g. sulphated bile salts, bromosulphthaleine) that may or may not be conjugated to glutathione prior to crossing the canalicular membrane. A mutation in the cMOAT gene (also known as ABCC2) that is responsible for the transcription of MRP2 results in a combined

conjugated and unconjugated (50:50) hyperbilirubinemia (50-400 µM)[54]. Dubin-Johnson syndrome

is inherited in an autosomal recessive manner, is extremely rare and is not fatal[40]. Phenotypically,

Dubin-Johnson syndrome is akin to Rotor syndrome[42], which has a similar patient presentation,

however, circulating conjugated bilirubin concentrations may be lower in this cohort.

1.4 Bilirubin and lipid status

1.4.1 Gilbert's Syndrome

As indicated in Table 1, many studies investigating Gilbert's Syndrome have been published recently.

A variety of studies have been conducted with the most rigorous approach adopted by Boon et

al.[13] and Wallner et al.[15] who age and gender matched their study populations. Maruhashi et

al.[55], recently investigated the largest age matched group of GS and control subjects (108 subjects

per group) and included male subjects only. Due to the heterogeneity of study design and

experimental rigor, detailed discussion is only made to studies with age and gender matching or

study within a particular gender.

Boon et al.[13] recently showed that total cholesterol (TC) and low density lipoprotein cholesterol

(LDL) concentrations were significantly lower in GS versus matched controls (0.83 and 0.91 mM,

respectively), the concentrations of which were negatively correlated with unconjugated bilirubin.

Oxidised LDL concentrations were lower in GS, as first reported by Tapan et al.[56], and strongly

associated with reduced circulating LDL concentrations and not with bilirubin, arguing against a role

for bilirubin in protecting against LDL oxidation *in vivo*. Interestingly, when oxLDL concentrations

were expressed relative to LDL concentrations, oxLDL concentrations were significantly elevated in

GS, indicating the LDL was more oxidised in hyperbilirubinemic subjects[13]. A possible

hypocholesterolemic effect in GS is further supported by Wallner et al.[15](unpublished data), who

also report a trend to lower TC (0.39 mM), LDL (0.34 mM) concentration in GS. These reductions

were reflected by significant decreases in the LDL 1 and 2 (medium and small LDL) sub-fractions,

however, no difference in the very small dense LDL particle (LDL-3) concentration existed. These

data fail to support data presented by Tapan et al.[56] who showed reduced sd-LDL in a cohort of male, age and BMI matched individuals, indicating the possibility of gender dependent perturbation of cholesterol homeostasis in GS. Such conclusions are further supported by the elegant experiments of Smiderle et al.[57], who showed premenopausal women possessing the 7/7 TA repeat polymorphism had significantly reduced TC concentrations, versus individuals with the 6/7 or 6/6 polymorphism. The 7/7 TA repeat polymorphism is associated with hyperbilirubinemia[58], however, bilirubin concentrations were not reported in this study. Interestingly, the effect of the 7/7 TA repeat polymporphism was not evident in post-menopausal women, arguing for an important role of reproductive hormones in regulating lipid status in synergy with bilirubin. Indeed, estrogen and bilirubin are substrates for UGT1A1[59] and compete with each other for glucuronidation[60]. Unconjugated bilirubin concentrations are greater in young female versus male Gunn rats (see section 1.4.3), and experience a further pronounced hypocholesterolemia compared to their male counterparts. Further large scale GS studies are clearly necessary to conclusively determine association between bilirubin and lipid status in male/female subjects and the possible interaction of estrogen with bilirubin and lipid status.

1.4.2 Clinical studies

Table 2 summarises a number of clinical studies that have reported an association between bilirubin and lipid profile. Breimer et al.[61] were the the first authors to publish the relationship between bilirubin and lipid profile in a large cohort of middle-aged British men. These data revealed lower concentrations of TC and higher concentrations of HDL in individuals with higher bilriubin concentrations. A number of subsequent studies described inverse relationships between bilirubin and TC[62-64] and non-HDL cholesterol[64,65] and a positive association with HDL concentrations[61,62,65-68]. Only one study reported decreasing HDL concentrations with increasing bilirubin[69]. The most striking trend to emerge from this analysis is that every study investigating healthy subjects reported a reduction in triacylglycerols with elevated bilirubin concentrations [62,64-69]. This finding is supported by a clear inverse realtionship between bilirubin

and VLDL concentrations[66]. Not surprisingly, most of these studies and others revealed inverse associations between bilirubin and adiposity related variables including BMI and waist circumference[62,64-70].

When the relationship between lipid status and bilirubin was explored in clinical subjects suffering kidney disease, bilirubin remained negatively correlated with TC in IgA nephropathy patients[71], however, was unrelated to lipid status in haemodiaysis patients[72]. Despite the lack of any relationship between bilirubin and lipid status in end stage renal disease, bilirubin was clearly related to cardiovascular mortality/events in persons dependent upon haemodialysis. These data provide strong support for bilirubin protecting from haemodialysis induced oxidative stress[73].

Finally, a relationship between weight loss and circulating bilriubin was recently published by Andersson et al.[74]. After four weeks of daily sibutramine administration, overweight and obese patients' circulating bilirubin concentrations increased in proportion with weight lost, most clearly in men. Similarly, reductions in triacylglycerols occurred in association with increased bilirubin. Whether, circulating bilirubin is dependent upon weight-loss (e.g. related to reduced free radical production or inflammation) or is responsible in part for weight loss remains to be determined.

A series of interesting studies were recently published that attempted to attribute causality of circulating bilirubin on changes in other biochemical and CVD related endpoints. McArdle et al. [64] investigated a large group other otherwise healthy Armish individuals and reported significant negative relationships between bilirubin and LDL, TC and BMI (Table 2). Similar, however weaker, relationships were found between bilirubin and waist circumference, systolic and diastolic blood pressure, triacylglycerols, C-reactive protein and carotid intima-media thickness. However, the use of Madelian randomization statistical analysis excluded a causal role for bilirubin in modifying all of these variables, and surprisingly revealed a causal role in determining brachial artery diameter and and cold pressor responses. Stender et al.[75] used an identical analytical approach the results of which indicated bilirubin was not causally related to a reduction in ischaemic heart disease or

myocardial infarction in multiple longitudinally studied cohorts. However, it should be noted that these authors corrected for BMI in their analysis, which may well have negated the effects of bilirubin, if indeed bilirubin affects lipid homeostasis, as hypothesised in this review. Finally, an insightful comment by Johansen and Hegele[76], described the potential power and limitations of the Madelian randomization approach and revealed that the assumptions of this analysis have likely been violated in the previously mentioned studies, because UGT1A1 also metabolises products other than bilirubin, which might influence cardiovascular pathophysiology (e.g. oestogen as discussed previously). Indeed, the assumption that UGT1A1 accurately predicts circulating bilirubin concentrations is debateable, with great variation in circulating bilirubin concentrations in UGT1A1 modified genotypes[58]. Furthermore, McArdle et al.'s[64] finding that UGT1A1 promotor polymorphism explained less than half (45%) of the variation in bilirubin concentrations in their cohort questions whether UGT1A1 genotype in an appropriate suggogate marker to analyse the causal effects of bilirubin *in vivo*. Therefore, a more robust model including other genetic determinants of circulating bilirubin (e.g. *hmox1* promoter polymorphisms), or specific investigation in mutant animal models (see 1.4.3 Gunn rat model) is required, to determine whether bilirubin, *per se*, influences cardiovascular risk and if it does, how it might accomplish this.

1.4.3   Gunn rat model

The Gunn rat is a model of human Crigler-Najjar syndrome type I (i.e. complete absence of bilirubin glucuronidation capacity). Gunn rats inherit a single point (frame shift) mutation in the UGT1A1 gene that truncates and inactivates UGT1A1[77]. Therefore, hepatic bilirubin glucuronidation and excretion is absent[78]. The lack of hepatic bilirubin conjugation increases circulating unconjugated bilirubin concentrations to between 50-200 µM in these animals[13,34]. Only four studies have reported circulating lipid profile parameters in Gunn rats. The studies of Fu et al.[79] and Sakamoto et al.[80] reported lower total and non-HDL fractions. Whereas lower total, HDL cholesterol and a trend towards lower triacylglycerol and LDL concentrations in aged female hyperbilirubinemic animals was recently reported[13]. These data were further extended by Wallner et

al.[15](unpublished data) showing that differences in circulating cholesterol species and triacylglycerols is more prominent in young female rats (versus male), revealing sexual dimorphism in the bilirubin effect. The hypocholesterolemia observed in female Gunn rats is striking, with Gunn animals reporting total cholesterol values one third of heterozygote and wild-type animals[13]. Furthermore, reduced body mass only exists in female Gunn rats (versus litter-mate controls)[13,15]. Two possible mechanisms could explain these observations, implicating either a role for UGT1A1 or unconjugated bilirubin in perturbing cholesterol metabolism/excretion. Reduced circulating cholesterol and triacylglycerol concentrations in these animals probably explains the reduction in body weight in them[13], the reason for which, until now, has remained a mystery. A more pronounced effect in female Gunn rats indicates an interaction between estrogen, bilirubin and lipid metabolism. It is possible that estrogen (moreso in female animals) competes with bilirubin for glucuronidation by UGT1A1[60], elevating bilirubin concentration[15], with bilirubin exerting an effect on cholesterol homeostasis. It is also equally possible that bilirubin competes with estrogen for conjugation[60], elevating estrogen concentrations[81]. Circulating estrogen levels in Gunn rats/Gilbert's syndrome have not yet been reported, however, could partly explain alterations in lipid status[82]. For example, peak estrogen concentrations in pre-menopausal women are associated with reduced total, LDL cholesterol and triacylglycerol levels and elevated HDL levels[83]. These exciting observations support a role for elevated circulating unconjugated bilirubin, competition for or impaired UGT1A1 activity in modulating lipid profile, adiposity, BMI and potentially cytokine concentrations in humans [13,15].

1.5 Modulation of lipid metabolism by bilirubin

1.5.1 Modulation of intestinal cholesterol secretion

The classic paradigm of cholesterol homeostasis involves a central role of the hepatobiliary secretion of cholesterol, either as such or after metabolism towards bile salts. Accordingly, cholesterol homeostasis in the body is considered to be regulated by cholesterol synthesis, biliary cholesterol secretion, intestinal cholesterol absorption (from dietary or biliary origin), and finally, fecal loss.

Recently, however, this classic concept has been challenged (see [84,85] for reviews). It has become clear that apart from hepatobiliary secretion, a major fraction of cholesterol in the intestinal lumen is derived from non-dietary and non-biliary origin. Rather, it has attributed to transintestinal cholesterol excretion, predominantly based on physiological studies in genetically manipulated mouse models (see[86,87] for review). Interestingly, these novel insights into cholesterol metabolism complement recent observations of a transintestinal pathway for unconjugated bilirubin disposal under conditions of permanent unconjugated hyperbilirubinemia. In 1963, Schmid and Hammaker [88] first demonstrated that Gunn rats with bile duct fistula continued to excrete via the feces radio-isotope labelled bilirubin, compatible with an non-hepatobiliary route of excretion. Hafkamp et al. performed kinetic studies with 3H-labelled unconjugated bilirubin in hyperbilirubinemic Gunn rats[33]**.** These studies demonstrated that in genetic conditions of deficient bilirubin glucuronidation the non-biliary, transintestinal route for bilirubin disposal from the body was quantitatively more important than the biliary route. Also, the transepithelial (non-biliary) route of bilirubin disposal could be augmented by strategies which formerly have been applied to reduce serum cholesterol, including treatment with the lipase inhibitor orlistat[34]. The mechanism by which either cholesterol of unconjugated bilirubin enters the intestinal lumen transintestinally has not yet been elucidated. Yet, the concurrence between the two hydrophobic compounds that transepithelial secretion into the lumen of the intestine constitutes a quantitatively important pathway for their disposal from the body provides a tempting concept that (part of) the interaction between bilirubin and cholesterol homeostasis may take place at the level of the intestine. It seems warranted to explore these options, particularly since recently stable isotope methodologies have been developed to address these research questions in relevant experimental animal models[89,90].

1.5.2 Modulation of lipid metabolism

1.5.2.1 Intrinsic and extrinsic pathways of lipoprotein synthesis

Ocadlik et al.[14] first described significantly reduced VLDL, IDL and LDL 3-7 sub-fraction concentrations, in addition to reduced triacylglycerol levels in male individuals with GS. This

observation provides an indirect indication that UCB affects the intrinsic pathway of lipoprotein synthesis. The hepatic production of VLDL is regulated by various key steps, including apolipoprotein B trafficking towards secretion or degradation, availability of lipid components (cholesterol, phosphatidylcholine, triacylglycerols) and the activity of the ==microsomal triacylglycerol transfer protein.== VLDL secretion is the major route by which the liver can systemically (re)distribute triacylglycerols in the body. The removal of triacylglycerols from VLDL, mediated by lipoprotein lipase, leads to the formation of IDL and subsequently LDL which is then recognised by LDL receptors on hepatocytes for removal and degradation. Ocadlik's[14] data support a hypothesis of reduced hepatic VLDL assembly in GS by a currently unknown mechanism. However, the observation that circulating IDL, LDL and particularly LDL subfraction 3-7 concentrations are lower in these subjects suggests that decreased VLDL concentrations lead to reduced levels of progressively triacylglycerol poor, cholesterol sub-fractions. High density lipoprotein concentrations do not seem to be affected or are mildly increased in many population studies[3,7,15,55]. These data suggest that ApoA-1 synthesis and lipidation by the ATP-binding cassette transporter A1 (ABCA1) in the peripheral tissues remain unaffected. Interestingly, HDL cholesterol concentrations are dramatically reduced in the hyperbilirubinemic Gunn rat[13,15]. However, unlike in humans, HDL constitutes the greatest sub-fraction of cholesterol in rodents. In UGT1A1 competent animals, HDL and LDL concentrations approximated 1.06 and 0.3 mM respectively. However, in Gunn rats HDL and LDL concentrations equalled 0.12 and 0.15 mM, indicating a significant bias towards lowering HDL concentrations[13]. The relevance of lower HDL concentrations in Gunn rodents for understanding the reduced LDL concentrations in Gilbert's syndrome remains unclear. However, it is tempting to speculate that the decreased CVD risk in GS patients is partly mediated by hitherto undefined mechanism(s) affecting serum lipid profile.

1.5.2.2 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a ligand-activated cytosolic protein belonging to the basic helix-loop-helix/Per-Arnt-sim (bHLH/PAS) family of transcription factors. The AhR is expressed in

many organs including the liver, gut, lungs in addition to lymphocytes[91,92]. Exogenous ligands for the AhR are mainly environmental pollutants, such as polycyclic and halogenated aromatic hydrocarbons, which include 3-methylcholanthrene, benzo[a]pyrene, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). These pollutants exert their toxic and carcinogenic responses through AhR-dependent expression of xenobiotic metabolising enzymes, especially members of the cytochrome P450 family 1A (CYP1A)[93].

The aryl hydrocarbon receptor has also been shown to play a role in physiology and development as *AhR* knock-out/null mice exhibit several defects, including abnormalities in the liver and immune system[94-96], as well as suppression of hepatic cholesterol biosynthesis[97], which indicates presence of endogenous ligands for AhR. Such ligands thus far identified, include kynurenic acid[98], 3-indoxyl sulphate[99], indirubin[100], biliverdin[92] and bilirubin[92]. The mechanism(s) by which endogenous ligands, especially biliverdin and bilirubin, regulate AhR-dependent cholesterol biosynthesis is poorly understood.

In the absence of a ligand AhR is localised to cytosol and associates with chaperone proteins. Ligand binding changes AhR conformation, in turn, releases it from chaperone proteins and allows translocation into the nucleus. Once in the nucleus, AhR heterodimerizes with the AhR nuclear translocator (Arnt) protein and forms an active transcription factor that interacts with the xenobiotic/dioxin responsive DNA elements (XRE/DRE), which in turn activates transcription of relevant gene[93]. Importantly, recent study indicates that AhR-dependent regulation of cholesterol biosynthesis is independent of AhR/Arnt heterodimer binding to the DRE of genes encoding crucial enzymes in cholesterol biosynthesis pathway[97].

Using genetic techniques, Tanos et al.[97] showed that *AhR* null mice possessed *increased* expression of genes regulating cholesterol biosynthesis including the rate-limiting enzyme of cholesterol synthesis HMG-CoA reductase, and others belonging to the mevalonate pathway including HMG-CoA synthase, lanosterol synthase and farnesyl-diphosphate farnesyltransferase.

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Furthermore, the authors showed that administration of the AhR ligand in wild-type animals *decreased* the expression of genes regulating cholesterol synthesis. Interestingly, the authors demonstrated that AhR can exert these effects independent of binding the dioxin response element and therefore, might regulate cholesterol metabolism by interacting with other transcription factors including the sterol regulatory element-binding protein. The translation of these findings using human cells provides a compelling argument for AhR in regulating cholesterol metabolism. Treatment of human hepatocytes with AhR ligand, β-naphthoflavone, reduced expression of cholesterol metabolising genes and was accompanied by reduced secretion of cholesterol into culture media[97]. Silencing of the *AhR* expression in HepB3 cells by way of siRNA showed that the degree of up-regulation of cholesterol metabolising genes correlated with the amount of AhR present. Despite these intriguing effects of AhR agonism on the cellular expression of cholesterol metabolising genes, no consistent effect on circulating lipid concentrations exist. This might be a consequence of the differential activation of low and/or high affinity AhR by a variety of endogenous and exogenous ligands. Despite this observation, the administration of the prototypic AhR ligand 2,3,7,8-Tetrachlorodibenzo-p-dioxin can increase *or* decrease circulating cholesterol and triacylglycerol concentrations[101,102]. These data are supported by Forgacs et al.[103] who showed higher concentrations of TCDD decreased the expression of cholesterol metabolising genes in both rats and mice. These data are accompanied by increases in hepatic total lipid, cholesterol, n-6 polyunsaturated fatty acids and triacylglycerol concentrations 72 hours after administration of TCDD to mice (significant) and also in rats (non-significant)[103]. The authors concluded that increased uptake of lipoproteins (via increased LDL, VLDL, ApoB48 receptor and CD38 expression) likely accounted for the steatosis associated with TCDD administration. Whether the administration of TCDD reflects in any way the effects of mildly elevated unconjugated bilirubin in GS, remains to be explored. However, it should be noted that Gunn animals that experience a range of UCB concentrations, similar to that observed in GS[13], do experience AhR activation and CYP1A1

induction[104]. Therefore, these animals would represent an excellent model to study the effects of bilirubin on AhR mediated perturbation of lipid homeostasis, for translation into human studies.

1.5.3 Modulation of hepatic lipid excretion

Multidrug resistance Related Protein 2 (MRP2) mediated efflux of conjugated bilirubin into the canaliculus and the effect of this organic anion on biliary lipid excretion and gall stone formation remains an important and popular research topic. Black gall stones are commonly formed when bilirubin excretion is increased (hyperbilirubinbilia), in concert with *enhanced* biliary bilirubin deconjugation and reduced biliary solubilisation. As a consequence bilirubin can precipitate as a calcium salt and can form aggregates in conjunction with cholesterol, lecithin and mucin glycoproteins[105]. Increased excretion of organic anions (including conjugated bilirubin) across the canalicular membrane is also associated with the uncoupling of hepatic lipid transport that could be a predisposing factor for gall stone formation. Intriguingly, men with Gilbert's syndrome are also more likely to suffer black gall stone formation[106]. This may coincide with *reduced* bilirubin conjugation and the hepatic formation of bilirubin monoglucuronide. Current opinion suggests that bilirubin monoglucuronide (with one free carboxylate group) forms a calcium salt with other bilirubin molecules and is thus associated with black gall stone formation[105]. These data provide an interesting context to explore the possible role of bilirubin on hepatic lipid excretion, which might contribute to altered lipid status in Gilbert and Dubin-Johnson syndromes.

Verkade recently summarised six factors that influence the coupling of lipid excretion (phospholipid and cholesterol) to bile salt excretion.[107] These factors include that; 1) intracanalicular bile salts induce lipid excretion; 2) this effect is concentration dependent; 3) hydrophobic bile salts quantitatively extract more lipids into the canaliculus; 4) greater bile salt independent bile flow decreases lipid excretion; 5) reduced expression of Mdr2 decreases lipid excretion and 6) increased canalicular concentration of hydrophilic organic anions (including bilirubin conjugates) decreases lipid excretion.

1.5.3.1 Increasing bilirubin excretion decreases (uncouples) hepatic lipid excretion

The most well known and explored mechanism implicating bilirubin in altered hepatic lipid excretion is that of 'uncoupling'. The uncoupling phenomenon is induced by multiple organic anions, including conjugated bilirubin and is associated with reduced biliary cholesterol and phospholipid excretion, with maintained bile salt efflux.[107] A variety of organic anions accomplish this by a mechanism that is not yet fully understood. The current hypothesis involves associations of hydrophilic organic anions with bile salt molecules or micelles, which limit either the tendency or capacity of bile salt to exert a phospholipid- and cholesterol-excretory stimulus[108]. One of the first studies to explore the impact of bilirubin on hepatic excretion was published by Apstein[109]. In this study Sprague Dawley (SD) and Gunn rats had their bile ducts cannulated and were subsequently allowed to recover. Bile was then collected from the rats continuously for 18 hours before they were infused with taurocholate to restore biliary bile acid, phospholipid and cholesterol excretion. Bilirubin ditaurate (BDT) or UCB was then infused and biliary bile salt, cholesterol, phospholipid and bilirubin excretion was quantified. In UGT1A1 competent SD rats, both UCB and BDT administration dramatically reduced biliary cholesterol and phospholipid excretion. However, only BDT lowered phospholipid excretion in Gunn rats. These data indicated that presence of organic anions not at the intracellular level of the hepatocytes, but rather in the canaliculus (either BDT or conjugated bilirubin), significantly impaired hepatic lipid excretion.

In 1993, Verkade et al.[110,111] further progressed the understanding of bilirubin mediated uncoupling of lipid excretion. Male Groningen Yellow (GY; analogous to TR-, MRP2 deficient) rats and wild-type Wistars had their bile duct, duodenum and venous circulation cannulated. Bile and duodenal cannulas were joined to maintain the enterohepatic circulation of biliary components. When BDT was subsequently infused in both animals, its excretion was strongly and negatively correlated to the lipid:bile acid ratio of bile (r=-0.97), supporting a dose dependent effect of canalicular BDT in uncoupling lipid excretion. Upon gel filtration chromatogragphy of bile, bilirubin co-eluted with bile salts and not with biliary phospholipids or cholesterol, implicating an

intracanalicular association between hydrophilic anions and bile salts mediating the uncoupling effect[110].

To assess whether organic anions, including BDT, exerted their uncoupling effects by interfering with biliary micellisation, Verkade et al.[112] studied their effects in model bile systems. In a system comprising of taurocholate, phospholipid and cholesterol the addition of BDT (5mM) did not affect mean vesicle particle size, in contrast to that of other organic anions, Rose Bengal (hydrophobic) and sulphated taurolithocholate (hydrophilic). Unfortunately, the effect of BDT on formation of micelles was not assessed, and therefore, it is currently unknown whether BDT uncouples lipid excretion by directly interfering with micelle formation.

Kajihara et al.[113] later explored the role of bilirubin ditaurate infusion on biliary cholesterol and phospholipid excretion in wild type SD rats. In their study, i.v. BDT infusion had no effect on bile acid excretion, but it decreased biliary cholesterol and phospholipid content and increased the cholesterol:phosopholipid ratio. However, interestingly, the authors also showed a reduction in IgA and protein excretion after BDT infusion that could impair biliary immunity and perhaps contribute to the increased risk of gall stone formation in persons with hyperbilirubinbilia[105].

These findings were quickly followed by a report showing that BDT infusion[114], decreased total biliary lipid excretion and increased the biliary cholesterol to phospholipid ratio, in both bile and the canalicular membrane, without affecting the membrane saturated to unsaturated fatty acid ratio. A decrease in canalicular membrane fluidity was shown, without altered MRP2 protein expression. The authors argued MRP2 activity was likely reduced due to decreased membrane fluidity, which also has the potential to reduce canalicular lipid secretion as indicated previously[107].

1.5.3.2 *Effects of decreased bilirubin excretion on hepatic lipid excretion*

Although the ability of *increased* conjugated bilirubin to uncouple hepatic lipid excretion has been extensively studied (ie. canalicular bilirubin concentrations ~10-20 mM), little is known about the effects of *impaired* bilirubin conjugation/excretion (ie. canalicular bilirubin concentrations <1-2 mM)

on lipid export. The following paragraphs summarise the effects of inhibition of bilirubin conjugation/excretion on biliary lipid excretion in an attempt to answer this question.

1.5.3.2.1 Effects of reduced UGT1A1 activity

Studying the effects of pharmacological inhibition or genetic mutation in UGT1A1 on biliary lipid excretion represents a very attractive and physiologically relevant approach to studying the underlying perturbation in lipid status seen in Gilbert's syndrome (Table 1). To date, only one study has directly assessed basal biliary lipid excretion in the Gunn rat (versus SD rats). In this study, Apstein originally sought to explore the effects of i.v. bilirubin administration in the above animals (see previous section). This study showed that baseline biliary lipid excretion after taurocholate administration was not different between the groups. However, it should be noted that these results were obtained under non-physiological conditions, in that bile had been diverted for some time before experimentation and that the entero-hepatic circulation remained permanently disrupted. Contrasting, however indirect, evidence shows that the baseline biliary total cholesterol and phospholipid (PL) excretion in Gunn rats (TC 11.1 $nmol.min^{-1}kg^{-1}$; PL 116 $umol.min^{-1}kg^{-1}$)[34] is dramatically lower than that in wild-type Wistar rats (TC 64.4 $nmol.min^{-1}kg^{-1}$; PL 569 $umol.min^{-1}kg^{-1}$)[110]. It is important to note that the animals in these two studies were of similar weight and identical methods were used to quantify biliary lipids. However, the Gunn rats had consumed a high fat diet for seven weeks prior to the analysis. Furthermore, the bile composition in Gunn rats was assessed whilst animals were anaesthetised, which retards small bowel locomotion and bile acid absorption/transport back to the liver (which is coupled to biliary lipid secretion). Although, the effect of anaesthetics clearly confound these results, differences in the ratio of biliary phospholipid:cholesterol remained evident between the groups. For example, phospholipid:bile acid excretion ratios equalled 0.124 in Wistar rats and 0.159 in Gunn rats. Clearly this ratio was *increased* in anesthetised Gunn rats because bile acid excretion was reduced due to anaesthetisation. However, the cholesterol:bile acid ratios showed the opposite effect with *reduced* cholesterol excretion per unit of bile acid, even though bile acid excretion was much lower in Gunn rats (Wistar:

0.0140; Gunn 0.0105). These data indicate either reduced cholesterol synthesis and/or excretion in Gunn rats independent of reduced bile acid excretion encountered during anaesthesia. Although consumption of a high fat diet would be expected to increase biliary lipid excretion, surprisingly the ratio of cholesterol:bile acid excretion remained below that of wild type Wistar rats. These data, although far from conclusive, tentatively suggest that impaired UGT1A1 activity may reduce cholesterol biosynthesis/excretion and support studies showing marked reduction in circulating cholesterol species in Gunn rats[13,15].

1.5.3.2.2 Effects of reduced MRP2 activity

Studying the effects of pharmacological inhibition/competition or genetic mutation in MRP2 on biliary lipid excretion represents a novel approach to exploring the possibility of altered lipid status in Dubin-Johnson syndrome. It should be noted that an assessment of lipid status in Dubin-Johnson syndrome has not been conducted, however, data obtained from animal studies strongly supports the possibility of altered biliary lipid excretion in them. For example, Verkade[110] studied biliary lipid excretion in GY rats, who are deficient in MRP2, versus normal Wistar controls and found that biliary bile acid and phospholipid concentrations were higher in GY rats and is accompanied by significantly reduced bile flow. Importantly, the increased biliary phospholipid concentration in GY bile was accompanied by increased absolute excretion after correction for reduced bile flow, however, total bilirubin output remained non-significantly lower in GY animals. Interestingly, hepatocytes with dysfunctional/mutated MRP2 up-regulate the expression of MRP3[115,116], which also has affinity for bilirubin glucuronides and transports them back into the circulation. These observations are compatible with a compensatory effect, which aims to improve excretion of bilirubin from the hepatocyte[116] and suggest that impaired MRP2 activity, the canalicular transport protein for bilirubin glucuronide, increases hepatic phospholipid excretion.

Verkade et al.[111] then showed that decreased total bile flow in GY rats was associated with increased biliary bile acid concentration. Furthermore, the bile acid independent bile flow (BAIF) was significantly lower, compared to controls, in GY animals. In comparison to Wistar rats, decreased

BAIF in GY rats was associated with increased cholesterol and phospholipid concentrations in bile at similar bile acid concentrations. These data clearly implicate a role for reduced BAIF in increasing lipid excretion in MRP2 deficient rats, and is most evident when the physiological system is dependent on *de novo* bile acid synthesis.

Increased total lipid:bile acid excretion in GY rats indicated that reduced MRP2 activity (supported by elevated hepatic tissue bilirubin concentration in GY rats) is associated with improved coupling of lipid excretion[111]. From these data it is reasonable to assume that the coupling of lipid excretion could be mediated by decreased biliary organic anion concentration (including conjugated bilirubin)[107]. However, bilirubin concentration in the bile is similar between GY and Wistar animals[111]. Therefore, increased biliary lipid:bile acid might be associated with decreased concentrations of other MRP2 substrates, including glutathione, which is known to exert a potent osmotic effect and is probably responsible for the decreased BAIF in GY rats[117]. Therefore, decreased BAIF would increase exposure time of bile acids to the canalicular membrane which could increase lipid excretion[107].

Despite these results that link MRP2 dysfunction to increased biliary lipid excretion, little evidence exists to support lowered circulating lipid concentrations in these animals. So far, lipid analysis in Dubin-Johnson patients has not been published. Interestingly, in male MRP2 knockout mice, cholesterol concentrations were surprisingly greater compared to MRP2 competent animals. No difference in triacylglycerol levels were observed, and cholesterol and triacylglycerol levels were similar in female animals[118]. Plasma cholesterol and bile acid concentration are also elevated in the MRP2 mutant Eisai hyperbilirubinemic rat[119]. The mechanism responsible for increased circulating bile acid could be mediated by reduced sulphated bile acid conjugation/excretion into the bile by MRP2[120], resulting in spill-over into the circulation. The confirmation of a possible mechanism responsible for increased cholesterol efflux into the blood, however, remains to be elucidated.

The relevance of these findings to human conditions of impaired bilirubin excretion is currently far from conclusive, however, generally support a role for increased biliary lipid excretion in Dubin-Johnson syndrome, the human model of MRP2 insufficiency. Whether the same conclusion can be applied to persons with Gilbert's syndrome remains less likely, because most prior studies implicate a role for MRP2, and not conjugated bilirubin synthesis (UGT1A1), in uncoupling lipid excretion. Despite this, cholesterol:bile acid excretion ratios appear to be reduced in Gunn rats, and indicate reduced excretion of cholesterol into bile. Revealing whether this effect is mediated by reduced hepatic transport or *de novo* synthesis will represent a critical breakthrough in understanding the possible role of bilirubin, or its excretory pathway in modulating lipid homeostasis.

To prove (or disprove) the hypothesis that hepatic lipid homeostasis is perturbed in GS and contributes to decreased circulating lipid parameters the following questions require answering.

1) Is cholesterol and/or phospholipid biliary/fecal excretion lower in Gunn rats?

2) Does bilirubin administration or UGT1A1 knockout/inhibition contribute to 'uncoupling' of biliary lipid excretion, impaired cholesterol biosynthesis, altered cholesterol absorption or transepithelial intestinal cholesterol transport?

3) Is altered biliary or intestinal lipid homeostasis involved in the reported beneficial effect of GS towards CVD risk? and

4) Are the beneficial CVD and the potential lipid homeostatic effects caused by increased concentrations of unconjugated bilirubin or via loss of UGT1A1 function?

1.6 Relevance to CVD protection in the general population

Many observational studies exploring various population groups indicate a continuous positive relationship between coronary heart/related cardiovascular disease risk and blood cholesterol concentrations. A prospective meta-analysis with more than 90,000 subjects found a 12% reduction in all-cause mortality and a 19% reduction in coronary mortality per mM reduction in LDL cholesterol. Furthermore, a 23% reduction in the incidence of experiencing a major coronary event for the first time, and a 17% reduction in the incidence of stroke for the first time existed, per mM

LDL reduction. An LDL cholesterol reduction of 2 mM might be expected to reduce risk of vascular events by as much as 40%[121]. In the Multiple Risk Factor Intervention Trial investigating ~360 000 men, every 1 mM reduction in blood TC was associated with a ~50% lower risk of death from coronary heart disease, irrespective of blood cholesterol at baseline[122].

When considering the cholesterol lowering effects in subjects with diabetes over more than 4 years, an analysis of 14 randomised trials showed a 9% reduction in all-cause mortality and a 21% reduction in the incidence of major vascular events per mM LDL reduction[123]. The results of 58 randomised trials of cholesterol reduction by any means show for an LDL cholesterol reduction of 1.0 mM the risk of ischaemic heart disease (IHD) events was reduced by 11% in the first year of treatment, 24% in the second year, 33% in years three to five, and by 36% thereafter. A 51% reduction in IHD events was also reported after two or more years of LDL reduction treatment. After several years a reduction of 1.8 mM LDL would reduce IHD events by an estimated 61%[124].

These data convincingly show the benefit of reducing total and LDL cholesterol on cardiovascular events. Using the available highly controlled cross sectional studies in GS and control subjects [13,15] these authors showed a reduction of 0.39-0.83 mM in TC and a 0.34-0.91 mM reduction in LDL. Considering that GS manifests itself in adolescence with the lipid lowering effects lasting throughout the lifespan[15], bilirubin's secondary effects on cholesterol homeostasis could additionally explain a significant proportion of IHD protection observed in individuals with elevated bilirubin[125].

1.7 Additional mechanisms to explain CVD protection

In addition to the possible lipid lowering effects of bilirubin, it should be noted that bilirubin also induces cell cycle arrest in vascular smooth muscle cells (VSMCs) *in vitro* and *in vivo*. Currently, however, only data in conditions of significant hyperbilirubinemia support this conclusion. Stoekius et al. [126] recently reported that bilirubin (100 µM) inhibits Raf/ERK/MAPK phosphorylation, induces RB hypophosphorylation, leading to reduced YY1 transcription factor release and growth

arrest, but not apoptosis, in cultured human VSMCs. This report is supported by data in the Gunn rat [127], which shows reduced neointima formation after carotid artery balloon injury. In these experiments, Gunn rats possessed a bilirubin concentrations approximating 200 µM [127], well beyond physiological concentrations seen in controls and Gilbert's syndrome individuals. Ollinger et al. also showed similar results as Stoeckius et al.[126] however, also showed bilirubin's effects were independent of Ahr. When investigating the cell cycle effects of bilirubin, both groups implicated reduced MAPK signalling, leading to hypophosphorylation of RB and accumulation of cells in the G0/G1 cell cycle phase. Interestingly, these data are supported by clinical investigations showing that healthy individuals with elevated bilirubin concentrations have reduced carotid intima-media thicknesses [128]. The relevance of these data to prevention of CVD appears to indicate inhibition of endothelial dysfunction, smooth muscle proliferation and vascular inflammation, which can lead to progressive luminal obstruction and, potentially, atheroma development.

1.8   Future directions

Collectively, the associations described above indicate that bilirubin homeostasis may be related to lipid homeostasis and risk of CVD. It seems warranted to determine the interaction between bilirubin and lipid (patho)physiology in greater detail, since it may offer new targets and strategies for the prevention and treatment of CVD (see Figure 1). So far, published studies on lipid homeostasis in animal models for hyperbilirubinemia are scarce, as are studies on bilirubin homeostasis in animal models of hyperlipidemia, atherosclerosis and CVD. We propose to study and manipulate bilirubin and lipid homeostasis and determine the reciprocal effects, initially in well characterized animal models. Simultaneously, the effects of congenital variations in human bilirubin physiology, including GS and CN syndrome, on plasma lipids require further characterization.

1.9   Conclusions and perspective

This article has reviewed the relationship between circulating bilirubin and lipoprotein status in animal and human studies and discussed the potential effects of elevated UCB concentrations on

lipoprotein metabolism and excretion. It is acknowledged that the catabolism of heme (by *hmox*) can influence the circulating bilirubin concentration (in addition to impaired excretion), and therefore, other products of *hmox* including CO, might additionally mediate cardiovascular protection *in vivo* [129]. Assuming that most human studies were conducted in fasted subjects, an effect of elevated circulating UCB on lipid absorption is probably not justified or indeed possible to deduce. Fasted subjects, however, rely heavily upon intercellular stores and metabolism/mobilisation of lipoproteins, to maintain energy balance. Therefore, the perturbation of lipid status, particularly in studies that measured circulating cholesterol sub-fractions, would indicate an effect on endogenous lipoprotein metabolism. The most likely target organs would include the liver, intestine and fat pads, where UCB would accumulate, perhaps preventing lipid export or cholesterol metabolism. Bilirubin also has the potential to affect lipid metabolism, via aryl hydrocarbon receptor agonism, however, studies aimed at exploring bilirubin/AhR/hepatic lipid metabolism have not been conducted. Finally, the capacity of bilirubin excretion to affect bile acid induced lipid excretion was reviewed and concluded, from the available literature, that reduced bilirubin conjugation in Gilbert's syndrome (and therefore excretion) may affect hepatic lipid excretion. However, studies in Gunn rats and humans are required to conclusively determine a mechanism of action for bilirubin or UGT1A1.

In summary, although the mechanisms underpinning hypocholesterolemia and hypotriglyeridemia in GS remain to be discovered (Figure 1), the majority of studies confirm lowered circulating lipid concentrations in persons with higher bilirubin concentrations. Reduced IHD in persons with elevated UCB could be partly attributed to the antioxidant capacity of UCB. However, reduced LDL concentrations, and not bilirubin, robustly predict lower oxLDL concentrations, which is biomarker of CVD risk, in GS[13]. Furthermore, circulating LDL and TC are clearly related to risk of IHD and, therefore, the interaction of bilirubin with lipid metabolism must be considered when assessing protection from IHD in GS and in clinical studies. Collectively, these findings suggest that bilirubin is a physiological hypolipidemic agent that protects from cardiovascular disease.

Acknowledgement

Table 1. Summary of lipid status in the human condition of Gilbert's syndrome and individuals with UGT1A1 promotor polymorphisms.

Author	Experimental Model	Variables measured	Summary effect of hyperbilirubinemia[a]
Boon et al.[13]	22 GS 22 Control *Cross-sectional; age, gender and BMI matched*	TC, TAG, HDL, LDL, ox-LDL, BMI	↓TC*, ↓TAG[#], ↓LDL*, ↑HDL:LDL*, ↓ox-LDL (absolute), ↑ox-LDL (relative to LDL)
Bulmer et al.[3]	9 GS 12 Control *Cross sectional; age, height, weight matched*	TC, TAG, HDL, LDL, BMI	↑HDL:LDL[#]
Lin et al.[58]	1888 subjects 820 (6/6) 0.63 mg/dL TBIL 769 (6/7) 0.75 mg/dL TBIL 191 (7/7) 1.14 mg/dL TBIL *Longitudinal study*	LDL, HDL	No reported effect Great variability in [cholesterol] and [bilirubin] within groups
Lippi et al.[130]	163 GS 2047 Control *Cross-sectional study*	TC, TAG, HDL, LDL, TOTAL/HDL	No reported effect
Maruhashi et al.[55]	108 GS 108 Control *Cross-sectional study, all male, age matched*	TC, TAG, HDL, LDL, ox-LDL, BMI	↓ox-LDL (absolute)
Ocadlik et al.[14]	40 GS 60 Control *Cross-sectional study, similar age and BMI*	TC, TAG, HDL, LDL+sub-fractions, VLDL, IDL	↓TRIGS*, ↓LDL sub-fractions 3-7*, ↓VLDL* ,↓IDL* (↓TRIGS*, ↑LDL 1,2*, ↓LDL 3-7*)
Smiderle et al.[57]	155 pre-menopausal women 50 (6/6), 72 (7/6), 19 (7/7) *Cross sectional study*	TC, TAG, HDL, LDL	↓TC*, ↓LDL* 6/7, 7,7 vs. 6/6 No effect in post-menopausal women

Tapan et al.[131]	25 GS 53 Control *Cross sectional; all male, age, BMI matched*	TC, TAG, HDL, LDL, BMI	↓ BMI[#]
Tapan et al.[56]	42 GS 52 Control *Cross sectional; all male, age, BMI matched*	TC, TAG, HDL, LDL, sd-LDL, ox-LDL, BMI	↓ TC[#], ↓ sd-LDL*, ↓ ox-LDL*
Vitek et al.[7]	50 GS 38 Control *Longitudinal study; similar age*	TC, HDL, LDL	↑ TC*, ↑ LDL* (many more males in GS group)
Vitek et al.[27]	33 GS 25 Control *Cross sectional; similar gender*	TC, TAG, HDL, LDL	↓ TAG*
Wallner et al.[15] (unpublished data)	38 GS 38 Controls *Cross sectional, age and gender matched*	TC, TAG, LDL+sub-fractions, HDL, SAA, BMI	↓ TC[#], ↓ LDL[#], ↓ LDL sub-fractions 1-2*, ↓ SAA[#], ↓ BMI*
Yesilova et al.[11]	17 GS 15 Control *Cross sectional, age matched, similar gender distribution*	TC, TAG, HDL, LDL	↓ TAG[#], ↑ LDL[#]

[a] Significance of effect indicated by *P<0.05*, [#]*P<0.15*.

Abbreviations: BMI, body mass index; GS, Gilbert's Syndrome; MS, metabolic syndrome; OR, odds ratio; SAA, Serum amyloid A; TBIL, total bilirubin; TC, total cholesterol; TAG, triacylglycerols; sd-LDL, small dense LDL; ox-LDL, oxidized LDL, WC, waist circumference. Summary in parentheses indicate correlative/regression analysis.

Table 2. Summary of the relationship between bilirubin and lipid status in clinical studies.

Author	Experimental Model	Variables measured	Summary effect of hyperbilirubinemia[a]
Andersson et al.[74]	10198 overweight/obese subjects *Longitudinal study*	TAG, HDL, LDL, BW	(↓TAG*,↑HDL*,↑LDL*,↓BW*) ↑UCB* with >2% weight loss ↑UCB* with 1% weight loss in men vs. women
Bhuiyan et al. [65]	777 subjects *Cross-sectional study*	TAG, non-HDL, HDL, BMI	(↓TAG*,↓non-HDL*,↑HDL*, ↓BMI*)
Breimer et al. [61]	7685 male subjects *Longitudinal study*	TC, HDL, BMI	↓TC*, ↑HDL*
Chin et al.[71]	1458 IgA nephropathy patients *Longitudinal study*	TC	↓TC*
Chen et al.[72]	750 Hemodialysis patients *Longitudinal study*	TC, TAG, HDL, LDL, BMI	No reported effect
Choi & Choi[62]	12342 subjects *Cross-sectional study*	TC, TAG, HDL, LDL, BMI, WC	↓TC*, ↓TAG*, ↑HDL*, ↓ WC*, ↓BMI*; ↓OR for MS in males and females in ↑ bilirubin group; ↓ OR for abdominal obesity in ↑ bilirubin group
Ko et al.[66]	907 male subjects 601 female subjects *Cross-sectional study*	TC, TAG, LDL, HDL, VLDL, WHR, BMI	↓TAG*, ↑HDL*, ↓VLDL*,↓ BMI* (↓TAG*, ↓VLDL*, ↓BMI[#])
Kwon et al.[67]	5266 female subjects *Cross-sectional study*	TAG, HDL, BMI, WC	↓TAG*, ↑HDL*, ↓ WC*, ↓ BMI*; ↓OR for MS in all ↑ bilirubin groups (versus lowest bilirubin group)

Horsfall et al.[70]	504206 subjects *Longitudinal study*	BMI	↓BMI*
Hwang et al.[69]	2298 male subjects 2492 female subjects *Cross-sectional study*	TC, TAG, HDL, LDL, WC, BMI, BF	(↓TAG*, ↓HDL*, ↓LDL*, ↓BMI*, ↓WC*, ↓BF*), ↓OR for MS in females
Hwang et al.[68]	1013 male subjects 1294 female subjects *Cross-sectional study*	TC, TAG, HDL, LDL, WC, BMI	↓BMI*, (↑HDL*, ↓TAG*, ↓BMI*, ↓WC*)
McArdle et al.[64]	460 male subjects 408 female subjects *Cross-sectional study*	TC, TAG, HDL, LDL, WC, BMI	(↓TC*, ↓TAG#, ↓LDL*, ↓BMI*, ↓WC#) No causal effect of bilirubin concluded
Temme et al.[63]	5949 male subjects 5353 female subjects *Longitudinal study*	TC, BMI, BW	↓ TC#, ↑/↓ BMI*, ↑/↓ BW*; M/F respectively

[a] Significance of effect indicated by *$P<0.05$*, #*$P<0.15$*.

Abbreviations: BMI, body mass index; BW, body weight; MS, metabolic syndrome; OR, odds ratio; TC, total cholesterol; TAG, triacylglycerols; sd-LDL, small dense LDL; ox-LDL, oxidized LDL, WC, waist circumference; WHR, waist to hip ratio. Summary in parentheses indicate correlative/regression analysis.

Table 3. Circulating lipid variables in Gunn versus non-jaundiced rats.

Author	Experimental Model	Variables measured	Summary effect of hyperbilirubinemia[a]
Boon et al.[13]	7 Gunn 5 Wistar *56 weeks old, female*	TC, TAG, HDL, LDL, mass	↓TC*, ↓HDL*, ↓HDL:LDL*, ↓mass*
Fu et al.[79]	5 Gunn 7 Wistar *5 weeks old, male*	TC, glucose, mass	↓TC*, ↓ glucose[#], ↓mass*
Sakamoto et al.[80]	8 Gunn 8 Wistar *8-11 weeks old, male*	Non-HDL, HDL	↓non-HDL[§]
Wallner et al. [15] (unpublished data)	20 Gunn 20 Wistar *12 weeks old, male and female*	TC, TAG, HDL, LDL, mass	↓TC*, ↓HDL*, ↓LDL*, differences most significant in females, ↓mass* in female Gunn rats only

[a] Significance of effect indicated by *P<0.05, [#]P<0.15, [§] *extrapolated from graphical data*
Abbreviations: TC, total cholesterol; TAG, triacylglycerols.

Figure Headings

Figure 1. Diagrammatic representation of the digestive tract/organs, identifying potential sites of action and effects of elevated circulating unconjugated bilirubin on lipid homeostasis in Gilbert's syndrome individuals (**A:** decreased hepatic cholesterol synthesis; **B:** decreased hepatic cholesterol excretion; **C:** increased intestinal cholesterol excretion).

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