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Sekreční dráha bilirubinu a její poruchy

Bilirubin secretory pathway and its disorders

Disertační práce

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Abstrakt

Identifikace a funkční charakterizace řady přenašečových systémů na sinusoidální a kanalikulární membráně hepatocytů významně přispěly k objasnění molekulární podstaty dědičných hyperbilirubinémií. Porucha regulace hepatobiliárních transportních systémů se rovněž podílí na vzniku žloutenky u mnoha získaných jaterních chorob. Předkládaná práce rozšiřuje současné znalosti metabolické dráhy degradace hemu s důrazem na nové poznatky o mechanismu transportu bilirubinu v hepatocytech, vyplývající z objasnění molekulární podstaty Rotorova syndromu.

První předkládaná práce se zabývá antioxidačním působením bilirubinu v jaterní tkáni u modelu obstrukční žloutenky. Ve druhé práci jsme v rámci charakterizace nemocných s dědičnými formami konjugované hyperbilirubinémie zachytili několik nových mutací v genu *ABCC2*, jehož deficit podmiňuje Dubin-Johnsonův syndrom. V klíčové třetí práci o Rotorově syndromu jsme ukázali, že příčinou hyperbilirubinémie Rotorova typu je digenní porucha jaterního vychytávání konjugovaného bilirubinu podmíněná deficitem OATP1B1 a OATP1B3. Ukazuje se, že přímo do žluče je secernována pouze část bilirubinu konjugovaného v hepatocytech. Zbytek je nejprve vyloučen do krve prostřednictvím transportéru MRP3 a teprve následně vychytán zpět sinusoidálními transportéry OATP1B1 a OATP1B3. Ve čtvrté práci jsme potvrdili, že při konjugované hyperbilirubinémii provázející pokročilá stádia cholestatických onemocnění jater dochází ke snížení exprese rotorovských transportérů. Dále jsme zjistili, že exprese proteinů OATP1B inverzně koreluje s hladinou konjugovaného i celkového bilirubinu v séru. Je tedy velmi pravděpodobné, že pokles exprese až absence OATP1B1 a OATP1B3 je vedle zvýšení exprese MRP3 dalším mechanismem přispívajícím ke zvýšení konjugované složky hyperbilirubinémie v terminálních stádiích jaterních chorob provázených zejména obstrukčním typem cholestázy.

Klíčová slova: Bilirubin, hyperbilirubinémie, žloutenka, cholestáza

Abstract

Identification and functional characterization of numerous transport systems at the sinusoidal and canalicular membrane of hepatocytes have significantly expanded our understanding of bilirubin metabolism and contributed to elucidation of molecular basis of hereditary jaundice. Moreover, dysregulation of hepatobiliary transport systems could explain jaundice in many acquired liver disorders. This thesis is focused on the new aspects of bilirubin handling in hepatocytes based on elucidation of the molecular basis of Rotor syndrome.

The first study is focused on the antioxidative properties of bilirubin in liver tissue in a model of obstructive cholestasis. In the second part of the thesis we present several novel mutations in *ABCC2,* the gene associated with Dubin-Johnson syndrome, identified in patients selected for the Rotor locus mapping study. In the key third study concerned with Rotor syndrome we demonstrated that biallelic inactivating mutations causing complete absence of transport proteins OATP1B1 and OATP1B3 result in disruption of hepatic reuptake of bilirubin, which is the molecular basis of Rotor-type jaundice. These results indicate that apart from secretion of conjugated bilirubin into bile, a significant fraction of bilirubin glucuronide is secreted via MRP3 into sinusoidal blood and subsequently reuptaken by sinusoidal transporters OATP1B1 and OATP1B3. We further confirmed that Rotor proteins are down-regulated in advanced stages of cholestatic liver disorders. We demonstrated that OATP1Bs expression inversely correlates with serum levels of conjugated and total bilirubin. We suppose that aside from increased MRP3 expression, down-regulation of OATP1B1 and OATP1B3 contributes to conjugated hyperbilirubinemia in advanced liver diseases with predominantly obstructive type of cholestasis.

Keywords: Bilirubin, hyperbilirubinemia, jaundice, cholestasis

CONTENT

ABBREVIATIONS

ABC	ATP-binding cassette sub-family
ABCB1	ATP-binding cassette, subfamily B, member 1
ABCB4	ATP-binding cassette, subfamily B, member 4
ABCB11	ATP-binding cassette, subfamily B, member 11
ABCC1	ATP-binding cassette, subfamily C (CFTR/MRP), member 1
ABCC2	ATP-binding cassette, subfamily C (CFTR/MRP), member 2
ABCC3	ATP-binding cassette, subfamily C (CFTR/MRP), member 3
ABCC4	ATP-binding cassette, subfamily C (CFTR/MRP), member 4
ABCC6	ATP-binding cassette, subfamily C (CFTR/MRP), member 6
ABCC7	ATP-binding cassette, subfamily C (CFTR/MRP), member 7
ABCC13	ATP-binding cassette, subfamily C (CFTR/MRP), member 13, pseudogene
ABCG2	ATP-binding cassette, subfamily G, member 2
ABCG5/ABCG8	ATP-binding cassette, sub-family G, member 5/8
ATP	adenosine triphosphate
ATP8B1	ATPase, aminophospholipid transporter, class I, type 8B, member 1
BDL	bile duct ligation
BIND	bilirubin-induced neurological dysfunction
BRIC 1-3	Benign Recurrent Intrahepatic Cholestasis 1-3
BSEP	bile salt export pump
BSP	bromosulphophtalein
CFTR	cystic fibrosis transmembrane conductance regulator
DAMPs	damage-associated molecular patterns
DHEAS	dehydroepiandrosterone sulphate
DJS	Dubin-Johnson syndrome
FXR	farnesoid X-activated receptor
GSH	reduced glutathione
GSSG	oxidized GSH
GST	glutathione-S-transferase (EC2.5.1.18)
gtBPREM	glucuronosyltransferase phenobarbital response enhancing motif
GS	Gilbert syndrome
HGNC	HUGO Gene Nomenclature Committee
HNF1α	hepatocyte nuclear factor 1α
HNF3β	hepatocyte nuclear factor 3β
HMOX	heme oxygenase (EC1.14.99.3)

HMOX-1	heme oxygenase, isoform 1
HMOX-2	heme oxygenase, isoform 2
Hmox2-ps1	heme oxygenase 2, pseudogene 1 (Rattus norwegicus)
Hmox-3	putative heme oxygenase 3
ICP	intrahepatic cholestasis of pregnancy
IR-1	inverted repeat DNA element with 1 nucleotide spacing
LPAC	Low Phospholipid-Associated Cholelithiasis Syndrome
LXR	liver X receptor
MDR1	multidrug resistance protein 1
Mdr2	rodent multidrug resistance protein 2
MDR3	multidrug resistance protein 3
MRP1	multidrug resistance-associated protein 1
Mrp1	rodent multidrug resistance-associated protein 1
MRP2	multidrug resistance-associated protein 2
MRP3	multidrug resistance-associated protein 3
MRP4	multidrug resistance-associated protein 4
MRP6	multidrug resistance-associated protein 6
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NTCP	Na+-taurocholate cotransporting polypeptide/solute carrier family 10 member 1
OATP	organic anion-transporting polypeptide
Oatp	rat organic anion-transporting polypeptide
OATP1B1	organic anion-transporting polypeptide 1B1
OATP1B3	organic anion-transporting polypeptide 1B3
OCT	organic anion transporter
OCTN	organic zwitterion/cation transporter
OMIM	electronic database Online Mendelian Inheritance in ManTM
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PFIC 1-3	Progressive Familial Intrahepatic Cholestasis 1-3
Pgp	P-glycoprotein subfamily of ABC superfamily
PXR	pregnane X receptor
RES	reticuloendothelial system
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species

RS	Rotor syndrome
RXR	retinoid X receptor
SLC	solute carrier superfamily
SLC10A1	solute carrier superfamily 10 (sodium/bile acid cotransporter), member 1
SLC22A	solute carrier family 22 (organic cation transporter), member 1
SLCO	solute carrier organic anion transporter family
SLCO1B1	solute carrier organic anion transporter family member 1B1
SLCO1B3	solute carrier organic anion transporter family member 1B3
SNP	single nucleotide polymorphism
UCB	unconjugated bilirubin
UGT	uridine diphosphate glucuronosyltransferase (EC2.4.1.17)
UGT1A1	uridine diphosphate glucuronosyltransferase 1A

1. INTRODUCTION

1.1 Heme degradation pathway

1.1.1 Sources of heme and mechanism of its breakdown

Bilirubin is the end product of heme breakdown. About 80% of bilirubin originates from degradation of erythrocyte haemoglobin in the reticuloendothelial system (RES); the remaining 20% comes from inefficient erythropoiesis in bone marrow and degradation of other heme proteins (Stadeler G., 1864, London I.M. et al., 1950, Berk P.D. et al., 1979). Under physiological conditions, senescent red blood cells are engulfed by macrophages of RES; globin is converted into amino acids while the porphyrin ring of heme is split during oxidation process mediated by heme oxygenase (HMOX) system (Tenhunen R. et al., 1968). HMOX cleaves the heme ring at the alpha-methine bridge to form either biliverdin or, if the heme is still attached to a globin, verdoglobin (Evans J.P. et al., 2008). The overall reaction scheme is as follows:

$$\text{Heme b } (Fe^{3+}) + 3O_2 + 4(NADPH + H^+) \rightarrow \text{biliverdin} + Fe^{3+} + CO + 4NADP^+ + 3H_2O$$

Two isoforms of HMOX have been described. Heme oxygenase 1 (HMOX1, HO-1; 33 kDa), also known as heat shock protein HSP32, is the predominant isoform in the liver and spleen, inducible in response to oxidative stress (Müller R.M. et al., 1987). Heme oxygenase 2 (HMOX2, HO-2; 36 kDa) is a constitutive isoform expressed under homeostatic conditions (McCoubrey W.K. Jr. and Maines M.D., 1994). HMOX2 is primarily found in brain and testis. Both HMOX1 and HMOX2 are catalytically active (Maines M.D., 1997, Kikuchi G. et al., 2005). The third rat heme oxygenase, putative heme oxygenase 3 (Hmox2-ps1, Hmox3, HO-3), is thought to be a pseudogene derived from *Hmox2* gene (McCoubrey W.K. Jr. et al., 1997, Hayashi S. et al., 2004).

Linear tetrapyrrole biliverdin, ferric iron (Fe^{3+}) and carbon monoxide (CO) represent the end products of the oxidation process. Subsequently, biliverdin is converted by biliverdin reductase to bilirubin.

1.1.2 Bilirubin uptake

Water insoluble, unconjugated bilirubin (UCB) bound to albumin is transported to the liver where it is removed from the plasma. The exact mechanism of UCB uptake is unknown; however, passive transmembrane diffusion seems to be combined with active transport mediated by several sinusoidal transporters. Within the cytoplasm of hepatocytes, bilirubin is bound to major cytosolic proteins, glutathione-S-transferases (GSTs, formerly ligandin or Y-protein) and transported to the endoplasmic reticulum (ER) where conjugation with glucuronic acid takes place (Levi A.J. et al., 1969). Binding to GSTs reduces the efflux of bilirubin from hepatocytes and inhibits non-specific diffusion into various subcellular compartments (Kamisaka K. et al., 1975).

1.1.3 Conjugation of bilirubin

Conjugation of bilirubin is a two step process with a transfer of two glucuronic acid groups sequentially to the propionic acid groups of the bilirubin. The major product is bilirubin diglucuronide (Fig. 1). The reaction is catalysed by the enzyme uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1), a member of an enzyme family in the ER and nuclear envelope of hepatocytes (Burchell B., 1977, Burchell B., 1981, Roy-Chowdhury J. et al., 1986, Bosma P.J. et al., 1994). In addition to the liver, UGT activity has been detected in the small intestine and kidney (Kokudo N. et al., 1999, Fisher M.B. et al., 2001).

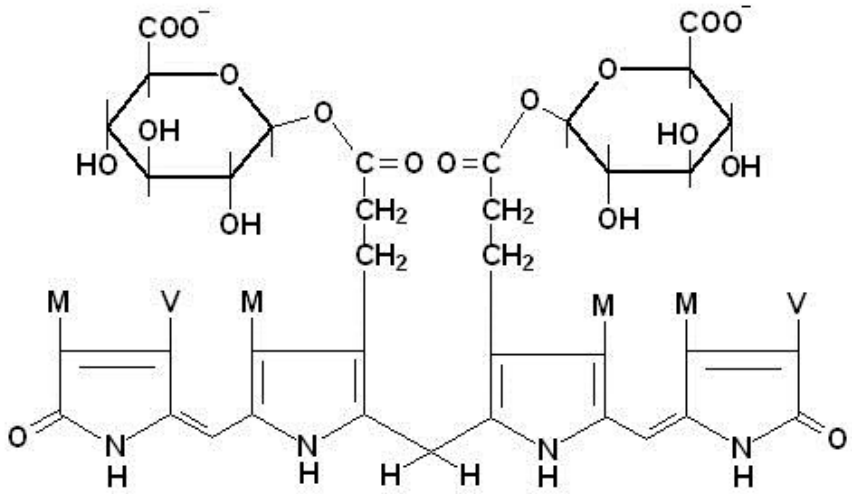

Fig. 1: **Structure of bilirubin diglucuronide.** Glucuronic acid is attached via ester linkage to the two propionic acid groups of bilirubin to form an acylglucuronide. M and V: methyl and vinyl groups. Adapted from: Murray K. et al., 2003.

UGT1A1 gene is a part of a complex locus encoding 13 UDP-glucuronosyltransferases. The locus spans approximately 200 kbp and contains a series of thirteen unique alternate promoters and first exons, followed by four common exons No. 2 - 5. Theoretically, each of the unique first exons is spliced to the first of the four shared exons. The unique first exons encode different substrate binding domains whereas the other functional domains encoded by the shared exons 2 – 5 are the same. In reality, only 9 of the 13 predicted *UGT1A*s are active genes encoding functional enzymes; four are non-functional pseudogenes (Burchell B. et al., 1998, Tukey R.H. and Strassburg C.P., 2000, Gong Q.H. et al., 2001, Owens I.S. et al., 2005, Mackenzie P.I. et al., 2005) (Fig. 2).

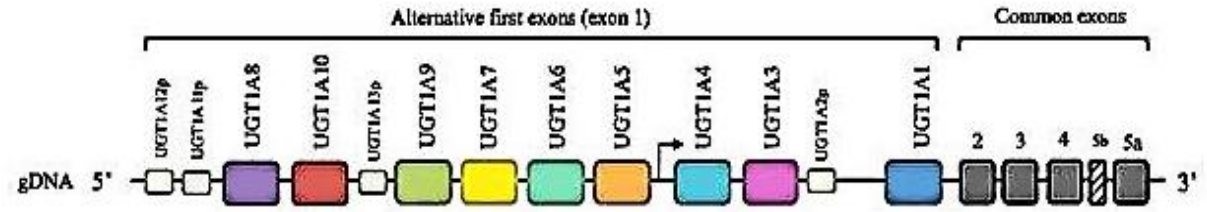

Fig. 2: **The human *UGT1A* locus.** The locus of *UGT1A* contains multiple alternative first exons. Each unique first exon has its own promoter site. The individual exons for each isoform are combined with the common exons 2-4 and 5a by splicing out any intervening sequence. Exons 2-4 and 5a are therefore present in every *UGT1A* isoform. Adapted from: GKBPharm® The Pharmacogenomics Knowledgebase (http://www.pharmgkb.org/gene/PA420?tabType=tabVip).

1.1.4 Canalicular excretion of conjugated bilirubin

Conjugated bilirubin is transported into bile against a concentration gradient. The energy for this unidirectional active transport is derived from adenosine triphosphate (ATP) hydrolysis by the canalicular ATP-binding cassette (ABC) protein identified as the multidrug resistance-associated protein MRP2/cMOAT and possibly, to a lesser extent, by ABC efflux transporter ABCG2 (Nishida T. et al., 1992, Paulusma C.C. et al., 1996, Haimeur A. et al., 2004, Vlaming M.L. et al., 2009). Transport mechanism mediated by MRP2 is supposed to be rate-limiting for the entire process of hepatic bilirubin metabolism. Maximum bilirubin secretory capacity (Tmax) into bile depends on bile salt-dependent and non-bile salt-dependent components. Bile acids increase the trafficking of vesicles containing MRP2 and the bile salt export pump (BSEP) from the Golgi apparatus to the canalicular domain of hepatocytes (Gatmaitan Z.C. et al., 1997).

1.1.5 Transporters of biliary lipids and pigments in hepatocytes

To secrete bile and excrete various metabolites either of endogenous or exogenous origin, liver cells must transport bilirubin, bile salts, phospholipids, and other substrates from portal blood to bile against a concentration gradient. Hepatocytes express polarized transport systems at the basolateral (sinusoidal) and apical (canalicular) plasma membrane domains (Fig. 3).

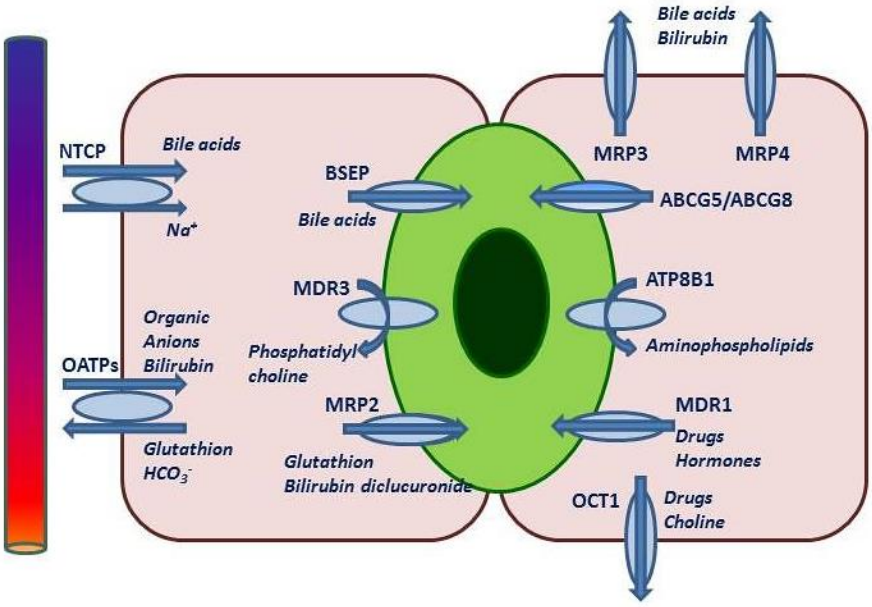

Fig. 3: **Membrane transporters in human hepatocytes.** Transporters expressed at the basolateral membrane are responsible for the uptake of bile salts (NTCP), amphiphilic subtrates (OATPs), and organic cations (OATPs, OCT1). MRP3 and MRP4 transporting bile salts and anionic conjugates are expressed at low levels in normal hepatocytes but are up-regulated during cholestasis. Transporter proteins at the canalicular domain of hepatocytes are responsible for the biliary secretion of bile salts, cholesterol, phosphatidylcholine, bilirubin conjugates, and glutathione. These transporters include BSEP, MRP2, MDR1, MDR3 and ABCG5/G8. ATP8B1 acts as aminophospholipid flippase. Its activity is essential to maintain membrane lipid asymmetry responsible for canalicular membrane stability.

1.1.5.1 Basolateral transport proteins

Basolateral transport systems involved in blood secretion and liver uptake of small molecules belong to the solute carrier (SLC) superfamily and MRP subfamily of the ATP-binding cassette (ABC) transporter superfamily, respectively. In general, ABC transporters are considered to be responsible for efflux of substrates, while SLC transporters mediate uptake of substrates into cells. SLC proteins include the Na^+-dependent transporter for the uptake of bile salts (NTCP; gene symbol *SLC10A1*), transporters for amphiphilic substrates such as organic anion-transporting polypeptides (OATPs; gene family *SLCO*, former *SLC21A*), and organic

cation transporters (OCTs; gene family *SLC22A*) (Hagenbuch B. and Meier P.J., 1996, Kullak-Ublick G.A. et al., 2000, Roth M. et al., 2012).

The Na^+-taurocholate cotransporting polypeptide (NTCP) represents the major bile salt uptake system of hepatocytes, whose driving force, the Na^+ concentration gradient, is maintained by Na^+/K^+-ATPase. Except for unconjugated and conjugated bile salts, this protein also transports sulphated steroids and thyroid hormones (Hagenbuch B. et al., 1991, Meier P.J. et al., 1997). NTCP deficiency has recently been described as a new inborn error of metabolism (Vaz F.M. et al., 2014, MIM not available yet). The entity is characterized by a relatively mild clinical phenotype with conjugated hypercholanemia.

Organic anion-transporting polypeptides (OATPs in humans, Oatps in rodents) are multispecific ATP- and Na^+-independent uptake transporters expressed in numerous epithelial cells throughout the body, transporting predominantly large and hydrophobic organic anions, such as conjugated and unconjugated bile salts, bilirubin, cardiac glycosides, thyroid and steroid hormones (estrogens and neutral steroids), selected organic cations, anti-HIV drugs, statins, and wide range of chemotherapeutics (Roth M. et al., 2012, Meier P.J. et al., 1997, Sissung T.M. et al., 2010). Based on their amino acid sequence identity, the different OATPs cluster into families (with more than 40% amino acid sequence identity) and subfamilies (more than 60% amino acid identity) (Hagenbuch B. and Meier P.J., 2004). The human and rodent OATPs form 6 families (OATP1-6) and 13 subfamilies. So far, eleven human OATPs have been identified (Hagenbuch B. and Stieger B., 2013) (Tab.1).

OATP1B1 (also termed OATP-C, OATP2, SLC21A6 or LST-1, gene *SLCO1B1*) and OATP1B3 (OATP8, SLC21A8 or LST3, gene *SLCO1B3*), the major OATP1Bs in humans, are highly homologous proteins with similar genomic organization into 15 exons, of which 12 are identical in length. Both proteins are glycosylated and have close secondary structures with 12 predicted transmembrane helices with both termini located intracellularly (König J. et al., 2000a, König J. et al., 2000b). Expression of *SLCO1B1* and *SLCO1B3* is restricted to human hepatocytes and the corresponding protein products are localized to the basolateral (sinusoidal) membrane (Abe T. et al., 1999, König J. et al., 2000b) (Fig. 4). While OATP1B1 is expressed throughout the lobule, OATP1B3 is predominantly localized to the centrilobular zone (König J. et al., 2000a, Ho R.H. et al., 2006).

Tab. 1: **The human members of the organic anion transporting superfamily**
Adapted from: Hagenbuch B. and Stieger B., 2013.

New gene symbol	New protein name	Predominant substrates	Tissue distribution	Human gene locus	Sequence Accession ID	Splice variants
SLCO1A2	OATP1A2	Bile salts, organic anions and cations	Brain (endothelial cells), kidney (apical), intestine (apical), liver (cholangiocytes), eye (ciliary body)	12p12	NM_021094 NM_134431	2
SLCO1B1	OATP1B1	Bile salts, organic anions	Liver (hepatocytes)	12p	NM_006446	1
SLCO1B3	OATP1B3	Bile salts, organic anions	Liver (hepatocytes)	12p12	NM_019844	1
SLCO1C1	OATP1C1	Thyroid hormones, BSP	Brain (blood-brain barrier), testis (Leydig cells)	12p12.2	NM_017435 NM_001145944 NM_001145945 NM_001145946	4
SLCO2A1	OATP2A1	Prostaglandins	Ubiquitous	3q21	NM_005630	
SLCO2B1	OATP2B1	estrone-3-sulphate, DHEAS, BSP	Liver (hepatocytes), placenta, intestine (apical), eye (ciliary body)	11q13	NM_007256 NM_001145211 NM_001145212	3
SLCO3A1	OATP3A1	estrone-3-sulphate, prostaglandin	Testis, heart, brain, ovary	15q26	NM_013272 NM_001145044	2
SLCO4A1	OATP4A1	Taurocholate, triiodothyronine, prostaglandin	Ubiquitous	20q13.33	NM_016354	
SLCO4C1	OATP4C1	Digoxin, ouabain, thyroid hormones, methotrexate	Kidney (basolateral)	5q21.2	NM_180991	
SLCO5A1	OATP5A1			8q13.3	NM_030958 NM_001146008 NM_001146009	3
SLCO6A1	OATP6A1		Testis	5q21.1	NM_173488	

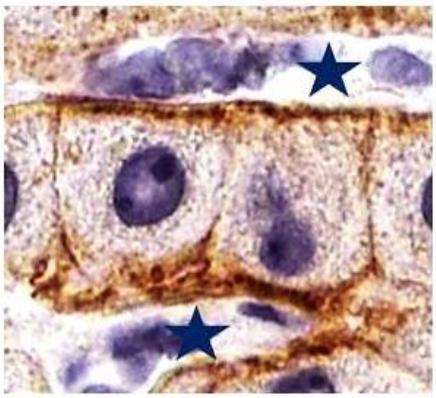

Figure 4: **Immunohistochemical expression of OATP1B1/3 transporter proteins.** Positive reaction localized to the sinusoidal membrane of hepatocytes stains brown. Asterisks mark sinusoidal space with Kupffer cell nuclei. Formalin-fixed liver tissue; MDQ mouse monoclonal anti-OATP2 antibody (ab15442, Abcam, Cambridge, UK). Original magnification x400.

Expression of OATP1B proteins can be regulated at the transcriptional and/or protein level (Roth M. et al., 2012). Constitutive OATP1B1 expression appears to be dependent on HNF1α, while OATP1B3 is likely regulated by HNF3β (Jung D. and Kullak-Ublick G.A., 2003, Vavricka S.R. et al., 2004).

Several polymorphisms in *SLCO1B1* and *SLCO1B3* have been identified. Some of them may affect kinetics and disposition to transport various OATP1B substrates of either endogenous or exogenous origin (Kalliokoski A. et al., 2008, Kalliokoski A. and Niemi M., 2009, Niemi M. et al., 2011, Schwarz U.I. et al., 2011). The polymorphism p.N130D, found in OATP1B1*1b and *15 haplotypes, seems to be responsible for developing severe hyperbilirubinemia in neonates (Büyükkale G. et al., 2011) and the OATP1B1*15 haplotype is associated with higher serum bilirubin levels in healthy adults (Ieiri I. et al., 2004, Zhang W. et al., 2007). In addition, recent study has shown that two non-coding *SLCO1B3* variants may contribute to idiopathic mild unconjugated hyperbilirubinemia (Sanna S. et al., 2009). These data, together with the observations of other groups (Cui Y. et al., 2001, Briz O. et al. 2003), support the concept that OATP1Bs are involved in liver uptake of unconjugated bilirubin.

Organic cation transporter OCT1 is a member of the SLC22 family which contains 13 functionally characterized human plasma membrane proteins, each with 12 predicted α-helical transmembrane domains. The family comprises organic cation transporters (OCTs), organic zwitterion/cation transporters (OCTNs), and organic anion transporters (OATs) (Koepsell H., 2013). OCT1 operates as an uniporter mediating facilitated diffusion and is responsible for Na^+-independent transport of a variety of small organic cations, both endogenous and exogenous, including drugs, choline, or monoamine neurotransmitters, down their electrochemical gradients (Jonker J.W. and Schinkel A.H., 2004, Nies A.T. et al., 2011). Except for sinusoidal membrane of human hepatocytes, OCT1 protein is localized to the luminal membrane of lung epithelial cells (Koepsell H., 1998, Lips K.S. et al., 2005) and weak expression of OCT1 mRNA has been

demonstrated in other tissues, such as heart, skeletal muscles, kidney, brain, placenta, and basophilic leukocytes (Gorboulev G. et al., 1997, Zhang L. et al., 1997, Koepsell H. et al., 2007).

The basolateral domain of hepatocytes also possesses several ATP-dependent efflux pumps that constitute the multidrug-resistance protein (MRP) subfamily (ABCC) (Müller M. and Jansen P.L., 1997, Hooiveld G.J. et al., 2001). The ABCC proteins transport organic anions with broad substrate specificity. MRP2/ABCC2 is the only representative to be localized apically (see below), whereas MRP1, 3, 4, 5 and 6 are sinusoidal or lateral proteins (Kool M. et al., 1997).

MRP1 (gene *ABCC1*; Mrp1 in rodents) was the first transporter of the ABCC subfamily identified in the liver. The level of MRP1/Mrp1 mRNA and protein in resting hepatocytes is very low (Müller M. and Jansen PL, 1998), however, mRNA levels of *Mrp1* are considerably increased after endotoxin administration and partial hepatectomy (Vos T.A. et al., 1998, Vos T.A. et al., 1999). Furthermore, *MRP1* mRNA and protein levels are increased in human hepatoblastoma HepG2 cells (Roelofsen H. et al., 1997). Physiologically important substrates for MRP1 include glutathion S-conjugates such as leukotriene C_4, bilirubin- and estrogen-glucuronides, taurolithocholate 3-sulphate, and oxidized glutathione (GSSG) (König J. et al., 1999a). In addition, transport of several cationic drugs in the presence of reduced glutathione (GSH) has been observed (Renes J. et al., 1999).

MRP3 (gene *ABCC3*) is the basolateral transporter of bile salts, glucuronide and anionic conjugates including glutathione (König J. et al., 1999b, Zeng H. et al., 2000, Lee Y.M. et al., 2004). It is expressed predominantly in the centrilobular hepatocytes, however, Northern blotting of various human tissues indicated the expression of MRP3 in the colon, bile duct epithelium, gallbladder, pancreas, and kidney (Cherrington N.J. et al., 2002). Under physiologic circumstances MRP3 expression in the liver is low. The expression rate is up-regulated during cholestasis or, independently of any cholestatic manifestation, in individuals with Dubin-Johnson syndrome (DJS) and after repeated administration of ethinylestradiol (König J. et al., 1999b, Donner M.G. and Keppler D., 2001, Ruiz M.L. et al., 2013).

MRP4 (gene *ABCC4*) is an inducible basolateral transporter co-transporting GSH, taurine and glycine conjugates of cholic acid. It is also a high-affinity transporter of sulphated bile salts, the sulphate conjugate of dihydroepiandrosterone, eicosanoids, uric acid, and signalling molecules, such as cAMP and cGMP (Ritter C.A. et al., 2005, Russel F.G. et al., 2008). Although MRP4 expression in the liver is low, it can be induced by bile salts in cholestasis (Denk G.U. et al., 2004, Borst P. et al., 2007).

MRP6 (gene *ABCC6*), constitutively expressed at high levels on the lateral surface of hepatocytes, was demonstrated to transport the anionic cyclopentapeptide BQ123, an endothelin receptor antagonist (Madon J. et al., 2000). *ABCC6* deficiency is the primary cause for chronic and acute forms of ectopic mineralization in humans and dystrophic cardiac calcification in mice (Le Saux O. et al, 2012).

1.1.5.2 Canalicular transport proteins

Most canalicular transporters for biliary lipids and pigments belong to the ATP-binding cassette (ABC) transporter superfamily. ABC transporters are transmembrane proteins binding ATP and utilizing the energy of ATP hydrolysis to drive the transport of various molecules across cell membranes and non-transport-related processes such as translation of RNA and DNA repair (Hyde S.C. et al., 1990, Dean M. et al., 2001). Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding domains, also known as nucleotide-binding folds (NBFs). The NBFs contain characteristic motifs (Walker A and B), separated by approximately 90–120 amino acids. ABC transporters also contain an additional element, the signature C motif, located just upstream of the Walker B site. The functional protein typically contains two NBFs and two transmembrane (TM) domains (Fig. 5). The TM domains contain 6–11 membrane-spanning α-helices and provide the specificity for the substrate (Hyde S.C. et al., 1990, Dean M. et al., 2001). In eukaryotes, the transport mediated by ATP pumps is mostly unidirectional from the cytoplasm to the outside of the cell or into intracellular compartments (ER, mitochondria, peroxisomes).

The human genome carries 48 ABC genes, arranged in seven subfamilies, designated A to G (HUGO Gene Nomenclature Committee, http://www.genenames.org/genefamilies/ABC). With respect to bile formation, two subclasses of the ABC superfamily are particularly important: the P-glycoprotein (Pgp) subfamily (ABCB, also known as MDR family of ABC transporters) and the multidrug-resistance protein (MRP) subfamily (ABCC, see above). ABCB subfamily is unique to mammals and comprises four full-transporters and seven half-transporters. Subfamily C includes the cystic fibrosis gene (CFTR, gene *ABCC7*), 11 other genes that encode proteins associated with multidrug resistence, and one pseudogene (*ABCC13*) (Dean M. et al., 2001, Borst P., Elferink R.O., 2002, Vasiliou V. et al., 2009).

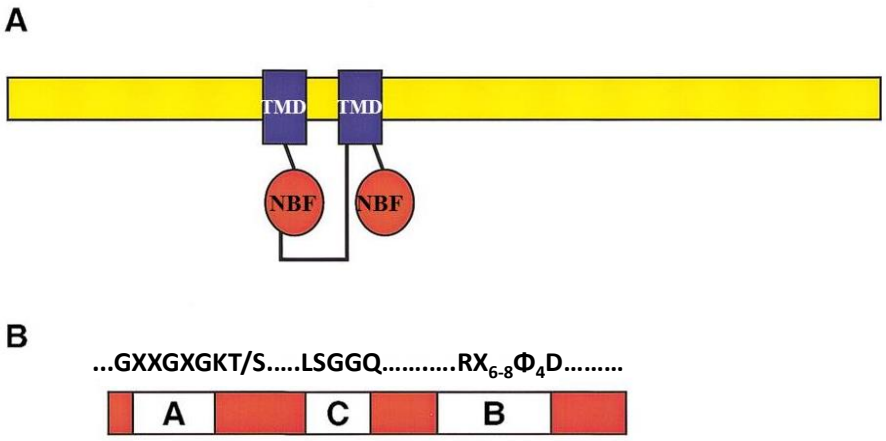

Fig. 5: **Diagram of a typical ABC transporter protein.** A. Diagram of the structure of a representative ABC protein is shown with a lipid bilayer in yellow, the transmembrane domains (TMD) in blue, and the nucleotide binding folds (NBF) in red. B. The NBF of an ABC gene contains the Walker A and B motifs found in all ATP-binding proteins. In addition, a signature or C motif is also present. The most common amino acids found in these motifs are shown above the diagram (X: any amino acid, Φ: hydrophobic amino acid). Adapted from: Dean M. et al., 2001.

Bile salt export pump (BSEP, gene *ABCB11*) is a ~160 kDa protein with 12 putative membrane spanning domains responsible for ATP-dependent transport of predominantly monovalent conjugated bile acids across the hepatocyte canalicular membrane. It is exclusively expressed in the liver and localized in canalicular microvilli and subcanalicular vesicles (Gerloff T. et al., 1998). Expression of BSEP is sensitive to the flux of bile salts through hepatocyte. The BSEP promoter contains an inverted repeat DNA element (IR-1) that represents a binding site for the farnesoid X receptor (FXR), a nuclear receptor for bile salts. FXR requires heterodimerization with retinoid X receptor (RXR), and after binding retinoic acid and bile salts, the complex regulates the transcription of several genes involved in bile salts homeostasis (Ananthanarayanan M. et al., 2001, Plass J.R et al., 2002). Mutations in the BSEP transporter gene may cause an autosomal recessive cholestatic disorder Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2, MIM #601847), characterized by severe jaundice, hepatomegaly and high plasma levels of bile acids and aminotransferases (Strautnieks S.S. et al., 1998). A milder non-progressive form of PFIC2, Benign Recurrent Intrahepatic Cholestasis type 2 (BRIC2, MIM #605479), is associated with intermittent episodes of cholestasis and gallstone formation. The cholestatic attacks vary in severity and duration and patients are asymptomatic between episodes, both clinically and biochemically (van Mill S.W.C. et al., 2004).

MDR1 P-Glycoprotein (gene *ABCB1*) transports steroid hormones, hydrophobic peptides, amphiphilic cationic drugs, bile salts, and many metabolites and toxins of endogenous or exogenous origin. In the gut, MDR1 pumps from the enterocytes towards the lumen, limiting the uptake of hydrophobic drugs (Dean M. et al., 2001).

MDR3 (gene *ABCB4*) is supposed to act as a floppase translocating phospholipids from the inner to the outer leaflet of the lipid bilayer of the canalicular membrane (Van der Bliek A.M. et al., 1987, Smith A.J. et al., 1994). Mutations in *ABCB4* cause Progressive Familial Intrahepatic Cholestasis type 3 (PFIC3, MIM #602347), an autosomal recessive cholestatic disorder characterized by the impaired formation of mixed micelles and by more hydrophilic bile with potent detergent properties resulting in membrane damage (de Vree J.M.L. et al., 1998). Except for PFIC3, there is evidence that a biallelic or monoallelic *ABCB4* defects may cause or predispose to a wide spectrum of human liver diseases, such as Low Phospholipid-Associated Cholelithiasis Syndrome (LPAC, MIM#600803), Intrahepatic Cholestasis of Pregnancy (ICP, MIM #147480), drug-induced liver injury, transient neonatal cholestasis, adult biliary fibrosis, or cirrhosis (Jacquemin E. et al., 2001, Rosmorduc O. and Poupon R., 2007, Davit-Spraul A. et al., 2010, Wendum D. et al., 2012).

MRP2 (gene *ABCC2*) transports conjugates of endogenous and xenobiotic compounds including bilirubin glucuronates and contributes to the bile formation by transporting glutathione (Nishida T. et al., 1992, Paulusma C.C. et al., 1996, Keppler D. et al. 1997, Jedlitschky G. et al., 1997). Under physiologic conditions MRP2 is expressed at the apical (canalicular) membrane of hepatocytes (Fig. 6). Much lower expression has been observed in the kidney, duodenum, ileum, brain and placenta (Cherrington N.J. et al., 2002, Macias R.I. et al., 2009). Absence or deficiency of MRP2 results in DJS (see below).

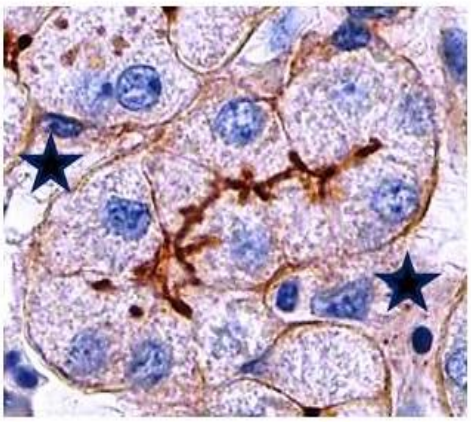

Fig. 6: **Immunohistochemical expression of MRP2 transport protein in hepatocytes.** Positive reaction localized to the apical (canalicular) domain of hepatocytes stains brown. Asterisks mark sinusoidal space with Kupffer cell nuclei. Formalin-fixed tissue; Anti-MRP2 mouse monoclonal antibody (clone M2Ⅲ-6, Kamiya, Seattle, WA). Original magnification x200.

Familial intrahepatic cholestasis type 1 transporter (FIC1, gene *ATP8B1*), a member of the type 4 subfamily of P-type ATPases (P4 ATPase), is a flippase mediating translocation of aminophospholipids from the outer (exoplasmic) to the inner (cytoplasmic) leaflet of plasma membranes (Groen A. et al., 2011). In most eukaryotic cells phosphatidylcholine and sphingolipids are concentrated in the exoplasmic leaflet, whereas the aminophospholipids phosphatidylserine and phosphatidylethanolamine are largely confined to the cytoplasmic leaflet of the plasma membrane. FIC1 thus helps in maintaining a detergent-resistant state of canalicular membranes. *ATP8B1* deficiency with loss of membrane cholesterol and physiological phospholipid asymmetry of the canalicular membrane has highly variable phenotypic manifestation ranging from Progressive Familial Intrahepatic Cholestasis type 1 (PFIC1, formerly Byler disease, MIM#211600) with onset in early infancy to a milder intermittent, non-progressive form known as Benign Recurrent Intrahepatic Cholestasis type 1 (BRIC1, Summerskill-Walshe syndrome, MIM #243300) (Bull L.N. et al., 1997, Bull L.N. et al., 1998).

The half-transporters ABCG5 and ABCG8, both members of the ABCG - subfamily, form a heterodimeric transporter mediating the biliary excretion of cholesterol and plant sterols. Each half-transporter carries a single ATP-binding cassette. ABCG5 and ABCG8 are encoded by two genes on chromosome 2, which are expressed predominantly in the liver, intestine, and gallbladder (Graf G.A. et al., 2002). Mutations in both alleles of either *ABCG5* or *ABCG8* result in sitosterolemia (MIM#210250), a rare inherited disorder characterized by up to 100-fold increase in serum levels of plant sterols (Patel S.B. et al., 1998).

1.1.6 Fate of bilirubin in the gastrointestinal tract

As conjugated bilirubin reaches the terminal ileum and the large intestine, the molecules of glucuronic acid are cleaved by bacterial β-glucuronidases and the pigment is subsequently reduced by the fecal flora to a group of colorless tetrapyrrolic compounds called urobilinogens. Urobilinogen can be metabolized to stercobilinogen and stercobilin. Most of the urobilinogens are oxidized to colored urobilins and excreted in the feces. A small fraction of urobilinogens is re-absorbed and re-excreted through the liver to constitute the enterohepatic urobilinogen cycle. Some urobilinogen is re-absorbed and subsequently excreted in the urine along with urobilin (Fahmy K. et al., 1972, Watson C.J., 1977).

1.2 Pathophysiology of hyperbilirubinemia and jaundice

Normal serum bilirubin concentration in adults is less than 17 μmol/l. Hyperbilirubinemia denotes increased serum bilirubin levels above 20 μmol/l. Jaundice (icterus), a yellow discoloration of tissues, namely the sclera of the eyes and skin, is the clinical manifestation of hyperbilirubinemia noticeable at serum bilirubin levels exceeding 30-50 μmol/l. Hyperbilirubinemia, either of conjugated or unconjugated type, may exist as an isolated pathologic finding. However, in most cases hyperbilirubinemia and jaundice present as a part of cholestasis, characterized by a decrease in bile flow due to impaired secretion by hepatocytes or to obstruction of bile flow through intra- or extrahepatic bile ducts. Cholestasis is associated with retention of bile salts, free cholesterol, biliary pigments, phospholipids, and other constituents normally excreted into bile.

Depending on the predominant type of bile pigment in the plasma, hyperbilirubinemia is classified into three major categories: unconjugated (indirect) hyperbilirubinemia – premicrosomal type, unconjugated (direct) hyperbilirubinemia – postmicrosomal type, and mixed hyperbilirubinemia (Vitek L., 2009).

1.2.1 Predominantly unconjugated hyperbilirubinemia (premicrosomal type)

Three basic pathophysiologic mechanisms may result in predominantly unconjugated hyperbilirubinemia: bilirubin overproduction, impaired bilirubin uptake, and impaired conjugation (Tab. 2).

Haemolysis and haemolytic anemias are the most frequent causes of bilirubin overproduction. Since an efficient system of biotransformation and secretion of conjugated bilirubin by the liver may compensate mild overproduction of bilirubin, hyperbilirubinemia accompanying chronic haemolytic anemias rarely exceeds 70 μmol/l. However, during acute haemolytic crisis serum bilirubin may reach considerably higher levels.

Disruption of bilirubin uptake by hepatocytes may be caused by impaired function of basolateral transporter systems due to immaturity of the transporters, inherited absence or deficiency of the transport proteins or bilirubin interference with other substrates at the sinusoidal domain of hepatocytes, respectively.

Tab. 2 : **Causes of predominantly unconjugated hyperbilirubinemia**

Increased bilirubin production	Haemolysis (intravascular or extravascular)
	Impaired red blood cell synthesis (megaloblastic, sideroblastic, iron deficiency anemia, lead poisoning)
Impaired hepatic bilirubin uptake	Congestive heart failure
	Portosystemic shunts
	Drugs (rifampin, probenecid)
Impaired bilirubin conjugation	Crigler-Najjar syndrome I and II
	Gilbert's syndrome
	Neonates
	Hyperthyroidism
	Ethinyl estradiol

Except for mutations in *UGT1A1* with decreased expression and partial or even complete inactivation of the enzyme, impaired conjugation of bilirubin may be caused by acquired deficiency of UGT1A1 associated with diffuse hepatocellular damage or by the enzyme inhibition mediated by various endogenous and exogenous substrates, including drugs and hormones.

1.2.2 Predominantly conjugated hyperbilirubinemia (postmicrosomal type)

The causes of predominantly conjugated hyperbilirubinemia include multiple acquired and inherited disorders (Tab. 3). Conjugated hyperbilirubinemia is a frequent finding associated with intrahepatic and/or extrahepatic cholestasis. On the other hand, inherited forms of predominantly conjugated hyperbilirubinemia, characterized by an isolated elevation of serum bilirubin levels without other symptoms of cholestasis, are rare and include primarily Dubin-Johnson and Rotor syndrome (see below).

In addition, defects in intestinal bilirubin metabolism and transport, such as impaired reduction of bilirubin to urobilinogen or prolongation of bilirubin transit time in the intestinal lumen, associated with increased enterohepatic circulation of bilirubin, may also result in hyperbilirubinemia of postmicrosomal type (Vitek L., 2009).

Tab. 3: **Causes of predominantly conjugated hyperbilirubinemia**

Extrahepatic cholestasis (biliary obstruction)	Choledocholithiasis
	Intrinsic and extrinsic tumors
	Primary sclerosing cholangitis
	AIDS cholangiopathy
	Acute or chronic pancreatitis
	Strictures
	Parasitic infections
Intrahepatic cholestasis	Primary biliary cirrhosis
	Drugs and toxins
	Sepsis/hypoperfusion
	Infiltrative diseases
	Total parenteral nutrition
	Pregnancy
	Sarcoidosis
	Viral hepatitis (fibrocholestatic)
Hereditary	Rotor syndrome
	Dubin-Johnson syndrome

1.2.3 Mixed type of hyperbilirubinemia

Combination of various etiopathogenetic factors associated with the development of pre- and postmicrosomal hyperbilirubinemia may result in proportional elevation of both unconjugated and conjugated serum bilirubin levels. Characteristically, disorders with diffuse damage of liver parenchyma, such as steatohepatitis, infectious hepatitis, cirrhosis, toxic and metabolic causes (haemochromatosis, Wilson´s disease, porphyria, etc.), diffuse neoplastic infiltration of liver parenchyma and circulatory disturbances, may result in mixed type of hyperbilirubinemia.

1.2.4 Bilirubin handling proteins in cholestasis

Up- and down-regulation of a broad range of transport systems involved in bile formation can explain impaired liver uptake and excretion of the biliary constituents resulting in cholestasis and jaundice, which accompanies some hereditary and many common acquired liver disorders. In hereditary cholestasis syndromes such as PFIC1-3, mutations in the genes encoding transporter proteins are the primary causes of cholestasis (Thompson R. and Jansen P.L., 2000). Alteration of hepatobiliary transport systems in acquired forms of cholestasis may be a

consequence of retention of potentially toxic biliary constituents (extra- and intrahepatic bile duct obstruction or destruction) or may be caused by pro-inflammatory cytokines (sepsis-induced cholestasis), hormones (intrahepatic cholestasis of pregnancy, oral contraceptive-induced cholestasis), or drugs. These factors can influence transporter mRNA and/or protein expression, lead to retrieval of transporter protein from the membrane to submembranous compartments, or directly inhibit protein function (Trauner M. et al., 1999, Lee J. and Boyer J.L., 2000, Wagner M. and Trauner M., 2005, Geier A. et al., 2007, Zollner G. and Trauner M., 2008). A general pattern of response to cholestatic liver injury is initiated by down-regulation of the basolateral membrane bound transporters (NTCP, OATPs). This mechanism limits bile acid uptake, thus preventing hepatocellular bile acid overload. Expression of several canalicular export pumps is relatively unaffected (BSEP) or even up-regulated (MDR1). Decreased expression of MRP2 in sepsis or in obstructive cholestasis is followed by up-regulation of several MRP homologues (particularly MRP3) at the basolateral membrane of hepatocytes that may extrude bile salts back to the sinusoidal blood and systemic circulation (Belinsky M.G. et al., 2005, Geier A. et al., 2007, Nies A.T. and Keppler D, 2007). Most of these changes are believed to represent compensatory mechanisms providing alternative excretory routes that may help prevent accumulation of potentially toxic bile components and other substrates in the liver (Zollner G. et al., 2003, Kullak-Ublick G.A. et al, 2004, Zelcer N. et al., 2006).

1.3 Bilirubin toxicity and antioxidant properties

Bilirubin is generally regarded as a potentially cytotoxic waste product. Patients with profound unconjugated hyperbilirubinemia are at risk for the development of bilirubin-induced neurological dysfunction (BIND) and kernicterus (Hervieux J., 1847, Schmorl G., 1903, Shapiro S.M., 2005, Shapiro S.M., 2010, Johnson L. and Bhutani V.K., 2011). The exact mechanisms of brain injury and bilirubin encephalopathy are still a matter of investigation. Toxic effects of unconjugated bilirubin (UCB) are explained by inhibition of DNA synthesis (Schiff D. et al., 1985). UCB may also uncouple oxidative phosphorylation and inhibit adenosine triphosphatase (ATPase) activity of brain mitochondria (Mustafa M.G. et al., 1969, Diamond I. and Schmid R., 1967). Bilirubin mediated inhibition of various enzyme systems, RNA synthesis and proteosyntesis in the brain and liver, and/or alteration of carbohydrate metabolism in the brain can also contribute to its toxicity (Strumia E., 1959, Flitman R. and Worth N.K., 1966, Greenfield S. and Nandi Majumdar A.P., 1974, Katoh R. et al., 1975). Moreover, UCB may trigger apoptosis of neuronal and glial cells via the mitochondrial pathway, an effect more pronounced in immature nerve cells (Rodrigues C.M. et al., 2002, Falcão A.S. et al., 2007a, Brites D., 2011). Vulnerability of immature nerve cells may be caused by the lower levels of mitochondrial enzyme oxidizing UCB and by the decreased expression of the multidrug resistance-associated protein 1 (MRP1) that protects human neuroblastoma SH-SY5Y cells, neurons, and astrocytes from UCB-induced cytotoxicity (Hansen T.W. and Allen J.W., 1997, Falcão A.S. et al., 2007b, Corich L. et al., 2009, Gazzin S. et al., 2011). Hence, respiratory chain dysfunction and decreased antioxidant defences in "young" neurons are supposed to be other important factors responsible for the susceptibility of premature infants to brain damage from UCB (Brito M.A. et al., 2008, Vaz A.R. et al., 2010, Brites D., 2011).

Nevertheless, recent data have indicated the potent antioxidant properties of mild or moderately elevated serum bilirubin levels, both conjugated and unconjugated, with substantial positive clinical consequences and especially their protective effects on atherogenesis and cancerogenesis (Stocker R. et al., 1987, Temme E.H. et al., 2001, Keshavan P. et al., 2004, Ollinger R. et al., 2007). A direct link between low serum bilirubin levels and peripheral vascular disease and the protective effects of mild or moderate unconjugated hyperbilirubinemia on atherosclerosis were confirmed in numerous studies and clinical trials (Siow R.C. et al., 1999, Mayer M., 2000, Vitek L. and Schwertner H.A., 2008, Lin J.P. et al., 2010).

Bilirubin, at micromolar concentrations *in vitro*, efficiently scavenges reactive oxygen species (ROS), such as singlet oxygen, peroxyl and superoxide anion radicals, and in the

presence of hydrogen peroxide bilirubin may serve as a substrate for peroxidases (Stocker et al., 1987). Additionally, bilirubin protects low density lipoproteins against oxidation processes and decreases carbonylation of proteins (Neuzil J. and Stocker R., 1993, Wu T.W. et al., 1996).
In vitro data have established the existence of biliverdin-bilirubin redox amplification cycle that may contribute efficiently to cellular protection against oxidative damage (Sedlak T.W. and Snyder S.H., 2004, Stocker R., 2004) (Fig. 7). Furthermore, bilirubin may act as an inhibitor of NADPH-oxidase activity (Taillé C. et al., 2004, Lanone S. et al., 2005, Datla S.R. et al., 2007).

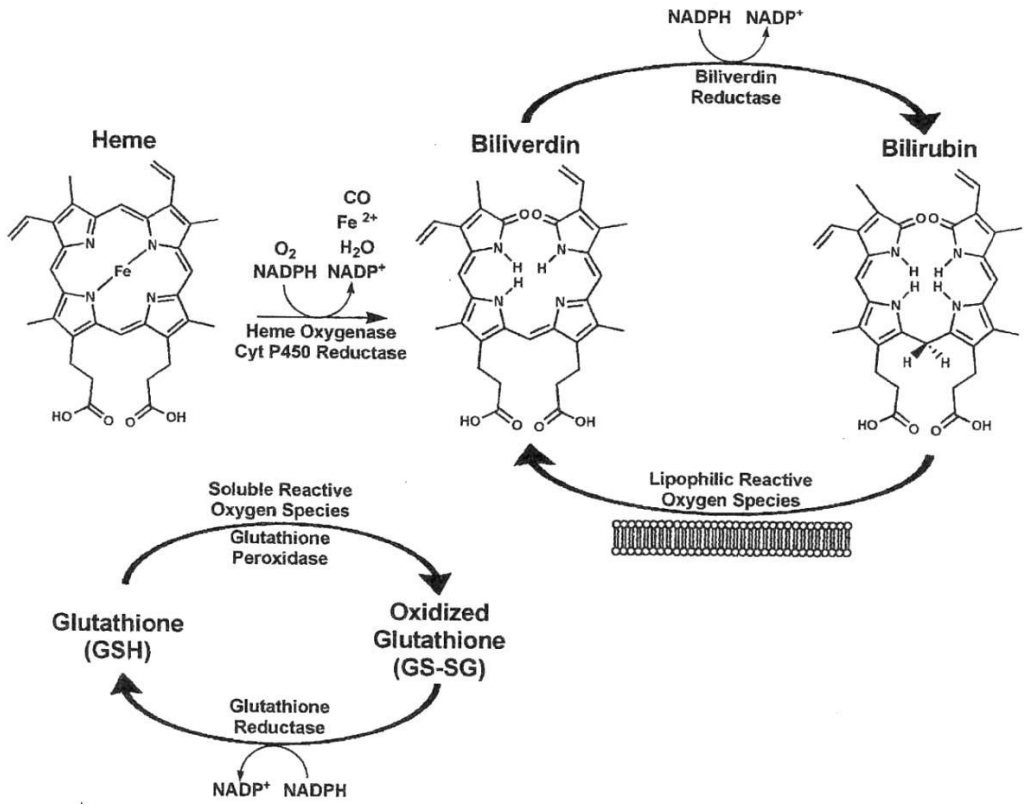

Fig. 7: **Oxidation-reduction cycles for bilirubin and GSH.** ROS may act directly on bilirubin, leading to its oxidation to biliverdin. Biliverdin-reductase catalyzes the reconversion of biliverdin to bilirubin, permitting bilirubin to detoxify a 10 000-fold excess of oxidants. Soluble oxidants are detoxified by GSH, a bilirubin independent cycle that requires two enzymes, GSH peroxidase and GSH reductase. Adapted from: Sedlak T.W. and Snyder S.H., 2004.

Apart from antioxidative properties, antiinflammatory, antiproliferative, cytostatic and pro-apoptotic effects of bilirubin are well-known and have been demonstrated in both *in vitro* and *in vivo* studies (Keshavan P. et al., 2004, Wang W.W. et al., 2004, Lanone S. et al., 2005, Ollinger R. et al., 2007).

1.4 Inherited forms of hyperbilirubinemia

1.4.1 Inherited forms of predominantly unconjugated hyperbilirubinemia

Conjugation of bilirubin in the ER is catalyzed by the enzyme UGT1A1. Mutations in *UGT1A1* can lead to decreased expression and partial or even complete inactivation of the enzyme (Sugatani J. et al., 2002). By contrast, expression of *UGT1A1* can be increased by phenobarbital (PB) administration. PB response activity is delineated to a 290-bp distal enhancer module sequence (-3483/-3194) (gtBPREM – glucuronosyltransferase phenobarbital response enhancing motif) of the human *UGT1A1*. gtBPREM is activated by the nuclear orphan receptor, human constitutive active receptor (hCAR). CAR is a cytoplasmic receptor which, after treatment with inducers such as PB, translocates into the nucleus, forms a heterodimer with the RXR and activates the PB response enhancer element (Sugatani J. et al., 2001).

Three types of inherited, predominantly unconjugated hyperbilirubinemia with different levels of UGT1A1 activity are recognized: Crigler-Najjar syndrome type I, type II and Gilbert syndrome.

Crigler-Najjar syndrome type I (CN1, MIM#218800), the most deleterious form, described in 1952 by Crigler and Najjar (Crigler J.F. Jr. and Najjar V. A., 1952), is characterized by complete or almost complete absence of UGT1A1 enzyme activity with severe jaundice (Ritter J.K. et al., 1992). Icterus occurring shortly after birth is complicated by bilirubin encephalopathy (kernicterus). Until the introduction of phototherapy and plasmapheresis, kernicterus was fatal in almost all cases during the first two years of life or caused serious brain damage with permanent neurologic sequelae. Intermittent phototherapy is life-long and it results in a thorough elimination of water-soluble photoisomers of unconjugated bilirubin via bile. However, the effectiveness of phototherapy may decrease gradually with age and patients are at higher risk of sudden brain damage (Karon M. et al., 1970, Jansen P.L., 1999). Although new treatment modalities such as hepatocyte or hepatic progenitor cell transplantation have already been used to treat CN1 patients, liver transplantation is still considered to be the only definitive treatment for CN1 (Matas A.J. et al., 1976, Shevell M.I. et al., 1987, Fox I.J. et al., 1998).

Crigler-Najjar syndrome type II (Arias syndrome, CN2, MIM#606785), described by Arias in 1962 (Arias I.M., 1962), is characterized by reduced UGT1A1 enzyme activity with a moderate degree of non-haemolytic jaundice. Bilirubin levels do not exceed 350 μmol/l and CN2 is only rarely complicated by kernicterus (Gollan J.L. et al., 1975). Virtually all the mutations responsible for the syndrome are autosomal recessive, as in CN1, but several observations have

also suggested the possibility of autosomal dominant pattern of inheritance (Hunter J.O. et al., 1973, Labrune P. et al., 1989, Moghrabi N. et al., 1993).

An important clinical difference between CN type I and type II is the response to PB treatment, with no effect in type I (complete loss of the UGT1A1 enzyme activity) and a decrease of serum bilirubin levels by more than 30% in CN type II (some residual UGT1A1 activity is preserved). Moreover, bilirubin glucuronides are present in bile in CN2. However, the method of choice for the diagnosis of CN syndrome is mutation analysis of *UGT1A1* (Seppen J. et al., 1994).

Gilbert syndrome (GS, MIM#143500), described in 1901 by Augustine Gilbert and Pierre Lereboullet (Gilbert A. and Lereboullet P., 1901), is characterized by fluctuating mild, unconjugated hyperbilirubinemia < 85 μmol/l without overt haemolysis, usually diagnosed around puberty, and aggravated by intercurrent illness, stress, fasting or after administration of certain drugs (Nixon J.C. and Monahan G.J., 1967, Schmid R., 1995). The clinical diagnosis of GS can be established if the patient has a mild, predominantly unconjugated hyperbilirubinemia and normal activity of liver enzymes. The reduced caloric intake test and PB stimulation test have low diagnostic specificity in GS subjects (Thomsen H.F. et al., 1981). Histological findings in GS are mild, with a slight centrilobular accumulation of pigment with lipofuscin-like properties (Barth R.F. et al., 1971). Ultrastructurally, hepatocytes reveal hypertrophy of the smooth endoplasmic reticulum (Dawson J., 1979). Since the morphological picture of GS is completely non-specific and the disorder is benign, liver biopsy is not indicated.

GS is characterized by reduced levels of UGT1A1 activity to about 25-30% caused by homozygous, compound heterozygous, or heterozygous mutations in the *UGT1A1* with autosomal recessive transmission (Black M. and Billing B.H., 1969).

GS is the most frequent hereditary jaundice affecting nearly 5-10% of the Caucasian population (Owens D. and Evans J., 1975). The genetic basis of GS was first disclosed in 1995 (Bosma P.J. et al., 1995) as presence of the allele UGT1A1*28, characterized by insertion of TA in the TATAA box (A[TA]$_7$TAA) in the proximal promoter of *UGT1A1*. UGT1A1*28 has been identified as the most frequent mutation in Caucasian GS subjects (Hsieh T.Y. et al., 2007). The insertion is responsible for reduction of transcription of *UGT1A1* to 20% from normal and for a decrease of hepatic glucuronidating activity by 80% in a homozygous state (Burchell B. and Hume R., 1999). In Caucasians and African Americans, the frequency of UGT1A1*28 allele is about 35-40%, but it is much lower in Asians, including Koreans (13%), Chinese (16%), and Japanese (11%) (Beutler E. et al., 1998, Ki C.S. et al., 2003, Ando Y. et al., 1998, Takeuchi K. et al., 2004). Moreover, in the majority of Caucasian GS subjects, expression of UGT1A1 is further decreased by the presence of the second mutation T>G in gtPBREM (Sugatani J. et al., 2001,

Sugatani J. et al., 2002). In addition to the mutations in the promoter, GS may be caused by mutations in structural regions of the *UGT1A1*. In Asians, other variants, such as UGT1A1*6 characterized by a missense mutation involving G to A substitution at nucleotide 211 (c.211G>A) in exon 1 (also known as p.G71R), UGT1A1*7 (p.Y486YD), UGT1A1*27 (p.P229PQ), and UGT1A1*62 (p.F83FL) have been detected (Ando Y. et al., 1998, Beutler E. et al., 1998, Sugatani J. et al., 2001, Ki C.S. et al., 2003, Takeuchi K. et al., 2004).

In addition to biochemical defect leading to reduced glucuronidation, other factors, such as impaired hepatic (re)uptake of bilirubin or an increased load of bilirubin, seem to be necessary for clinical manifestation of GS (Burchell B. and Hume R., 1999, Kadakol A. et al., 2000, Udomuksorn W. et al., 2007).

GS is benign and GS carriers present with no liver disease. However, the mutations in the *UGT1A1* identical to those recognized in GS subjects may contribute to the development of prolonged neonatal hyperbilirubinemia in breast-fed infants (Monaghan G. et al., 1999, Maruo Y. et al., 2000).

Moreover, since the process of glucuronidation is an important step in elimination of numerous endogenous and exogenous substrates, GS subjects may be more susceptible to the adverse effects of some drugs metabolized by UGT1A1, such as indinavir, atazanavir (Burchell B. et al., 2000, Zucker S.D. et al., 2001, Maruo Y. et al., 2005, Rotger M. et al., 2005, Zhang D. et al., 2005) or irinotecan (Ando Y. et al., 1998, Iyer L. et al., 2002, Strassburg C.P., 2008).

1.4.2 Inherited forms of predominantly conjugated hyperbilirubinemia

Two types of hereditary conjugated jaundice are known as Dubin-Johnson syndrome and Rotor syndrome. Both are characterized by the presence of mixed, predominantly conjugated hyperbilirubinemia, with conjugated bilirubin more than 50% of total bilirubin.

Dubin-Johnson syndrome (DJS, MIM#237500), a benign autosomal recessive disorder described in 1954 by Dubin and Johnson (Dubin I.N. and Johnson F.B., 1954) and Sprinz and Nelson (Sprinz H. and Nelson R.S., 1954), is characterized by fluctuating mild, predominantly conjugated hyperbilirubinemia with typical manifestation in adolescence or young adulthood. Most patients are asymptomatic except for occasional slight abdominal pain and fatigue. Urine excretion of total coproporphyrin in 24 hours is normal, but 80% are represented by coproporphyrin I. Biliary excretion of anionic dyes including bromosulphophtalein (BSP), indocyanine green and cholescintigraphy radiotracers is delayed with absent or delayed filling of the gallbladder (Shani M. et al., 1970). BSP clearance in DJS subjects is normal at 45 min with

the second peak at 90 min (Erlinger S. et al., 1973). Liver histology in DJS shows an accumulation of a distinctive melanin-like lysosomal pigment in an otherwise normal liver that gives the organ a characteristic dark pink or even black colour. The pigment is positive in periodic acid-Schiff (PAS) reaction and Masson-Fontana reaction with marked autofluorescence. In contrast to melanin, DJS pigment does not reduce neutral silver ammonium solution (Swartz H.M. et al., 1987). The amount of pigment may vary and possible transient loss may occur in coincidence with other liver diseases (Hunter F.M. et al., 1964, Watanabe S. et al., 1982). The molecular mechanism in DJS is absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT caused by homozygous or compound heterozygous mutation in *ABCC2* on chromosome 10q24 (Paulusma C.C. et al., 1996, Wada M. et al., 1998, Toh S. et al., 1999). The *ABCC2* mutation alters not only MRP2-mediated transport of conjugated bilirubin but also transport of many anionic substrates as well as a wide range of drugs, such as chemotherapeutics, uricosurics, antibiotics, leukotrienes, GSH, toxins, and heavy metals. Absence of MRP2 may result in impaired elimination and in subsequent renal toxicity of the substrates mentioned above (Uchiumi T. et al., 1998, Hulot J.S. et al., 2005, Jedlitschky G. et al., 2006, Ahmed S. et al., 2008, Pedersen J.M. et al., 2008).

A rare type of hereditary mixed hyperbilirubinemia resulting from simultaneous occurence of mutations characteristic for DJS and GS was classified as ***dual hereditary jaundice*** (Cebecauerova D. et al., 2005).

Rotor syndrome (RS, MIM #237450), described in 1948 by Rotor, Manahan, and Florentin (Rotor B. et al., 1948), is a rare familial disorder with autosomal recessive transmission, characterized by mild, predominantly conjugated hyperbilirubinemia with delayed excretion of anionic dyes without re-increase of their concentration. Total urinary coproporphyrin excretion is significantly increased and the proportion of coproporphyrin I in urine is approximately 65% of the total in homozygotes and 43% in heterozygotes (Wolkoff A.W. et al., 1976, Wolpert E. et al., 1977). Apart from predominantly conjugated hyperbilirubinemia and jaundice, clinical findings and liver tests are normal. By histopathological examination, the liver tissue does not display any marked architectural or cytomorphological abnormalities and pigment is not present. Molecular genetic analysis excluded pathogenic mutations in *ABCC2* and confirmed that RS is not allelic variant of DJS (Hřebíček M. et al., 2007). The prognosis is excellent, and no treatment is necessary.

In 1975, a case of hereditary predominantly conjugated hyperbilirubinemia with markedly reduced BSP hepatic storage capacity was described and a new syndrome was named ***Hepatic Uptake and Storage Disease*** (conjugated hyperbilirubinemia type III, MIM#237550)

(Dhumeaux D. and Berthelot P., 1975). However, re-examination of Rotor subjects revealed defective hepatic uptake and storage in these individuals (Wolpert E. et al., 1977). Hence, RS and Uptake and Storage Disease with similar BSP kinetics are considered to be the same entity (Berthelot P. and Dhumeaux D., 1978).

Parts of this chapter have been published in review articles: Sticova E, Jirsa M. **New insights in bilirubin metabolism and their clinical implications.** World J Gastroenterol. 2013; 19(38): 6398-407, and Jirsa M, Sticová E. **Neonatal hyperbilirubinemia and molecular mechanisms of jaundice.** Vnitr Lek. 2013;59(7):566-71 (***Enclosure 1 and 2***).

2. AIMS

This thesis is focused on the molecular aspects of bilirubin metabolism and transport and inherited forms of hyperbilirubinemia with emphasis on predominantly conjugated type of hereditary hyperbilirubinemia syndromes.

Our objectives were:

1. To evaluate the role of bilirubin tissue concentration on mediating oxidative stress in cholestatic liver
2. To characterize at the molecular level subjects with predominantly conjugated type of hereditary jaundice
3. To determine the molecular basis of predominantly conjugated Rotor-type jaundice
4. To assess the role of Rotor proteins OATP1B1 and OATP1B3 in pathogenesis of jaundice

3. RESULTS AND COMMENTARY

3.1 The role of bilirubin tissue concentration on mediating oxidative stress in the cholestatic liver

Enclosure 3: Muchova L, Vanova K, Zelenka J, Lenicek M, Petr T, Vejrazka M, Sticova E, Vreman HJ, Wong RJ, Vitek L. **Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress.** J Cell Mol Med. 2011;15(5):1156-65.

3.1.1 Summary of results

The role of bilirubin and bile acids on mediating oxidative stress in Wistar and hyperbilirubinemic Gunn rats following bile duct ligation (BDL) was evaluated. The results of this study demonstrate that:

a) bilirubin increases peroxyl radical scavenging capacity in plasma, but not in liver homogenates in BDL rats
b) intracellular bilirubin levels in hepatocytes are relatively decreased compared to plasma in BDL animals
c) bilirubin production is decreased and lipid peroxidation is increased after BDL
d) taurocholic acid increases lipid peroxidation in the liver homogenates and decreases intracellular bilirubin levels in HepG2-rNtcp cells

3.1.2 Commentary

Results of numerous *in vitro* and *in vivo* studies have demonstrated an important role of oxidative stress in the pathogenesis of cholestatic liver diseases (Lemonnier F. et al., 1987, Sokol R.J. et al., 1991, Singh S. et al., 1992, Parola M. et al., 1996, Pastor A. et al., 1997). *In vitro* potentially toxic bile acids accumulating within hepatocytes during cholestasis damage liver cells by a mechanism that depends on generation of ROS and impairment of mitochondrial respiration and electron transport, respectively (Sokol R.J. et al., 1995, Sokol R.J. et al., 2001). Moreover, cholestasis initiates an inflammatory response in the liver tissue resulting in accumulation of neutrophilic leukocytes, an additional important source of damaging ROS (Saito J.M. et al., 2000, Gujral J.S. et al., 2003). Thus, increased production of ROS may result in severe

hepatocellular and bile duct injury that occurs during cholestasis regardless of the cause (Copple B.L. et al., 2010) (Fig. 8).

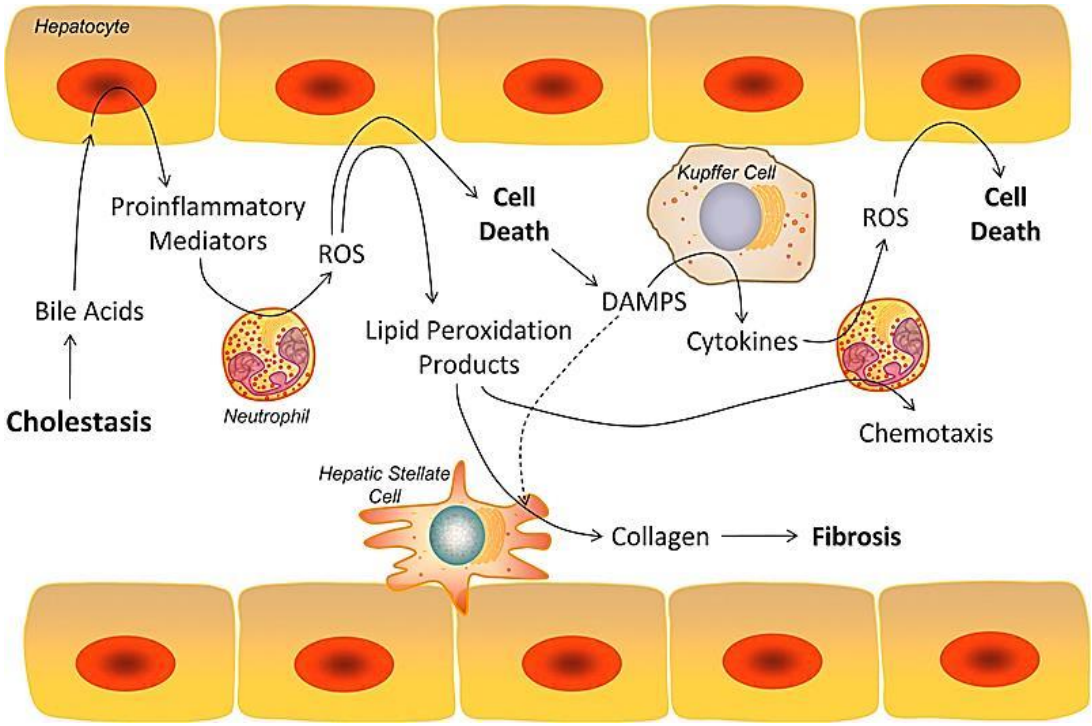

Fig. 8: **Bile acids and oxidative stress in the pathogenesis of cholestasis.** During cholestasis, bile acids stimulate hepatocytes to produce proinflammatory mediators that activate neutrophils. Neutrophil leukocytes release ROS that kill hepatocytes, which release damage-associated molecular patterns (DAMPs). DAMPs may amplify inflammation and liver injury by stimulating release of cytokines from Kupffer cells or even directly activating neutrophils. DAMPs may also promote fibrosis by activating Toll-like receptors on hepatic stellate cells. ROS also causes moderate lipid peroxidation the products of which are chemotactic for neutrophils and activate hepatic stellate cells and stimulate them to produce collagen. Adapted from: Copple B.L. et al., 2010.

Negative impact of bile acids and ROS on liver cells during cholestasis may be counteracted by several potent antioxidant systems in hepatocytes, including bilirubin. The results of this study indicate that bilirubin levels and the antioxidant capacity in plasma are increased in obstructive cholestasis; however, bilirubin in the liver tissue is relatively decreased compared to plasma. The lowering of intracellular bilirubin can be explained by a combination of several possible mechanisms:

a) bile acids-mediated down-regulation of the expression and activity of HMOX

b) increased consumption of bilirubin through biliverdin reductase catalytic cycle in the setting of increased oxidative stress (Sedlak T.W. and Snyder S.H., 2004, Stocker R., 2004)
c) alteration in transporter expression in cholestasis (Wagner M. and Trauner M., 2005, Geier A. et al., 2007, Zollner G. and Trauner M., 2008)

Thus, despite the fact that plasma bilirubin is a marker of cholestasis and hepatocyte dysfunction, plasma bilirubin concentrations may not reflect actual tissue bilirubin metabolism.

3.1.3 Conclusion

To induce cell injury in hepatocytes, that are normally highly resistant to oxidative stress and lipid peroxidation (Mathews W.R. et al., 1994, Hong J.Y. et al., 2009), a combination of increased ROS formation, especially due to accumulation of bile acids during cholestasis, and impairment of the antioxidant defense systems is necessary. Consequently, the increase in bile acids/bilirubin ratio in obstructive cholestasis may be implicated in the oxidative stress-mediated cholestatic liver injury.

3.2 Characterization of subjects with predominantly conjugated type of hereditary jaundice at the molecular level

Enclosure 4: Sticova E, Elleder M, Hulkova H, Luksan O, Sauer M, Wunschova-Moudra I, Novotny J, Jirsa M. **Dubin-Johnson syndrome coinciding with colon cancer and atherosclerosis.** World J Gastroenterol. 2013;19(6):946-50.

3.2.1 Summary of results

We analyzed six individuals with long-term predominantly conjugated hyperbilirubinemia. Molecular genetic alterations in *ABCC2* identified in the analyzed probands are summarized in Tab. 4.

Tab. 4: **Molecular genetic alterations in *ABCC2* identified in six analyzed probands**

Proband	DNA alteration	Protein alteration	Pathogenicity	MAF
1	Heterozygous **c.2360_2366delCCCTGTC**	p.Pro787LeufsX7	Pathogenic	
	Heterozygous **c.3258+1G>A**	abnormal splicing	Pathogenic	
2	Heterozygous c.1249G/A	p.Val417Ile	SNP rs2273697	0.174
	Heterozygous c.1446C/G	no	SNP rs113646094	0.003
	Heterozygous **c.2213C>G**	p.Ala738Gly	Likely pathogenic	
	Heterozygous **c.2310C>G**	p.Ser770Arg	Likely pathogenic	
3	Homozygous c.116G/A	p.Tyr39Phe	SNP rs927344	0.004
	Heterozygous c.1249C/G	p.Val417Ile	SNP rs2273697	0.174
4	Heterozygous c.2009T/C	p.Ile670Thr	SNPrs17222632	0.005
	Heterozygous **c.2741G>A**	p.Ser914Asn	Uncertain	
	Homozygous c.3563T/A	p.Glu1188Val	SNP rs17222723	0.043
	Heterozygous c.4290G/T	p.Val1430Val	SNP rs1137968	0.043
	Heterozygous c.4544G/A	p.Cys1515Tyr	SNP rs8187710	0.070
5	Heterozygous c.1249G/A	p.Val417Ile	SNP rs2273697	0.174
6	Heterozygous c.3972C/T	no	SNP rs3740066	0.288

MAF – minor allele frequency in GenBank dbSNP

3.2.2 Commentary

Proband 1: Molecular genetic analysis of *ABCC2* identified a heterozygous deletion c.2360_2366delCCCTGTC in protein coding exon 18, predicted to cause a reading frame shift and premature termination of DNA translation at position 793 with protein alteration p.Pro787LeufsX7, and a heterozygous mutation c.3258+1G>A affecting the donor splice site of intron 23, predicted to cause abnormal splicing of mRNA. Considering the clinical picture and the results of laboratory tests, the two mutations are supposed to be in *trans* position. Unfortunately, no living relatives of the patient were found to confirm our presumption.

Moreover, histologic analysis of the percutaneous liver biopsy specimen disclosed an accumulation of a distinctive coarse brown black pigment in centrizonal and midzonal hepatocytes. The pigment was negative in Perls´ iron stain, with rudimentary autofluorescence, reducing Masson´s solution. Additionally, complete absence of MRP2 protein at the canalicular membrane of hepatocytes was demonstrated immunohistologically.

Thus, morphological and mutation analysis accordingly established the diagnosis of Dubin-Johnson syndrome in this proband.

Proband 2: Molecular genetic analysis of the *ABCC2* gene and *UGT1A1* promoter were performed and the following sequence variations have been detected:

The first sequence variantion c.1249G/A in exon 10 of *ABCC2* (dbSNP rs2273697) is a known frequent variantion with allelic frequency of the allele A around 0.174. The predicted consequence of this variant is a conservative amino acid substitution p.Val417Ile. The amino acid at position 417 is not conserved: isoleucine is found in dog, cow and mouse and other amino acids are present at the same position in lower organisms and plants. The allele c.1249A does not seem to affect splicing as predicted by the GeneSplicer software (http://ccb.jhu.edu/software/genesplicer/). Therefore the allele c.1249A is likely non-pathogenic.

The second sequence variation c.1446C/G does not change protein sequence and does not affect mRNA splicing (results obtained from GeneSplicer).

The third heterozygous mutation 2213C>G (p.Ala738Gly) was predicted as likely pathogenic by PredictSNP 1.0 software with 61% confidence of pathogenicity (http://loschmidt.chemi.muni.cz/predictsnp/).

The fourth sequence variant c.2310C>G found in heterozygous state is presumed to change the amino acid at the position 770. The amino acid substitution p.Ser770Arg is non-conservative and introduces positive charge to the protein. Serine at position 770, located in the first ATP-binding domain, that is critical for normal function of the protein, is highly conserved in animals and

plants. The mutation was predicted as pathogenic by PMut software (score 0.61, values over 0.5 are likely pathogenic, http://mmb2.pcb.ub.es:8080/PMut/), and by PredictSNP 1.0 software with 87% confidence of pathogenicity.

Homozygous state for both promoter variants $A(TA)_7TAA$ of TATA box and the c.-3279G allele were found in *UGT1A1*. Such combination represents the most common cause of Gilbert syndrome (GS) in Caucasians (Bosma P.J. et al., 1995, Sugatani J. et al., 2002).

Thus, the molecular findings indicate that hereditary hyperbilirubinemia in Proband 2 is caused by mutations in both *ABCC2* and *UGT1A1* and may correspond to dual hereditary jaundice (Cebecauerova D. et al., 2005).

Proband 3: Two known likely non-pathogenic sequence variations in *ABCC2* were disclosed: a less frequent homozygous variant c.116 G/A in exon 2 (dbSNP rs927344) with predicted amino acid substitution p.Tyr39Phe (non-pathogenic mutation with 83% confidence according to PredictSNP 1.0), and a heterozygous sequence variant c.1249 C/G (dbSNP rs2273697) in exon 10 with predicted protein alteration p.Val417Ile.

Proband 4: A novel heterozygous mutation c.2741G>A in exon 20 found in this patient is predicted to cause protein alteration p.Ser914Asn. Pathogenicity of this mutation is uncertain (deleterious mutation with confidence 51% according to PredictSNP 1.0). The same heterozygous mutation was found in one sibling of the proband 4.

The other four likely non-pathogenic DNA sequence variations are single-nucleotide polymorphisms c.2009T/C (p.Ile670Thr), dbSNP rs17222632 (rare but likely non-pathogenic with confidence 63% according to PredictSNP 1.0); c.3563T/A (p.Glu1188Val), dbSNP rs17222723; c.4290G/T (p.Val1430Val), dbSNP rs1137968; and c.4544G/A (p.Cys1515Tyr), dbSNP rs8187710.

Proband 5: Molecular genetic analysis of *ABCC2* disclosed a heterozygous sequence variation c.1249G/A in exon 10, dbSNP rs2273697, with predicted amino acid substitution p.Val417Ile.

Moreover, percutaneous liver biopsy was performed in this proband and preserved lobular architecture without pigment accumulation, as well as strong immunohistological expression of MRP2 at the canalicular membrane of hepatocytes excluded DJS.

Proband 6: The only sequence variant c.1446C>G found in exon 10 (dbSNP rs3740066) does not change the protein sequence.

3.2.3 Conclusion

In summary, we analyzed six unrelated index subjects with long-term predominantly conjugated hyperbilirubinemia. DJS was diagnosed in one subject (Proband 1). Presence of known or predictably pathogenic mutations in both alleles of the *UGT1A1* promoter and *ABCC2* in Proband 2 corresponds to dual hereditary jaundice. The diagnosis of DJS was not confirmed in the other four subjects and further investigations were necessary to clarify the cause of hyperbilirubinemia (see Chapter 3.3).

3.3 Molecular basis of predominantly conjugated Rotor-type jaundice

Enclosure 5: Van de Steeg E, Stránecký V, Hartmannová H, Nosková L, Hřebíček M, Wagenaar E, van Esch A, de Waart DR, Oude Elferink RP, Kenworthy KE, Sticová E, al-Edreesi M, Knisely AS, Kmoch S, Jirsa M, Schinkel AH. **Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver.** J Clin Invest. 2012;122(2):519-28.

3.3.1 Summary of results

The study has animal and human part.

The animal study was held by the group of Dr. Alfred Schinkel, The Netherlands Cancer Institute, Amsterdam:

a) experiments with the double knock-out mice with inactive Oatp1a/b and Abcc3 show that Abcc3 mediates bilirubin glucuronide excretion from the liver to sinusoidal blood and Oatp1a/b transporters mediate its hepatic reuptake
b) experiments with the *SLCO1B1* and *SLCO1B3* transgenic mice with liver specific expression of OATP1B1 and OATP1B3 generated on the background of the *Slco1a/b* deficiency indicate that either of the human OATP1B proteins reuptakes conjugated bilirubin from plasma to hepatocytes.

The parallel human mapping study conducted by the Czech group in 11 Rotor subjects from 8 different families (3 Saudi-Arabian, 1 Filipino and 4 Central European, including Probands 4, 5 and 6 with unexplained predominantly conjugated hyperbilirubinemia - see Chapter 3.2) demonstrates that biallelic inactivating mutations of both *SLCO1B1* and *SLCO1B3* genes located on chromosome 12 with complete simultaneous deficiency of the corresponding OATP1B1 and OATP1B3 proteins result in disruption of hepatic reuptake of bilirubin glucuronide. This mechanism explains Rotor-type hyperbilirubinemia.

3.3.2 Commentary

Aside from MRP2 mediated transport of conjugated bilirubin into bile, a significant fraction of bilirubin glucuronide is, under physiologic conditions, secreted into sinusoidal blood and subsequently reuptaken by hepatocytes for final biliary excretion. This process is mediated by sinusoidal transporters MRP3 and organic anion-transporting polypeptides OATP1B1 and

OATP1B3 (van de Steeg E. et al., 2010, Iusuf D. et al., 2012a). Since the expression of OATP1Bs is higher in the centrilobular hepatocytes (König J. et al., 2000a, Ho R.H. et al., 2006), the MRP3 - OATP1B1/3 loop is likely responsible for shifting (hopping) of conjugated bilirubin and other substrates from the periportal to the centrilobular zone of the liver lobule (Fig. 9). Such intralobular substrate transfer may protect periportal hepatocytes against elevated concentrations of various xenobiotics (Iusuf D. et al., 2012b). In addition, the OATP1B proteins mediate hepatic clearance of bilirubin conjugated in splanchnic organs and may represent an important alternative pathway in enterohepatic circulation (van de Steeg E et al., 2010).

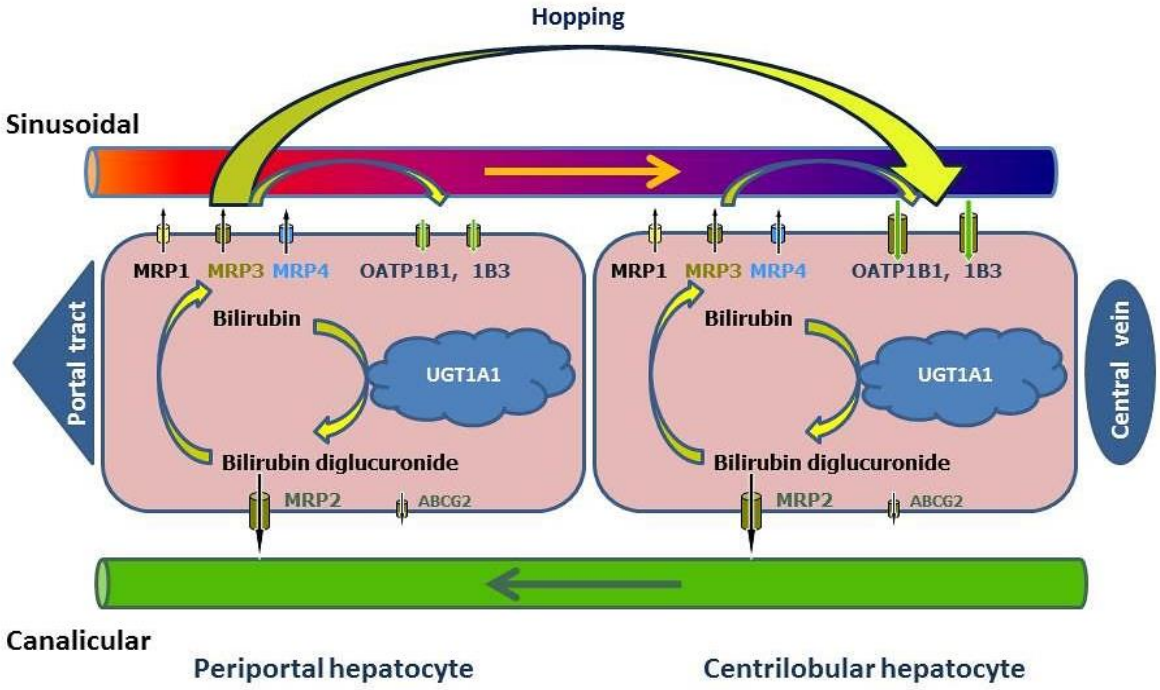

Fig. 9: **Hepatic cycle of conjugated bilirubin**. Bilirubin conjugated in the ER of hepatocytes is secreted into bile. This process is mediated by MRP2 with possible minor contribution of other transporters (ABCG2) at the canalicular membrane of hepatocytes. In addition, even under physiologic conditions, a substantial fraction of bilirubin conjugates is secreted by MRP3 and other MRP2 homologues at the sinusoidal membrane into the blood, from where they can be subsequently reuptaken by sinusoidal membrane - bound OATP1B1 and OATP1B3 transporters. The highest expression of OATP1B proteins has been demonstrated at the centrilobular hepatocytes. We hypothesize that MRP3-OATP1B1/3 substrate shifting from periportal to centrizonal hepatocytes may act as a protection of the periportal hepatocytes against elevated concentrations of various xenobiotics.

OATP1Bs may also contribute to liver uptake of UCB since complete absence of both OATP1Bs in RS is associated with elevated levels of UCB. Furthermore, results of functional studies demonstrate that OATP1B3, but not OATP1B1, may play an important role in the carrier-mediated uptake of foetal UCB by the placental trophoblast and contribute to elimination of UCB across the placental barrier (Briz O. et al., 2003, Macias R.I. et al., 2009).

Our human study clearly demonstrates that biallelic inactivating mutations of the human OATP genes *SLCO1B1* and *SLCO1B3* resulting in complete OATP1Bs deficiency, confirmed by absence of OATP1B1/3 immunostaining in liver biopsy, may explain predominantly conjugated hyperbilirubinemia of Rotor-type. Simultaneous and complete deficiency of OATP1B1/3 may also explain impairment of the BSP uptake and storage capacity in RS and in Hepatic Uptake and Storage Disease, respectively (Dhumeaux D. and Berthelot P., 1975, Wolpert E. et al., 1977, Berthelot P. and Dhumeaux D., 1978).

Except for 11 probands included in our study, during the years 2012 - 2013 we analyzed other three male subjects (1 Dutchman, 2 Turks) with predominantly conjugated hyperbilirubinemia, in which the diagnosis of RS was established by mutation analysis (two Turkish subjects) and by immunostaining of OATP1B1/3 transport proteins in two supplied liver biopsy specimens (the Dutch and one Turkish subject).

All thirteen RS individuals, whose mutation analysis had been performed, were homozygous for biallelic inactivating mutations in both *SLCO1B1* and *SLCO1B3.*
Three haplotypes were identified:

a) a biallelic nonsense mutation in *SLCO1B1* and a biallelic deletion of exon 12 in *SLCO1B3* were present in three subjects (haplotype R1-linked mutations)
b) a biallelic whole-gene deletion spanning both *SLCO1B1* and *SLCO1B3* was present in nine subjects (haplotype R2-linked mutations)
c) a nonsense mutation in *SLCO1B1* and a biallelic splice site mutation in *SLCO1B3* were present in one subject (haplotype R3-linked mutations)

Mutations in *SLCO1B1* and *SLCO1B3* detected in 13 RS subjects are summarized in Tab. 5.

Interestingly, biochemistry and genetic analysis of family members of Rotor subjects and control subjects indicate that presence of at least one wild-type (functional) allele of either *SLCO1B1* or *SLCO1B3* prevents Rotor-type hyperbilirubinemia (Jirsa M. et al., 2012). A combination of a mild mutation in one allele of either *SLCO1B1* or *SLCO1B3* with deleterious mutations affecting the remaining three alleles has not yet been documented.

Tab. 5: **Mutations in *SLCO1B* genes in RS subjects**

Proband	**Haplotype R1-linked mutations**		**Haplotype R2-linked mutations**		**Haplotype R3-linked mutations**	
	SLCO1B3 7.2-kb deletion	*SLCO1B1* c.1738C→T (p.R580X)	*SLCO1B3* 405-kb deletion	*SLCO1B1* c.481+1G→T splice site mutation	*SLCO1B3* c.1747+1G→A splice site mutation	*SLCO1B1* c.757C→T (p.R253X)
1	del/del	T/T				
2	del/del	T/T				
3	del/del	T/T				
4			del/del	–/–		
5			del/del	–/–		
6			del/del	–/–		
7			del/del	–/–		
8			del/del	–/–		
9			del/del	–/–		
10			del/del	–/–		
11					A/A	T/T
12			del/del	–/–		
13			del/del	–/–		

Since in Proband 3 the cause of predominantly conjugated hyperbilirubinemia had not been explained by mutation analysis of *ABCC2* (see Chapter 3.2) and the proband had not been included in the former Rotor study, we additionally analyzed *SLCO1B1* and *SLCO1B3* in this subject. No pathogenic mutation but seven known likely non-pathogenic sequence variations were disclosed (Tab. 6).

Tab. 6**: Mutations in *SLCO1B* genes in Proband 3**

Gene	**DNA alteration**	**Protein alteration**	**Pathogenicity**	**MAF**
SLCO1B1	Heterozygous c.388A/G	p.Asn130Asp	SNP rs2306283	0.378
	Homozygous c.571T/C	p.Leu191Leu	SNP rs4149057	0.367
SLCO1B3	Homozygous c.334T/G	p.Ser112Ala	SNP rs4149117	0.298
	Homozygous c.360-3 c/t		SNP rs3764009	0.298
	Homozygous c.699G/A	p.Met233Ile	SNP rs7311358	0.298
	Heterozygous c.767G/C	p.Gly256Ala	SNP rs60140950	0.066
	Homozygous c.1557A/G	p.Ala519Ala	SNP rs2053098	0.297

MAF – minor allele frequency in GenBank dbSNP

3.3.3 Conclusion

In this study we have elucidated the role of the transport proteins OATP1B1 and OATP1B3 in the metabolic pathway of heme degradation and disclosed still unknown hepatic cycle (the MRP3 - OATP1B1/3 loop) of conjugated bilirubin and many other substrates. In the human part of the study we demonstrated that biallelic inactivating mutations of both *SLCO1B1* and *SLCO1B3* with complete absence of transport proteins OATP1B1 and OATP1B3 at the sinusoidal membrane of hepatocytes result in Rotor-type hyperbilirubinemia.

3.4 The role of Rotor proteins OATP1B1 and OATP1B3 in the pathogenesis of jaundice

Enclosure 6: Sticova E, Lodererova A, Schinkel AH, van de Steeg E, Frankova S, Kollar M, Lanska V, Kotalova R, Dedic T, Jirsa M. **Down-regulation of OATP1B proteins correlates with hyperbilirubinemia in advanced cholestasis.** *(submitted)*

3.4.1 Summary of results

The last study is focused on the potential role of Rotor proteins in the pathogenesis of jaundice in common liver diseases. Our aim was to correlate immunohistochemical expression of OATP1B proteins in formalin-fixed paraffin-embedded liver tissue with serum bilirubin levels in advanced stages of primary hepatocellular and primary biliary liver diseases.

In the first part of the study five primary antibodies directed against OATP1B1 and/or OATP1B3 polypeptide were tested in frozen and paraffin embedded liver tissue using different immunohistochemical protocols. The results of the staining in formalin-fixed paraffin-embedded tissue are summarized in Tab. 7.

The most important findings are as follows:

a) the MDQ (ab15442) anti-OATP1B1 antibody cross-reacts with both OATP1B1 and OATP1B3 in frozen and paraffin-embedded liver tissue
b) the H-52 (sc-98981) anti-OATP1B3 antibody cross-reacts with both OATP1Bs with more specific detection of OATP1B3 in frozen and paraffin-embedded liver tissue
c) the ESL (ab15441) anti-OATP1B1 antibody specifically recognizes OATP1B1 only in frozen sections, but not in formalin-fixed paraffin-embedded tissue.

In the second part of the study, proportion of hepatocytes expressing both OATP1B1 and OATP1B3 was semi-quantitatively assessed with the MDQ antibody in formalin-fixed paraffin-embedded liver samples obtained from the patients with end-stage hepatocellular (n=21) and biliary liver diseases (n=31). Thereafter, the immunohistochemical OATP1Bs expression was correlated with serum bilirubin levels in these patients. *UGT1A1* promoter TATA-box and *SLCO1B1* rs4149056 genotyping was performed to rule out individuals genetically predisposed to hyperbilirubinemia.

Tab. 7: **Reactivity of primary antibodies in formalin-fixed paraffin-embedded liver tissue**

Primary antibody	Immunogen aa no.	Immunogen sequence	Pretreatment					
			Without	Proteinase K	pH6	pH8	pH9	High pH
OATP2 MDQ (Ab15442)	OATP1B1, 1-24	MDQNQHLNKTAEAQPSENKK TRYC	-	-	+	+	+	+
OATP2 ESL (Ab15441)	OATP1B1, 671-691	ESLNKNKHFVPSAGADSETHC	-	-	-	-	-	-
Oatp2 A-2 (sc-376424)	Oatp1b2, 611-660	ASFLPALFILILMRKFQFPGDID SSDTDPAEMKLTAKESKCTNV HRSPTM	-	-	-	-	-	-
OATP2 (LS-C8521,2)	OATP1B1, C-term	17 aa, sequence not provided	-	-	-	-	-	-
OATP8 H-52 (sc-98981)	OATP1B3, 651-702	FQGKDTKASDNERKKVMDEA NLEFLNNGEHFVPSAGTDSKT CNLDMQDNAAAN	-	-	+	+	+	+

Expression of the OATP1B proteins was decreased in end-stages of both groups of patients with significantly lower values in the group of primary biliary disorders (1.9±1.1 vs. 2.7±0.6; p=0.009). Inverse correlations between the immunohistological OATP1Bs expression score and serum total, conjugated and unconjugated bilirubin levels were observed in the advanced stages of primary biliary diseases. By contrast, no statistically significant correlation was found between the same parameters in the group of primary hepatocellular diseases.

3.4.2 Commentary

Human OATP1B1 and OATP1B3 are highly homologous proteins with similar genomic organization and close secondary structure (König J. et al., 2000a, König J. et al., 2000b, Cui Y. et al., 2003).

Cross-reactivity of the mouse monoclonal anti-OATP2 antibody [MDQ] (ab15442) with both human OATP1B1 and OATP1B3 has been already described in Western blot, immunoprecipitation and immunocytochemistry of transfected cells (Cui Y. et al., 2003). In our study, we confirmed cross-reactivity of MDQ antibody in *SLCO1B1*^{tg} and *SLCO1B3*^{tg} mouse and in human formalin-fixed paraffin-embedded liver tissue.

In the second part of the study we demonstrated reduced immunohistological OATP1B1/3 expression in terminal stages of liver diseases, which was more marked in the group of primary biliary disorders compared to the primary non-cholestatic parenchymal diseases. Our observations correspond to the results of the previously published experimental studies demonstrating down-regulation of OATP1B mRNA and/or protein in patients suffering from primary sclerosing cholangitis, primary biliary cirrhosis and biliary atresia (Oswald M. et al., 2001, Kojima H. et al., 2003, Zollner G. et al., 2003, Chen H.L. et al., 2008).

Decreased OATP1Bs expression can be explained by combination of several possible mechanisms encompassing reduced liver cell mass in cirrhosis and additional genetic and epigenetic mechanisms resulting in decreased number of the OATP1B1/3 expressing cells and reduced density of OATP1B proteins at the basolateral membrane of hepatocytes. Down-regulation of the basolateral bilirubin uptake systems mediated most likely by action of bile salts is supposed to represent an adaptive process protecting hepatocytes against accumulation of toxic biliary constituents during chronic cholestasis (Zollner G. et al., 2003, Geier A. et al., 2007).

3.4.3 Conclusion

Based on the results of our study we assume that down-regulation of OATP1B1 and OATP1B3 at the basolateral membrane of hepatocytes may contribute to conjugated hyperbilirubinemia in advanced liver diseases with predominantly obstructive type of cholestasis.

4. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Bile formation and secretion is a unique and vital function of the liver. Over the last several decades, the application of cellular and molecular techniques to the study of heme degradation pathway and bile formation has resulted in considerable expansion of our knowledge in this field. Mutations affecting the basolateral and apical membrane transporters responsible for accumulation of either conjugated or unconjugated forms of bilirubin and other substrates have been identified. Furthermore, molecular basis of several hyperbilirubinemia syndromes has been disclosed.

Except for Gilbert syndrome, the majority of inherited hyperbilirubinemia syndromes are rare autosomal recessive disorders with a low prevalence in the general population and, apart from CN syndrome type I and some cases of CN type II in neonatal period, mostly not requiring further therapy. Nonetheless, the enzyme and transport systems involved in bilirubin metabolism may play an important role in the elimination and disposition processes of many other endogenous and exogenous substrates including hormones, drugs, toxins, and heavy metals. Dysfunction or absence of these systems, including selected ABC transporters and OATPs, may alter pharmacokinetics and pharmacodynamics of many biologically active agents, affect penetration of the substrates into various tissues and lead to their intracellular accumulation with a subsequent increase of organ toxicity (Kalliokoski A. et al., 2008, Strassburg C.P., 2008, Kalliokoski A. and Niemi M., 2009, Niemi M. et al., 2011). In addition, the absence of the functional transport proteins involved in hepatobiliary and enterohepatic circulation may involve drug disposition, drug-drug or drug-food interactions and result in decreased effectiveness or even resistance to a diverse spectrum of chemotherapeutic agents and xenobiotics (Kiang T.K. et al., 2005, Shitara Y., 2011, Karlgren M. et al., 2012). Individuals with mutations in the responsible gene or genes with the fully expressed phenotype of the corresponding hyperbilirubinemia syndrome, as well as subjects carrying mutations without clinical manifestation of hyperbilirubinemia under normal conditions, may be more susceptible to the adverse effects of some drugs and metabolites (Sadée W. and Dai Z., 2005, Sissung T.M. et al., 2010). The early and precise diagnosis and identification of a genetic defect thus avoids unnecessary invasive diagnostic procedures and may help to prevent unwanted drug side-effects in the genetically predisposed subjects.

Apart from drug-induced injury, genetic transporter variants may cause or predispose to a wide spectrum of human liver diseases and determine susceptibility and progression in acquired cholestatic disorders (Jacquemin E. et al., 2001, Wagner M. et al., 2009, Davit-Spraul A. et al.,

2010). To prevent occurrence or recurrence of the disease and its severe complications, molecular genetic techniques may permit the screening of high-risk subjects and early initiation of long-term curative or prophylactic therapy.

Regardless of rare occurrence of the majority of inherited hyperbilirubinemia syndromes, in our case report concerned with DJS (Enclosure 4) we demonstrate that hereditary jaundice should be included in the differential diagnosis of liver diseases even in atypical age categories. Moreover, the coincidence of hereditary hyperbilirubinemia with another disease or pathologic stimulus can modify the clinical picture and results of laboratory tests, including histomorphology (Hunter F.M. et al., 1964, Watanabe S. et al., 1982, Cebecauerova D. et al., 2005).

Despite the fact, that heme degradation pathway is supposed to be one of the best studied metabolic pathways in human, elucidation of molecular basis of Rotor syndrome and disclosure of still unknown hepatic cycle of conjugated bilirubin and other substrates has clearly demonstrated that there are still many questions that remain unanswered. Thus, further studies of enzymes and hepatobiliary transport systems involved in bilirubin metabolism and bile formation and their regulation in health and disease are warranted.

5. SOUHRN VÝSLEDKŮ

1. Přispěli jsme k objasnění úlohy bilirubinu v patogeneze oxidativního stresu u modelu obstrukční žloutenky.
2. U pěti ze šesti analyzovaných probandů se podařilo objasnit příčiny převážně konjugované hyperbilirubinémie:
   - u jednoho probanda byl diagnostikován DJS, způsobený dvěma dosud nepopsanými patogenními mutacemi
   - u jednoho probanda byly zjištěny simultánní mutace v genech *ABCC2* a *UGT1A1*, které mohou být asociovány s tzv. duální hereditární žloutenkou
   - u třech probandů byly nalezeny mutace asociované s Rotorovským typem žloutenky

   U jednoho probanda nebyly mutační analýzou genů *ABCC2, SLCO1B1* a *SLCO1B3* prokázány žádné patogenní mutace asociované s RS či DJS.
3. Naše práce přinesla nové poznatky o úloze transportních proteinů OATP1B1 a OATP1B3 v metabolické dráze degradace hemu a odhalila dosud neznámý jaterní cyklus konjugovaného bilirubinu a celé řady dalších substrátů pro MRP3, OATP1B1 a OATP1B3. Zároveň jsme prokázali, že současná inaktivující mutace *SLCO1B1* a *SLCO1B3* s kompletní absencí transportních proteinů OATP1B1 a OATP1B3 vedoucí k poruše zpětného vychytávání konjugovaného bilirubinu hepatocyty je molekulární podstatou žloutenky Rotorova typu.
4. Potvrdili jsme, že snížená exprese transportních proteinů OATP1B1 a OATP1B3 může být jedním z patogenetických mechanismů přispívajících k rozvoji ikteru v pokročilých stádiích získaných jaterních onemocnění, zejména s obstrukčním typem cholestázy.

SUMMARY OF RESULTS

1. We contributed to clarification of the role of bilirubin in pathogenesis of oxidative stress in a model of obstructive jaundice.
2. We explained the cause of predominantly conjugated hyperbilirubinemia in five of the six analyzed index subjects:
   - DJS caused by two novel pathogenic mutations was identified in one proband
   - simultaneous mutations in *ABCC2* and *UGT1A1* promoter likely associated with dual hereditary jaundice were detected in one proband
   - mutations associated with Rotor type of jaundice were detected in three probands

   In one subject mutations associated with either RS or DJS were not demonstrated by mutation analysis of *ABCC2, SLCO1B1* and *SLCO1B3*.
3. We brought new insights in the role of transport proteins OATP1B1 and OATP1B3 in the heme degradation pathway and disclosed still unknown hepatic cycle of conjugated bilirubin and many other substrates. Additionally, we demonstrated that simultaneous inactivating mutations in *SLCO1B1* and *SLCO1B3* with complete absence of basolateral transport proteins OATP1B1 and OATP1B3 resulting in disturbed uptake of conjugated bilirubin by hepatocytes represent molecular basis of Rotor syndrome.
4. We confirmed that decreased expression of OATP1B1 and OATP1B3 in hepatocytes may contribute to the pathogenesis of jaundice accompanying advanced stages of acquired liver diseases, particularly with obstructive type of cholestasis.

6. LIST OF EXPERIMENTAL METHODS

6.1 General methods

Nucleic acid isolation from human cells and tissues

Restriction analysis of DNA

Polymerase chain reaction

DNA electrophoresis on agarose and polyacrylamide gel

Molecular cloning techniques

Direct sequencing of genomic DNA

Histochemistry and immunohistochemistry on frozen and paraffin sections

Electron microscopy

Experimental work with small laboratory animals

Statistical methods

6.2 Specific methods

6.2.1 Methods related to evaluation the role of bilirubin and bile acids on mediating oxidative stress

Animal and experimental protocol. Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and biliary trees were exposed through midline abdominal incisions. Microsurgical ligation of bile ducts and resections of extrahepatic biliary tracts were performed. Sham-operated rats underwent the same procedure without bile duct resection and ligation.

Histopathological analysis. Left lateral lobes of livers were fixed overnight in 10% buffered formalin (pH 7.4) at 4°C followed by a standard procedure for paraffin embedding. Serial sections (6 μm thick) were cut and stained with H&E, Shikata's orcein method, and elastic-van Gieson stain. Each slide was viewed using standard light microscopy.

6.2.2 Methods related to characterization of subjects with predominantly conjugated type of hereditary jaundice

Mutation analysis of ABCC2. Written informed consent was obtained from the patients before their genetic examination. *ABCC2* was analysed by direct sequencing of genomic DNA extracted from peripheral leucocytes. All 32 exons, with the adjacent parts of the intronic sequences, were amplified by PCR using the intronic oligonucleotide primers. Amplified fragments were gel-purified, extracted from the gel with QIA quick spin columns (Qiagene, Hilden, Germany), and sequenced on a Genetic Analyzer ABI 3130 (Life Technologies, Prague, Czech Republic). The obtained sequence was compared with the reference sequences GenBank NM 000392 (mRNA) and NT 030059 (genomic DNA). Exon 18 with suspected deletion c.2360_2366delCCCTGTC was cloned into a plasmid vector pCR4.1-TOPO (Invitrogen, Carlsbad, CA), and the wild-type and mutated alleles were sequenced separately. Presence of the second mutation in DJS subject (Enclosure 4) was confirmed by PCR - restriction fragment - lenght polymorphism analysis (*PCR-Bsh1236*I *RFLP).*

The pathogenicity of the sequence variants in *ABCC2* detected in the probands was predicted by the GeneSplicer software (http://ccb.jhu.edu/software/genesplicer/), Pmut software (http://mmb2.pcb.ub.es:8080/PMut/) or PredictSNP 1.0 software (http://loschmidt.chemi.muni.cz/predictsnp/).

Histopathological methods and ultrastructural analysis of liver in DJS subject. Sections of the formalin-fixed and paraffin-embedded liver tissue cut at 4-6 μm were stained with H&E, periodic acid-Schiff with diastase (PAS-D), Schmorl´s and van Gieson´s method. Special stainings (Gömöri, silver ammonium complex - Masson´s and Perls Prussian blue method) for pigment characterization were added.

For immunohistochemical analysis, 4-6 μm - thick sections were incubated with the anti-MRP2 mouse monoclonal antibody (clone M2III-6, Kamiya, Seattle, WA). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualisation and counterstaining with Harris´s hematoxylin was performed.

Ultrastructural analysis was performed on formalin-fixed liver sample osmicated, dehydrated in ascending ethanol solutions and embedded into Epon-Araldite mixture. Ultrathin sections were double stained with uranyl acetate and lead nitrate and then examined under a JEM 1200 EX electron microscope.

6.2.3 Methods related to Rotor study

Histopathological analysis. Sections of archival paraffin-embedded human liver tissue (formalin or Carnoy solution fixative; 4–6 μm thick) were stained with H&E, elastic-van Gieson stain and PAS-D techniques.

For OATP1B1 and OATP1B3 immunostaining, deparaffinized sections were treated in 10 mM sodium citrate buffer, pH 6.0, for 30 min at 96°C, and incubated with primary mouse anti-OATP1B antibody (clone MDQ; ab15442, Abcam, Cambridge, UK), 1:100 dilution, overnight at 4°C. Bound antibody was visualized with horseradish peroxidase/diaminobenzidine (EnVision), with hematoxylin counterstaining.

For MRP2 immunostaining, 4-6 μm thick sections were incubated with the anti-MRP2 mouse monoclonal antibody (clone M2III-6, Kamiya, Seattle, WA). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualisation and counterstaining with Harris´s hematoxylin was performed.

6.2.4 Methods related to evaluation the role of OATP1B1 and OATP1B3 transporters in pathogenesis of jaundice

Mutation analysis of UGT1A1 and SLCO1B1. *UGT1A1* TATA-box promoter polymorphism rs8175347 and the *SLCO1B1* c.521T>C (p.Val174Ala) coding polymorphism rs4149056 were genotyped by direct sequencing of genomic DNA extracted from peripheral leucocytes on the Applied Biosystems ABI 3130 genetic analyzer (Life Technologies, Prague, Czech Republic).

Histopathological analysis. The 4 μm thick paraffin sections of formalin-fixed mouse and human liver tissue were pretreated by incubation with Proteinase K (Dako, Glostrup, Denmark) or in citrate buffer - pH 6.0 (Dako), Tris/EDTA buffer pH 8.0 (Leica, Wetzlar, Germany), Tris/EDTA buffer pH 9.0 (Dako), and High pH buffer (Dako). Sections without pretreatment were also used in parallel. Subsequent incubations with primary antibodies recognizing either N- or C- terminus of OATP1B1 and/or OATP1B3 (dilution 1:50 and 1:100) were done overnight at +4° C. For detection of primary antibodies a two-step (Dako, Histofine) or a three-step (Vector, Laboratories, Burlingame, CA) visualization system was used. Counterstaining with Harris's hematoxylin was performed at the end.

To minimize the reactivity of the secondary anti-mouse antibody with endogenous immunoglobulin in the mouse tissue, sections of mouse livers were stained with the Dako ARK^{TM} (Animal Research Kit) Peroxidase (Dako).

Statistical analysis. Results were expressed as the mean ± SD. To calculate the statistical significance of the differences between the groups, the Mann-Whitney test was used. The relations between the parameters were estimated by the nonparametric Spearman´s correlation coefficient. An exponential model was used for significant correlations. Two-sided $p<0.05$ was considered statistically significant.

Detailed description of the methods listed above, all performed by the author, is a part of a methodology section of the articles enclosed to this thesis (Enclosures No. 3-6).

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8. LIST OF AUTHOR´S PUBLICATIONS AND PRESENTATIONS

8.1 Publications *in extenso* related to the topic of the Ph.D. thesis

1. Muchova L, Vanova K, Zelenka J, Lenicek M, Petr T, Vejrazka M, Sticova E, Vreman HJ, Wong RJ, Vitek L. Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress. J Cell Mol Med. 2011; 15(5):1156-65. **IF 4.124**
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Review articles

5. Sticova E, Jirsa M. New insights in bilirubin metabolism and their clinical implications. World J Gastroenterol. 2013; 19(38):6398-6407. Review. **IF 2.433**
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8.2 Publications *in extenso* not related to the topic of the Ph.D. thesis

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8.3 Author´s presentations related to the topic of the Ph.D. thesis

1. Sticová E, Imunohistochemická diagnostika jaterních chorob. XXXIX. Májové hepatologické dny, Karlovy Vary, 10.-13.5.2010. Oral presentation.
2. Sticová E, Elleder M, Hůlková H, Novotný J, Lukšan O, Jirsa M. Dubin-Johnsonův syndrom: koincidence s kolorektálním karcinomem a pokročilou aterosklerózou u 82-letého pacienta. XL. Májové hepatologické dny, Karlovy Vary, 16.-18.5.2012 a Dny molekulární patologie. Olomouc, 26.4.-27.4.2013. Poster.
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4. Sticová E. Vrozené vady metabolismu bilirubinu a žlučových kyselin. Bioptická diagnostika chorob jater pro denní praxi. Doškolovací kurz. Praha, 25.-27.11.2013. Oral presentation.
5. Sticova E, Lodererova A, Frankova S, Kollar M, Lanska V, Jirsa M. Down-regulation of OATP1B proteins contributes to hyperbilirubinemia in advanced cholestasis. Prague Hepatology Meeting, Prague, 11.-13.9.2014. Poster.

8.4 Author´s presentations not related to the topic of the Ph.D. thesis

1. Sticová E, Lukšan O, Mrázová I, Jirsa M. Úloha MMP9 v progresi jaterního poškození u modelu biliární léze. XXXVIII. Májové hepatologické dny, Karlovy Vary, 12.-14.5.2010. Poster.
2. Sticová E, Kozák T, Vernerová Z. Aggressive systemic mastocytosis with Charcot-Leyden crystals-associated crystal storing histiocytosis in bone marrow. 24th European Congress of Pathology. Praha. 8.-12.9. 2012. Poster.
3. Kollar M, Sticova E, Frankova S, Honsova E. Acute hepatitis E virus infection as a cause of graft hepatitis. 25th European Congress of Pathology. Lisabon, 31.8.- 4.9.2013. Poster.
4. Sticova E, Gkalpakiotis S, Gurlich R, Sach J. Pancreatic panniculitis and intraosseous fat necroses as the initial symptoms of pancreatic disease. AAF/OMI Satellite Symposium on selected issues in surgical pathology, 28.-29.3. 2014. Semmelweis University, Budapest, Hungary. Oral presentation.

9. ENCLOSURES

1. Sticova E, Jirsa M. New insights in bilirubin metabolism and their clinical implications. World J Gastroenterol. 2013; 19(38):6398-6407. Review.
2. Jirsa M, Sticová E. Vrozené hyperbilirubinémie a molekulární mechanizmy žloutenky. Vnitřní lékařství. 2013; 59(7):566-571. Review.
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REVIEW

New insights in bilirubin metabolism and their clinical implications

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Abstract

Bilirubin, a major end product of heme breakdown, is an important constituent of bile, responsible for its characteristic colour. Over recent decades, our understanding of bilirubin metabolism has expanded along with the processes of elimination of other endogenous and exogenous anionic substrates, mediated by the action of multiple transport systems at the sinusoidal and canalicular membrane of hepatocytes. Several inherited disorders characterised by impaired bilirubin conjugation (Crigler-Najjar syndrome type Ⅰ and type Ⅱ, Gilbert syndrome) or transport (Dubin-Johnson and Rotor syndrome) result in various degrees of hyperbilirubinemia of either the predominantly unconjugated or predominantly conjugated type. Moreover, disrupted regulation of hepatobiliary transport systems can explain jaundice in many acquired liver disorders. In this review, we discuss the recent data on liver bilirubin handling based on the discovery of the molecular basis of Rotor syndrome. The data show that a substantial fraction of bilirubin conjugates is primarily secreted by MRP3 at the sinusoidal membrane into the blood, from where they are subsequently reuptaken by sinusoidal membrane-bound organic anion transporting polypeptides OATP1B1 and OATP1B3. OATP1B proteins are also responsible for liver clearance of bilirubin conjugated in splanchnic organs, such as the intestine and kidney, and for a number of endogenous compounds, xenobiotics and drugs. Absence of one or both OATP1B proteins thus may have serious impact on toxicity of commonly used drugs cleared by this system such as statins, sartans, methotrexate or rifampicin. The liver-blood cycling of conjugated bilirubin is impaired in cholestatic and parenchymal liver diseases and this impairment most likely contributes to jaundice accompanying these disorders.

Key words: Hyperbilirubinemia; Hereditary jaundice; UGT1A1; ABCC2; Organic anion transporting polypeptide 1B1; Organic anion transporting polypeptide 1B3

Core tip: Experiments with *Oatp1a/1b*-null mice and *Oatp1a/1b*; *Abcc3* combination knockout mice plainly demonstrated that even under physiologic conditions a substantial portion of bilirubin glucuronides is not excreted directly into bile but is transported back to the blood by Abcc3. *Oatp1a/1b* activity accentuated in downstream (centrizonal) hepatocytes allows efficient reuptake of bilirubin conjugates, with a subsequent possibility being safely eliminated by excretion into bile. This and molecular findings in Rotor syndrome suggest that human transporters MRP3 and OATP1Bs form a sinusoidal liver-to-blood cycle which mediates shifting (hopping) of bilirubin and other substrates from periportal to centrizonal hepatocytes (References 18, 19, 22, 125).

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INTRODUCTION

Bilirubin is the end product of heme breakdown. About 80% of bilirubin originates from degradation of erythrocyte haemoglobin in the reticuloendothelial system; the remaining 20% comes from inefficient erythropoiesis in bone marrow and degradation of other heme proteins[1-4]. Water insoluble, unconjugated bilirubin (UCB) bound to albumin is transported to the liver where it is removed from the plasma. The exact mechanism of UCB uptake is unknown; however, passive transmembrane diffusion seems to be combined with active transport mediated by several sinusoidal transporters (see below). Within the cytoplasm of hepatocytes, bilirubin is bound to ligandin and transported to endoplasmic reticulum where conjugation with glucuronic acid takes place. Conjugation is catalysed by the enzyme uridine diphosphate glycosyltransferase 1A1 (UGT1A1; EC2.4.1.17), a member of an enzyme family in the endoplasmic reticulum and nuclear envelope of hepatocytes[5-8]. In addition to the liver, UGT activity has also been detected in the small intestine and kidney[9,10]. *UGT1A1* gene (ID: 54658) is a part of a complex locus encoding 13 UDP-glucuronosyltransferases[11]. The locus contains a series of thirteen unique alternate promoters and first exons, followed by four common exons No. 2-5. Theoretically, each of the unique first exons is spliced to the first of the four shared exons. The unique first exons encode different substrate binding domains whereas the other functional domains encoded by the shared exons 2-5 are the same[11-15]. In reality, only 9 of the 13 predicted *UGT1A*s are active genes encoding functional enzymes; four are nonfunctional pseudogenes.

The excretion of conjugated bilirubin into bile is mediated by an ATP-dependent transporter identified as the multidrug resistance-associated protein MRP2/cMOAT and, to a lesser extent, also by ATP-binding cassette (ABC) efflux transporter ABCG2. MRP2 is encoded by *ABCC2* and expressed under physiologic conditions at the apical (canalicular) membrane of hepatocytes and, to a much lesser extent, in the kidney, duodenum, ileum, brain and placenta[16]. Since the MRP2 mediated export represents an important step in detoxification of many endogenous and exogenous substrates, the absence of functionally active MRP2 prevents the secretion of these conjugates into bile. Absence of MRP2 mediated transport is followed by upregulation of the basolateral MRP2 homologues at the sinusoidal membrane of hepatocytes and conjugated bilirubin flow is redirected into sinusoidal blood[17]. Aside from MRP2 mediated transport of conjugated bilirubin into bile, recent studies have shown that a significant fraction of the bilirubin conjugated in the liver is, under physiologic conditions, secreted into sinusoidal blood and subsequently reuptaken by hepatocytes for final biliary excretion[18,19]. The process is mediated by sinusoidal transporters MRP3 and organic anion-transporting polypeptides OATP1B1 and OATP1B3. OATP1B transporters facilitate sodium-independent uptake of numerous endogenous and exogenous substrates[20,21]. Since expression of OATP1Bs is higher in centrilobular hepatocytes, the MRP3-OATP1B1/3 loop is likely responsible for shifting (hopping) of conjugated bilirubin and other substrates from the periportal to the centrilobular zone of the liver lobule (Figure 1). Such intralobular substrate transfer may protect periportal hepatocytes against elevated concentrations of various xenobiotics[22]. In addition, the OATP1B proteins mediate hepatic clearance of bilirubin conjugated in splanchnic organs and may represent an important alternative pathway in enterohepatic circulation[18].

OATP1Bs may also contribute to liver uptake of UCB since complete absence of both OATP1Bs in Rotor syndrome (RS, see below) is associated with elevated levels of UCB and single nucleotide polymorphisms in genes encoding OATP1B proteins have been shown to influence serum bilirubin level[23,24]. Furthermore, results of functional studies demonstrate that OATP1B3, but not OATP1B1, may play an important role in the carrier-mediated uptake of foetal UCB by the placental trophoblast and contribute to elimination of UCB across the placental barrier[25,26].

Mild or moderately elevated serum bilirubin seems to be beneficial: Bilirubin is known as a strong antioxidant[27,28] and the protective effects of bilirubin on atherogenesis and cancerogenesis have been demonstrated in both *in vitro* and *in vivo* studies[29-33]. On the other hand, patients with profound unconjugated hyperbilirubinemia are at risk for bilirubin encephalopathy (kernicterus)[34,35]. The toxic effects of bilirubin are explained by inhibition of DNA synthesis[36]. Bilirubin may also uncouple oxidative phosphorylation and inhibit adenosine triphosphatase (ATPase) activity of brain mitochondria[37,38]. Bilirubin mediated inhibition of various enzyme systems, RNA synthesis and protein synthesis in the brain and liver, and/or alteration of carbohydrate metabolism in the brain can also contribute to its toxicity[39-43]. The accumulation of bilirubin in plasma and tissues results in characteristic yellow discoloration of tissues known as icterus or jaundice.

Inherited disorders of bilirubin excretory pathway played the key role in understanding the individual steps of the bilirubin excretory pathway. Disrupted regulation of hepatobiliary transport systems explained jaundice in many acquired liver disorders[44-48]. Additional information was obtained from a number of animal models of hereditary jaundice. These include the Gunn rat and *Ugt1*(-/-) mouse mimicking the Crigler-Najjar syndrome type I [49-51], the Bolivian population of squirrel monkeys mimicking Gilbert syndrome (GS)[52,53] and mutant TR or GY (Groningen yellow) rats with organic anion excretion defect (TR -/-), Eizai hyperbilirubinuria rats (EHBR), mutant Corriedale sheep, and *Mrp2*(-/-) mice, all modelling the Dubin-Johnson syndrome (DJS)[54-58].

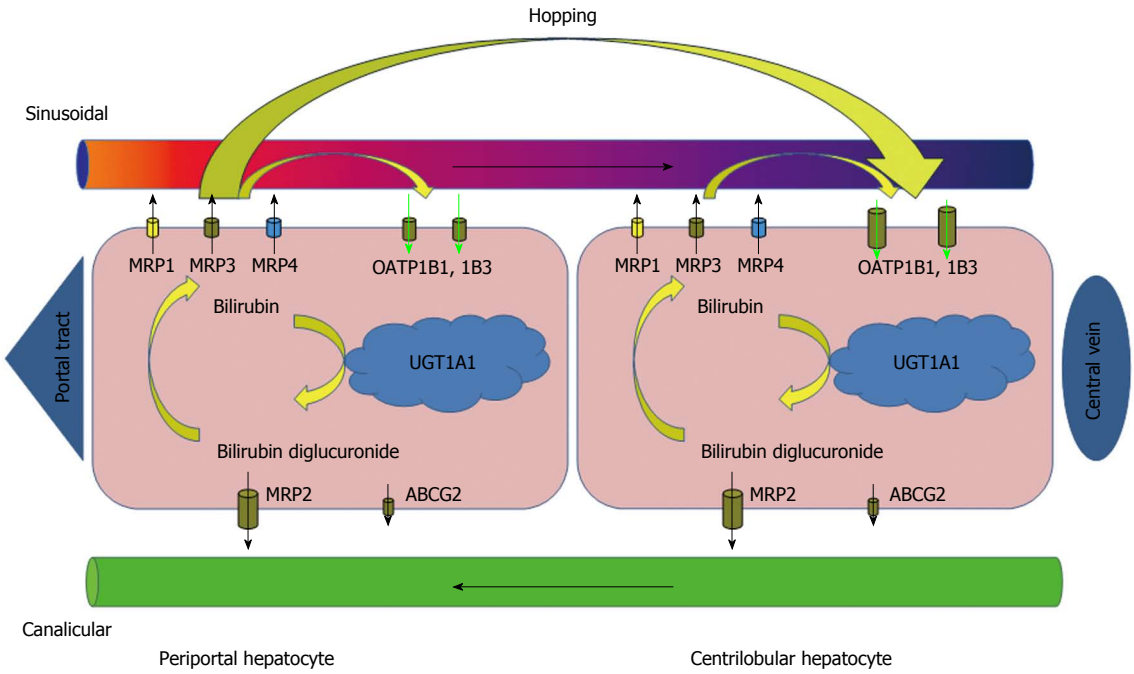

Figure 1 Liver cycle of conjugated bilirubin. Bilirubin conjugated in endoplasmic reticulum of hepatocytes is secreted into the bile. This process is mediated by MRP2/ABCC2 with possible minor contribution of other transporters (ABCG2) at the canalicular membrane of hepatocytes. In addition, even under physiologic conditions, a fraction of bilirubin conjugates is secreted by MRP3 across the sinusoidal membrane into the blood, from where they can be subsequently reuptaken by sinusoidal membrane-bound OATP1B1 and OATP1B3 transporters. The highest overall expression of OATP1Bs has been demonstrated at the centrilobular hepatocytes. The process of substrate shifting (hopping) from periportal to centrizonal hepatocytes may act as a protection of the periportal hepatocytes against elevated concentrations of various xenobiotics. MRP: Multidrug resistance-associated protein; OATP: Organic anion transporting polypeptide; UGT: Uridine diphosphate glucuronosyltransferase; ABC: ATP-binding cassette.

HEREDITARY PREDOMINANTLY UNCONJUGATED HYPERBILIRUBINEMIA

Conjugation of bilirubin in endoplasmic reticulum is catalysed by the enzyme UGT1A1. Mutations in *UGT1A1* can lead to decreased expression or partial or even complete inactivation of the enzyme[59]. By contrast, expression of *UGT1A1* can be increased by phenobarbital (PB) administration. PB response activity is delineated to a 290-bp distal enhancer module sequence (-3483/-3194) glucuronosyltransferase phenobarbital response enhancing motif (gtBPREM) of the human *UGT1A1*[59,60]. gtBPREM is activated by the nuclear orphan receptor, human constitutive active receptor (hCAR). CAR is a cytoplasmic receptor which, after treatment with activators such as PB, translocates into the nucleus, forms a heterodimer with the retinoid X receptor and activates the PB response enhancer element.

Three types of inherited, predominantly unconjugated hyperbilirubinemia with different levels of UGT1A1 activity are recognised: Crigler-Najjar syndrome type I (CN1), type II (CN2) and GS.

CN1 (MIM#218800), the most deleterious form, described in 1952 by Crigler and Najjar[61], is characterised by complete or almost complete absence of UGT1A1 enzyme activity with severe jaundice[62]. Icterus occurring shortly after birth is complicated by bilirubin encephalopathy (kernicterus). Until the introduction of phototherapy and plasmapheresis, kernicterus was fatal in almost all cases during the first two years of life or caused serious brain damage with permanent neurologic sequelae. Intermittent phototherapy is lifelong and it results in a thorough elimination of water-soluble photoisomers of unconjugated bilirubin *via* bile. The effectiveness of phototherapy may decrease gradually with age and patients are at higher risk of sudden brain damage[63].

Although new treatment modalities such as hepatocyte or hepatic progenitor cell transplantation have already been used to treat CN1 patients, liver transplantation is still considered to be the only definitive treatment for CN1[63-67]. Gene therapy seems to be a promising therapeutic possibility for the patients with CN1 in the near future[68,69].

CN2 (Arias syndrome, MIM #606785), described by Arias in 1962[70], is characterised by reduced UGT1A1 enzyme activity with a moderate degree of nonhemolytic jaundice. Bilirubin levels do not exceed 350 μmol/L and CN2 is only rarely complicated by kernicterus[71]. Virtually all the mutations responsible for the syndrome are autosomal recessive, as in CN1, but several observations have also suggested the possibility of autosomal dominant pattern of inheritance[72-74].

An important clinical difference between CN type I and type II is the response to PB treatment, with no effect in type I (complete loss of the UGT1A1 enzyme activity) and a decrease of serum bilirubin levels by more than 30% in CN type II (some residual UGT1A1 activity is preserved). Moreover, bilirubin glucuronides are present in bile in CN2. However, the method of choice for the diagnosis of CN syndrome is mutation analysis of

UGT1A1[75].

GS (MIM #143500), described in 1901 by Gilbert and Lereboulet[76], is characterised by fluctuating mild, unconjugated nonhemolytic hyperbilirubinemia < 85 μmol/L without overt haemolysis, usually diagnosed around puberty, and aggravated by intercurrent illness, stress, fasting or after administration of certain drugs[77,78]. Physical examination and the results of routine laboratory tests are normal apart from elevated serum bilirubin and jaundice. The clinical diagnosis of GS can be established if patients have a mild, predominantly unconjugated hyperbilirubinemia and normal activity of liver enzymes. The reduced caloric intake test and phenobarbital stimulation test have low diagnostic specificity in GS subjects[79]. Histological findings in GS are mild, with a slight centrilobular accumulation of pigment with lipofuscin-like properties[80]. Ultrastructurally, hepatocytes reveal hypertrophy of smooth endoplasmic reticulum[81,82]. Since the morphological picture of GS is completely non-specific and the disorder is benign, liver biopsy is not indicated.

GS is characterised by reduced levels of UGT1A1 activity to about 25%-30% caused by homozygous, compound heterozygous, or heterozygous mutations in the *UGT1A1* with autosomal recessive transmission[80].

GS is the most frequent hereditary jaundice affecting nearly 5%-10% of the Caucasian population[83]. The genetic basis of GS was first disclosed in 1995[84] as presence of the allele *UGT1A1*28*, characterised by insertion of TA in the TATAA box (A[TA]7TAA) in the proximal promoter of *UGT1A1*. *UGT1A1*28* has been identified as the most frequent mutation in Caucasian GS subjects[85]. The insertion is responsible for reduction of transcription of *UGT1A1* to 20% from normal and for a decrease of hepatic glucuronidation activity by 80% in a homozygous state[86]. In Caucasians and African Americans, the frequency of *UGT1A1*28* allele is about 35%-40%, but it is much lower in Asians, including Koreans (13%), Chinese (16%), and Japanese (11%)[87-89]. Moreover, in the majority of Caucasian GS subjects, expression of UGT1A1 is further decreased by the presence of the second mutation T>G in gtPBREM[59,60]. In addition to the mutations in the promoter, GS may be caused by mutations in structural regions of the *UGT1A1*. In Asians, other variants, such as *UGT1A1*6* characterised by a missense mutation involving G to A substitution at nucleotide 211 (c.211G>A) in exon 1 (also known as p.G71R), *UGT1A1*7* (p.Y486D), *UGT1A1*27* (p.P229Q), and *UGT1A1*62* (p.F83L) have been detected[60,87-90].

In addition to biochemical defect leading to reduced glucuronidation, other factors, such as impaired hepatic (re)uptake of bilirubin (see Rotor syndrome below for the possible mechanism) or an increased load of bilirubin, seem to be necessary for clinical manifestation of GS[86,91,92].

GS is benign and GS carriers present with no liver disease. However, the mutations in the *UGT1A1* identical to those recognised in GS subjects may contribute to the development of prolonged neonatal hyperbilirubinemia in breast-fed infants[93,94].

Moreover, since the process of glucuronidation is an important step in elimination of numerous endogenous and exogenous substrates, GS subjects may be more susceptible to the adverse effects of some drugs metabolised by UGT1A1, such as indinavir, atazanavir[95-99] or irinotecan[100-102].

HEREDITARY PREDOMINANTLY CONJUGATED HYPERBILIRUBINEMIA

Two types of hereditary conjugated jaundice are known as Dubin-Johnson and Rotor syndrome. Both are characterised by the presence of mixed, predominantly conjugated hyperbilirubinemia, with conjugated bilirubin more than 50% of total bilirubin.

DJS (MIM # 237500), a benign autosomal recessive disorder described in 1954 by Dubin *et al*[103] and Sprinz *et al*[104], is characterised by fluctuating mild, predominantly conjugated hyperbilirubinemia, with typical manifestation in adolescence or young adulthood. Most patients are asymptomatic except of occasional slight abdominal pain and fatigue. Urine excretion of total coproporphyrin in 24 h is normal, but 80% are represented by coproporphyrin I. Biliary excretion of anionic dyes including bromosulfophthalein (BSP), indocyanine green and cholescintigraphy radiotracers is delayed with absent or delayed filling of the gallbladder[105]. BSP clearance in DJS subjects is normal at 45 min with the second peak at 90 min[106]. Liver histology in DJS shows an accumulation of distinctive melanin-like lysosomal pigment in an otherwise normal liver that gives the organ a characteristic dark pink or even black colour. The pigment is positive in PAS and Masson-Fontana reaction with marked autofluorescence. In contrast to melanin, DJS pigment does not reduce neutral silver ammonium solution[103,107]. The amount of pigment may vary and possible transient loss may occur in coincidence with other liver diseases[108,109]. The molecular mechanism in DJS is absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT caused by homozygous or compound heterozygous mutation in *ABCC2* (gene ID: 1244) on chromosome 10q24[110-114]. The *ABCC2* mutation alters not only MRP2-mediated transport of conjugated bilirubin but also transport of many anionic substrates as well as a wide range of drugs, such as chemotherapeutics, uricosurics, antibiotics, leukotrienes, glutathione, toxins and heavy metals. Absence of MRP2/cMOAT may result in impaired elimination and in subsequent renal toxicity of the substrates mentioned above[115-120].

A rare type of hereditary mixed hyperbilirubinemia caused by the simultaneous presence of mutations characteristic for DJS and GS has been classified as dual hereditary jaundice[121]. Serum direct bilirubin concentrations in dual hereditary jaundice reach only 20%-50% of total bilirubin.

RS (MIM #237450), described in 1948 by Rotor *et al*[122],

is characterised by mild, predominantly conjugated hyperbilirubinemia with delayed excretion of anionic dyes without re-increase of their concentration. Total urinary coproporphyrin excretion is significantly increased and the proportion of coproporphyrin I in urine is approximately 65% of the total in homozygotes and 43% in heterozygotes[123,124]. By histopathological examination, the liver tissue does not display any marked architectural or cytomorphological abnormalities and pigment is not present.

The presence of homozygous mutations in both *SLCO1B1* and *SLCO1B3* neighbouring genes located on chromosome 12 with complete and simultaneous deficiency of proteins OATP1B1 and OATP1B3 has recently been identified as the molecular mechanism of the syndrome[125]. The complete absence of both transporters OATP1B1 and OATP1B3 has been confirmed by immunohistochemistry in all studied Rotor subjects. Interestingly, the presence of a single functional allele of either *SLCO1B1* or *SLCO1B3* prevented the jaundice.

RS does not require any therapy but, with regard to the impact of OATP1B transporters on pharmacokinetics of a broad spectrum of commonly used drugs such as penicillins, statins, sartans, rifampicin, methotrexate and many others, it is assumed that RS subjects and also those with the deleterious mutations in either of the *SLCO1B* genes, even without full clinical expression of the syndrome, may be at increased risk for drug toxicity[125-129].

BILIRUBIN HANDLING PROTEINS IN CHOLESTASIS

Animal models of obstructive and intrahepatic cholestasis help us to discover and understand the main principles of acquired defects in hepatobiliary transport of bile salts and other organic anions. Up and down regulation of these mechanisms can explain impaired liver uptake and excretion of the biliary constituents resulting in the cholestasis and icterus which accompanies many common acquired liver disorders[48,130,131]. A general pattern of response to cholestatic liver injury is initiated by downregulation of the basolateral membrane bound transporters NTCP and OATP1B1. The expression of several canalicular export pumps is relatively unaffected [bile salt export pump (BSEP), multidrug resistance protein 2 (MDR2)] or even upregulated (MDR1). Decreased expression of MRP2 in sepsis or in obstructive cholestasis is followed by upregulation of several MRP homologues at basolateral membrane of hepatocytes that may extrude bile salts back to the sinusoidal blood and systemic circulation. Most of these changes are believed to help prevent an accumulation of potentially toxic bile components and other substrates in the liver.

Similar patterns of expression of the bilirubin and bile salts handling proteins and mRNA are observed in cholestatic liver diseases in humans. At the stage Ⅰ and Ⅱ of primary biliary cirrhosis (PBC), expression and localisation of OATP1B1, OATP1B3, NTCP, MRP2, MRP3 and MDR3 are unchanged. At stage Ⅲ, immunostaining intensities of the sinusoidal uptake transporters and their mRNA levels decrease. Irregular MRP2 immunostaining suggests redistribution of MRP2 into intracellular structures in the advanced stages of PBC; however, at stage Ⅲ and Ⅳ, basolateral uptake transporters NTCP and OATP1B1 are downregulated. Expression of the canalicular export pumps for bile salts (BSEP) and bilirubin (MRP2) remains unchanged and the canalicular P-glycoproteins MDR1 and MDR3 and the basolateral efflux pump MRP3 are upregulated[132-135].

At the early-stages of cholestasis in extrahepatic biliary atresia, BSEP, MDR3, MRP2, NTCP/SLC10A1, SLCO1A2 and nuclear receptor farnesoid X receptor are downregulated. At the late-stages of cholestasis, farnesoid X receptor and BSEP levels returns to normal, MDR3 and MDR1 are upregulated and MRP2 is downregulated[136].

In primary sclerosing cholangitis, the level of OATP1B1 mRNA in liver tissue has been demonstrated to represent 49% of controls and the level of MRP2 mRNA dropped to 27% of controls[137].

CONCLUSION AND PERSPECTIVES

Over the last decades, molecular basis of hyperbilirubinemia syndromes has been elucidated and mutations affecting the basolateral and apical membrane transporters responsible for accumulation of either conjugated or unconjugated bilirubin have been identified.

Except for GS, the majority of inherited hyperbilirubinemia syndromes are rare autosomal recessive disorders with a low prevalence in the general population and, apart from CN syndrome type I and some cases of CN type Ⅱ in neonatal period, mostly not requiring further therapy. Nonetheless, the enzyme and transport systems involved in bilirubin metabolism may play an important role in the elimination and disposition processes of many other endogenous and exogenous substrates including hormones, drugs, toxins and heavy metals[102,138]. Dysfunction or absence of these systems, including selected ABC transporters and OATPs, may alter pharmacokinetics and pharmacodynamics of many biologically active agents, affect penetration of the substrates into various tissues and lead to their intracellular accumulation with a subsequent increase of organ toxicity[126,127,128]. In addition, the absence of the functional transport proteins involved in hepatobiliary and enterohepatic circulation may involve drug disposition, drug-drug or drug-food interactions and result in decreased effectiveness or even resistance to a diverse spectrum of chemotherapeutic agents and xenobiotics[139-141]. Individuals with mutations in the responsible gene or genes with the fully expressed phenotype of the corresponding hyperbilirubinemia syndrome, as well as subjects carrying mutations without clinical manifestation of hyperbilirubinemia under normal conditions, may be more susceptible to the adverse effects of some drugs and metabolites[142,143].

Clarifying the molecular genetic basis of hereditary hyperbilirubinemia syndromes together with the discoveries of the major systems essential for the metabolism and transport of bilirubin and other endogenous and exogenous substrates represent a substantial contribution to the current knowledge of the heme degradation pathway. Further investigation of how bilirubin transport proteins and their variations affect pharmacokinetics of drugs may be of significant clinical importance.

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Vrozené hyperbilirubinemie a molekulární mechanizmy žloutenky

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Souhrn: Úvod shrnuje klasickou dráhu degradace hemu a z ní odvozené rozdělení žloutenek. Následuje popis vrozených žloutenek známých jako Crigler-Najjarův, Gilbertův, Dubin-Johnsonův a Rotorův syndrom s důrazem na vysvětlení molekulární podstaty těchto metabolických poruch. Zvláštní pozornost je věnována nedávno objevenému molekulárnímu mechanizmu Rotorova syndromu spočívajícímu v neschopnosti jater zpětně vychytávat frakci konjugovaného bilirubinu vylučovaného primárně do krve a nikoliv do žluči. Snížená schopnost jater vychytávat konjugovaný bilirubin přispívá i k rozvoji hyperbilirubinemie u běžných onemocnění jater a žlučových cest a k toxicitě xenobiotik a léků využívajících transportní proteiny pro konjugovaný bilirubin.

Klíčová slova: hem – bilirubin – Gilbertův syndrom – Dubin-Johnsonův syndrom – Rotorův syndrom – OATP1B1 – OATP1B3

Neonatal hyperbilirubinemia and molecular mechanisms of jaundice

Summary: The introductory summarises the classical path of heme degradation and classification of jaundice. Subsequently, a description of neonatal types of jaundice is given, known as Crigler-Najjar, Gilbert's, Dubin-Johnson and Rotor syndromes, emphasising the explanation of the molecular mechanisms of these metabolic disorders. Special attention is given to a recently discovered molecular mechanism of the Rotor syndrome. The mechanism is based on the inability of the liver to retrospectively uptake the conjugated bilirubin fraction primarily excreted into the blood, not bile. A reduced ability of the liver to uptake the conjugated bilirubin contributes to the development of hyperbilirubinemia in common disorders of the liver and bile ducts and to the toxicity of xenobiotics and drugs using transport proteins for conjugated bilirubin.

Key words: heme – bilirubin – Gilbert's syndrome – Dubin-Johnson syndrome – Rotor syndrome – OATP1B1 – OATP1B3

Úvod

Bilirubin je konečným produktem degradace hemu, jehož hlavními zdroji jsou erytrocyty a buňky erytropoetické řady pohlcované makrofágy ve slezině a v krvetvorné kostní dřeni. Méně významným zdrojem jsou cytochromy v jaterních buňkách. Nekonjugovaný, ve vodě prakticky nerozpustný bilirubin, vázaný na albumin, je krví transportován do jater. Po disociaci vazby na albumin je bilirubin importován do hepatocytů, v jejichž cytosolu se váže na proteiny ze skupiny glutation-S-transferáz označované dříve názvem ligandin. Následuje konjugace se dvěma molekulami kyseliny glukuronové, která se odehrává v lumen endoplazmatického retikula za katalýzy uridindifosfátglukosiduronát bilirubin glukuronosyltransferázou UGT1A1 (EC 2.4.1.17) (obr. 1).

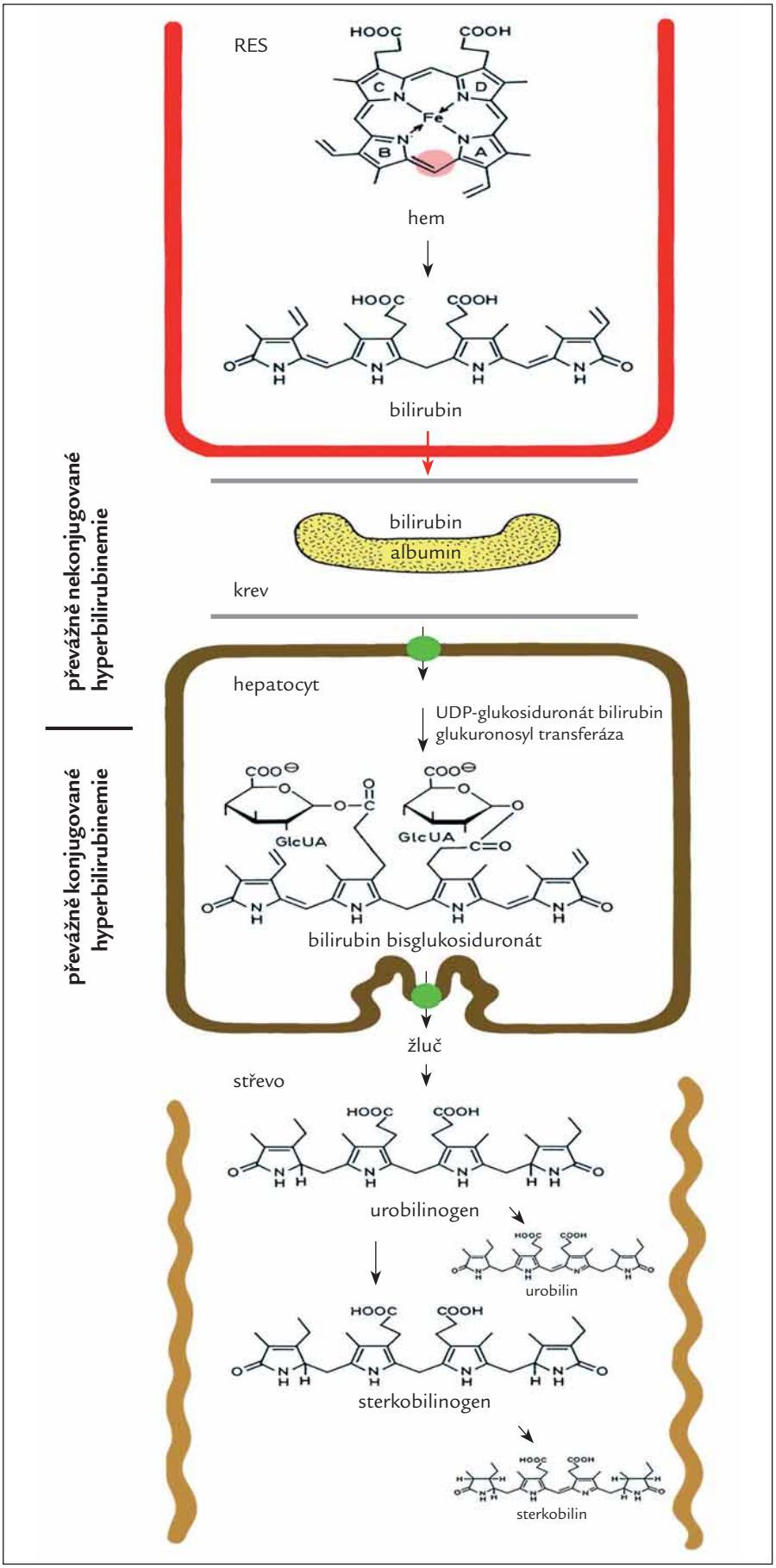
Gen *UGT1A1* lokalizovaný v komplexním genovém lokusu *UGT1A* [1] kóduje jediný pro bilirubin specifický konjugační enzym UGT1A1. Konjugovaný, ve vodě rozpustný bilirubin je zatím neznámým mechanizmem exportován z endoplazmatického retikula zpět do cytosolu, odkud migruje ke žlučovému pólu hepatocytu. Ze žlučového pólu je konjugovaný bilirubin aktivně secernován do žluči působením kanalikulárního ABC transportéru ABCC2/MRP2 fungujícího jako exportní pumpa multivalentních organických aniontů. Vedle této pumpy je pravděpodobná účast dalších transportérů s nižší afinitou ke konjugovanému bilirubinu (např. ABCG2), které při vyřazení genu *ABCC2* přejímají úlohu bilirubinové pumpy [2]. Ve střevě je konjugovaný bilirubin dekonjugován, zčásti resorbován a zčásti dále degradován působením bakteriální střevní flóry.

Dědičné poruchy metabolizmu jsou klíčem k poznání metabolických drah. Nejinak je tomu i v případě metabolické dráhy degradace hemu. Na základě biochemického vyšetření je možno rozlišovat převážně **nekonjugované** žloutenky, vyvolané buď nadprodukcí bilirubinu při hemolýze, nebo sníženou rychlostí jaterní konjugace, a převážně **konjugované** žloutenky, jejichž vyvolávající příčinou je onemocnění jaterního parenchymu, intrahepatální cholestáza nebo obstrukce žlučových cest (obr. 1).

Dědičné převážně nekonjugované hyperbilirubinemie jsou vyvolány mutacemi v genu pro UGT1A1 a zahrnují Crigler-Najjarův syndrom I. a II. typu a benigní hyperbilirubinemii Gilbertova typu. Dědičné převážně konjugované hyperbilirubinemie zahrnují Dubin-Johnsonův a Rotorův syndrom.

Crigler-Najjarův syndrom (CNS)

Crigler-Najjarův syndrom (CNS) [3] je raritní autozomálně recesivní porucha konjugace bilirubinu vyvolaná



Obr. 1. Odbourávání hemu na žlučová barviva a rozdělení hyperbilirubinemií podle převažujícího typu bilirubinu v séru [44].

mutacemi ve strukturálních oblastech genu *UGT1A1*. Podle koncentrace sérového bilirubinu se rozlišuje CNS I. typu (OMIM #218800) a CNS II. typu (OMIM #606785) [4].

Pro **CNS I. typu** jsou charakteristické hodnoty bilirubinu nad 350 μmol/l. Ostatní laboratorní nálezy, chromoexkreční testy, jakož i jaterní histologie jsou v mezích normy. Konjugační aktivita bilirubinu je prakticky nulová a bilirubinemie neklesá po podání fenobarbitalu. Příčinou jsou nonsense a některé missense mutace v exonech či jejich intronickém okolí, jejichž důsledkem je ztráta funkce či prakticky nulová exprese proteinu UGT1A1 [5]. Neléčená choroba je letální, postižení umírají zpravidla v dětském věku na jádrový ikterus. Terapie spočívá v celoživotní intermitentní fototerapii (někdy až po dobu 12 hod denně) nebo v transplantaci jater. Při fototerapii dochází k intenzivnímu vylučování ve vodě rozpustných fotoizomerů nekonjugovaného bilirubinu do žluči. Ve střevě probíhá částečná zpětná izomerace na nekonjugovaný bilirubin IXα, který vstupuje do enterohepatálního cyklu. Zvýšení účinnosti fototerapie je možno dosáhnout adjuvantní terapií, která spočívá v sekvestraci bilirubinu ve střevě (např. vazbou na neresorbovatelný fosforečnan vápenatý) nebo v urychlení střevní degradace bilirubinu. K transplantaci jater se přistupuje kolem 5. roku věku, neboť účinnost fototerapie s věkem postupně klesá a tím roste riziko náhlého poškození mozku [6].

CNS II. typu je benigní formou CNS. Bilirubinemie dosahuje hodnot 100–350 μmol/l. Stejně jako u CNS I. typu jsou ostatní laboratorní nálezy a jaterní morfologie normální. Aktivita UGT1A1 v játrech je snížena na 10–25 % normální hodnoty a hlavním produktem bilirubinu je bilirubin monoglukosiduronát. Na rozdíl od většiny případů CNS I. typu dochází u CNS II. typu po podání fenobarbitalu k výrazné indukci transkripce genu *UGT1A1*, která se projeví poklesem bi-

lirubinemie. CNS II. typu je podmíněn takovými mutacemi v genu *UGT1A1*, z nichž alespoň jedna nemá za následek kompletní inaktivaci či nulovou expresi kódovaného enzymu [5]. Průběh CNS II. typu je benigní a s výjimkou novorozeneckého období zpravidla nevyžaduje žádnou terapii.

Gilberův syndrom (GS)

Familiární nehemolytická žloutenka, popsaná jako benigní hyperbilirubinemie již v roce 1901 [7], je autozomálně recesivně dědičná metabolická odchylka podmíněná konstitutivním snížením rychlosti konjugace bilirubinu v játrech na hodnoty kolem 30 % [8,9]. Prevalence GS v kavkazské populaci je 5–10 % [10,11]. V evropské populaci je GS v převážné většině případů podmíněn homozygotním stavem pro variantu A(TA_7)TAA TATA-boxu v promotoru genu *UGT1A1* [12]. Normální sekvence TATA-boxu je A(TA_6)TAA. Inzerce TA má za následek snížení exprese strukturálně normálního enzymu UGT1A1 [12]. U většiny nositelů GS je exprese UGT1A1 dále snížena přítomností druhé mutace T>G v pozici –3 279 od začátku translace genu *UGT1A1* v tzv. gtPBREM (glucuronosyltransferase phenobarbital response enhancing motif) [13,14]. Tato mutace je příčinou zpomaleného poklesu bilirubinemie po podání fenobarbitalu, čehož bylo v minulosti využíváno diagnosticky v tzv. fenobarbitalovém testu. Vedle uvedených mutací v promotoru genu *UGT1A1* mohou GS vyvolat i mutace ve strukturálních oblastech genu. Nejčastější je heterozygotní mutace c.211G>A vyskytující se především v asijských populacích [15]. V homozygotním stavu vyvolává tato mutace CNS II. typu [16]. Fenotyp heterozygotů pro některé mutace vyvolávající CNS odpovídá fenotypu GS [17].

Klinicky se familiární nehemolytická žloutenka Gilbertova typu projevuje jako intermitentní ikterus sklér, popř. mírná žloutenka kůže a sliznic. K tomu se mohou přidružit neurotické symptomy, zvýšená únava, nechutenství bez poklesu tělesné hmotnosti a abdominální dyskomfort. Fyzikální vyšetření je zcela v normě stejně jako laboratorní nález s výjimkou hyperbilirubinemie nekonjugovaného typu zřídka přesahující 100 μmol/l. Bilirubinemie se zvyšuje při fyzické zátěži, stresu a hladovění, čehož bylo v minulosti využíváno k diagnostickému testu (tzv. test hladověním). U části nositelů GS byla pozorována snížená clearance bromsulfoftaleinu, indocyaninové zeleně a dalších aniontových barviv [4]. V bioptických vzorcích jater bylo pozorováno hromadění lipofuscinu v hepatocytech, a to především v centrilobulární zóně [4]. Ani testy jaterní chromoexkrece, ani výsledky jaterní biopsie nemají žádnou diagnostickou hodnotu, a proto tato vyšetření nejsou indikována. GS dnes není považován za chorobu, nýbrž za metabolickou odchylku, která nevyžaduje žádnou terapii ani dietní či režimová opatření. U nositelů GS byl naopak prokázán nižší výskyt kardiovaskulárních chorob a nádorových onemocnění, což je přičítáno antioxidačním vlastnostem bilirubinu [18].

Dubin-Johnsonův syndrom (DJS)

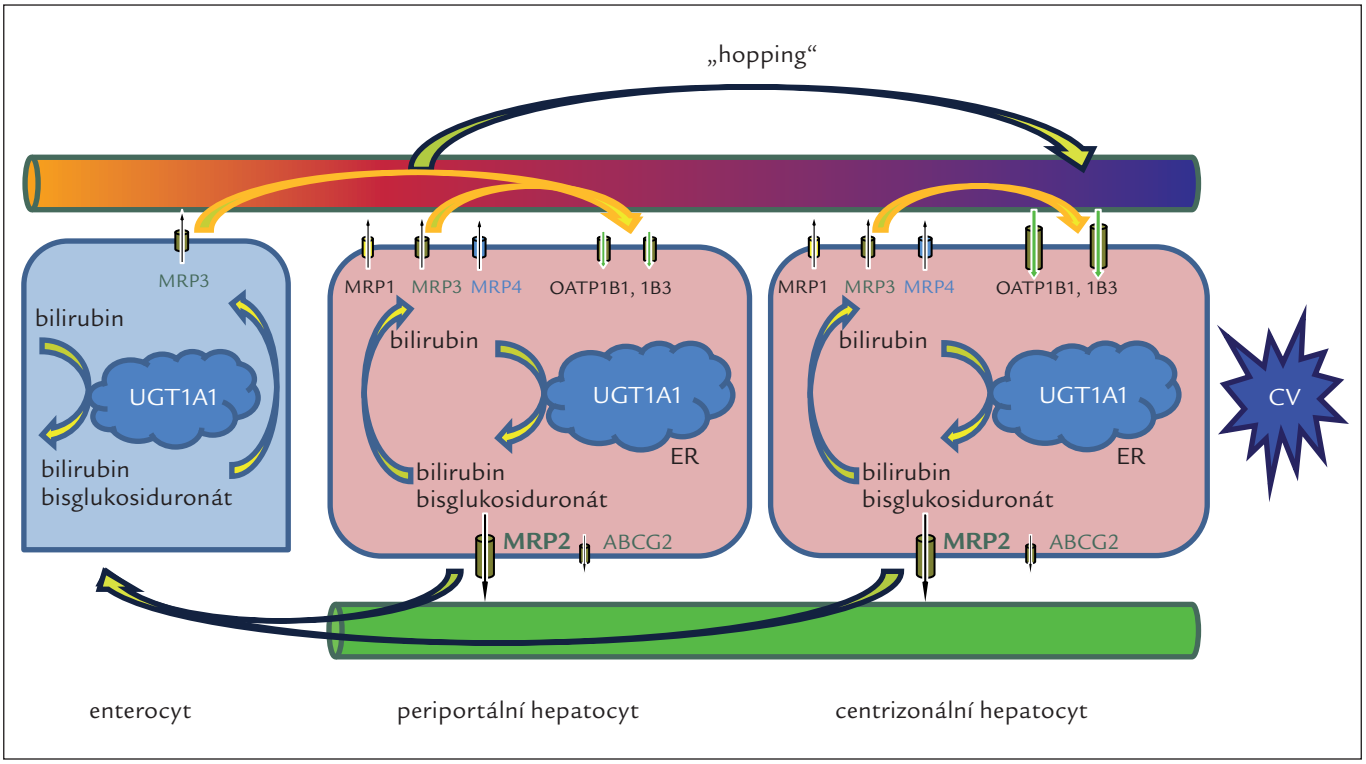
Dubin-Johnsonův syndrom (DJS, OMIM #237500) je vzácná benigní autozomálně recesivní konjugovaná hyperbilirubinemie. Genetickou příčinou DJS jsou mutace v genu *ABCC2/MRP2* [19]. Gen *ABCC2* je lokalizován na dlouhém raménku 10. chromozomu (10q24), zaujímá 45 kb a sestává z 32 protein kódujících exonů.

Pro klinický obraz je charakteristický kolísavý ikterus sklér a někdy i kůže bez dalších příznaků. Hladina celkového bilirubinu se pohybuje kolem 100 μmol/l, přičemž více než 50 % připadá na přímý bilirubin [20,21]. Ostatní laboratorní nálezy včetně odpadu celkových porfyrinů jsou normální, avšak izomer I koproporfyrinu představuje více než 50 % vylučovaného koproporfyrinu [22]. Vylučování bromsulfoftaleinu do žluči po i.v. podání je zpomaleno a v 90. min po podání dochází k opětovnému vzestupu jeho koncentrace [23]. Obdobnou kinetiku jako bromsulfoftalein mají indocyaninová zeleň a další organické anionty včetně radiofarmak používaných k cholescintigrafii, takže vizualizace jater i plnění žlučníku jsou opožděny [24–26]. Jaterní histologie je normální s výjimkou akumulace tmavého melanin-like pigmentu s výraznou autofluorescencí v hepatocytech. Pigment, jehož chemické složení dosud není známo, dává pozitivní PAS reakci a redukuje amoniakální roztok stříbra (Masson-Fontanova reakce), avšak na rozdíl od melaninu neredukuje neutrální roztok stříbra. Byly dokumentovány i případy DJS bez pigmentace jater [27,28]. Imunohistologicky lze ve většině případů prokázat absenci proteinu ABCC2 v kanalikulární membráně hepatocytů [29]. Výjimečně může být mutovaný nefunkční protein ABCC2 normálně exprimován [30].

Na našem pracovišti byly zachyceny nové mutace v genu *ABCC2* u jedinců s DJS [31,32] a zdokumentován první případ dosud nepopsané převážně nekonjugované dědičné žloutenky vyvolané přítomností mutací typických pro DJS a GS [31]. Podíl konjugovaného bilirubinu u této tzv. duální hereditární žloutenky činí pouze 20–50 % sérové koncentrace celkového bilirubinu.

Rotorův syndrom (RS)

Rotorův syndrom (RS, OMIM #237540) je velmi vzácná autozomálně recesivně dědičná forma převážně konjugované žloutenky, která je klinickým a základním laboratorním vyšetřením neodlišitelná od DJS. Stejně jako u DJS je u RS převažujícím vylučovaným koproporfyrinem izomer I. Na rozdíl od DJS je odpad celkových porfyrinů v moči zvýšen a poměr izomeru I koproporfyrinu k izomeru III bývá nižší než u DJS [33]. Vylučování aniontových barviv játry je pomalejší než u DJS a nedochází k opětovnému vzestupu jejich koncentrace [34]. Při cholescintigrafii se nezobrazí ani játra ani žlučník a zobra-



Obr. 2. Jaterní cyklus konjugovaného bilirubinu.

Cyklus zprostředkovávaný proteiny MRP3 a OATP1B se může odehrávat jak v periportálních, tak v centrizonálních hepatocytech, kde je exprese proteinů OATP1B nejvyšší. Nejvýznamnější je ale přesun („hopping“) konjugovaného bilirubinu a dalších substrátů MRP3 a OATP1B z periportálních do centrizonálních hepatocytů. Tento přesun nejen zvyšuje celkovou sekreční kapacitu jater pro bilirubin, ale může i chránit periportální hepatocyty před toxicitou vstřebaných xenobiotik, léků a jejich konjugátů. Proteiny OAT1B rovněž odpovídají za jaterní clearance bilirubinu konjugovaného ve splanchnické oblasti, zejména ve střevě, a konečně střevní konjugace spolu s jaterním vychytáváním konjugátů může představovat alternativní dráhu v enterohepatálním oběhu bilirubinu. Úplná absence proteinů OATP1B1 a OATP1B3 byla pozorovaná u RS.

CV – centrální véna, ER – endoplazmatické retikulum

zení žlučníku bylo pozorováno teprve po řadě hodin [24,35,36]. Jaterní histologie je zcela normální. Příčina Rotorova syndromu nebyla donedávna známa.

Na našem pracovišti se během 10 let jeho existence podařilo shromáždit klinické a laboratorní nálezy 11 nositelů Rotorova syndromu pocházejících ze 4 středoevropských, 3 arabských a 1 filipínské rodiny. Ve spolupráci se skupinou Ing. S. Kmocha z Ústavu dědičných metabolických poruch 1. lékařské fakulty UK a VFN v Praze se nám kombinací homozygotního mapování a mapování unikátních delecí podařilo identifikovat kandidátní lokus na chromozomu 12p12 obsahující dvojici genů *SLCO1B1* a *SLCO1B3*, jejichž všechny alely byly mutovány u všech 11 jedinců s RS [37]. Naopak všichni rodiče a sourozenci postižených nesli alespoň 1 normální alelu genu *SLCO1B1* nebo *SLCO1B3*.

Geny *SLCO1B1* a *SLCO1B3* kódují proteiny OATP1B1 a OATP1B3 exprimované v sinusoidální membráně jaterních buněk, které jsou odpovědné za jaterní vychytávání nekonjugovaného i konjugovaného bilirubinu, jakož i řady dalších endogenních látek, xenobiotik a léků. Odpověď na otázku, jak se konjugovaný bilirubin dostane do sinusoidální krve, poskytly experimenty s geneticky modifikovanými myšími liniemi provedené skupinou A. Schinkela v The Netherlands Cancer Institute v Amsterdamu [37]. Výsledky ukázaly, že významná frakce konjugovaného bilirubinu je za fyziologických podmínek secernována do krve prostřednictvím sinusoidální bilirubinové pumpy MRP3 homologní s MRP2. Zvýšená exprese proteinu MRP3 byla již dříve popsána jako hlavní kompenzační mechanizmus vylučování konjugovaného bilirubinu z cytosolu hepatocytů do krve u DJS [38] a při některých formách získané cholestázy, u nichž je blokována sekrece konjugovaného bilirubinu do žluči (sepse, toxiny, obstrukce žlučových cest) [39]. V centrální zóně jaterního lalůčku, kde je exprese rotorovských proteinů OATP1B1 a OATP1B3 nejvyšší, dochází ke zpětnému a téměř kompletnímu vychytávání konjugovaného bilirubinu z krve a jeho následnému vyloučení do žluče (obr. 2).

Rotorovské proteiny se podílejí i na vychytávání bilirubinu konjugovaného mimo játra, zejména ve střevě. Pravděpodobně největší význam transportní smyčky tvořené pumpou MRP3 a rotorovskými proteiny (obr. 2) spočívá v ochraně portálních hepatocytů před

toxickým působením zvýšených koncentrací látek přicházejících do jater z portálního oběhu a ve zvýšení sekreční kapacity jater na podkladě přesunu části nálože organických aniontů a aniontových konjugátů z přetížených periportálních hepatocytů do méně zatěžovaných hepatocytů v centrální zóně [40].

Molekulární mechanizmy žloutenky a jejich farmakologický význam

Poznání molekulárního mechanizmu žloutenky u RS má význam pro vysvětlení patofyziologie žloutenky u běžných chorob jater a žlučových cest. Při parenchymové žloutence dochází vedle poruchy konjugace a sekrece bilirubinu i k poruše jaterního vychytávání nekonjugovaného a patrně i konjugovaného bilirubinu ze sinusoidální krve. Na snížení vychytávání obou forem bilirubinu se může podílet snížená aktivita či exprese proteinů OATP1B1 a OATP1B3. U cholestázy či obstrukce žlučových cest může být konjugovaná hyperbilirubinemie potencována snížením exprese rotorovských proteinů. Tuto hypotézu podporují imunohistologické nálezy snížené exprese proteinů OATP1B1 a OATP1B3 u některých cholestatických jaterních chorob [41].

Jako zásadní se jeví význam jaterního cyklu bilirubinu a dalších organických aniontů pro vychytávání a metabolizmus xenobiotik a léků v jaterních buňkách. Rotorovské proteiny např. zprostředkovávají vychytávání nekonjugovaných žlučových kyselin, konjugovaných steroidů, hormonů štítné žlázy, bromsulfoftaleinu, indocyaninové zeleně, radiofarmak pro cholescintigrafii, benzylpenicilinu, rifampicinu, atorvastatinu, pravastatinu, rosuvastatinu, některých sartanů či metotrexátu [42]. Rovněž bylo prokázáno, že variace v rotorovských genech jsou asociovány se závažnými nežádoucími účinky léků, např. statinů [43].

Závěr

Dráha degradace hemu je považována za jednu z nejjednodušších a nejlépe prostudovaných metabolických drah u člověka. Cesta k poznání molekulární podstaty RS však ukázala, že i v takto detailně prostudované metabolické dráze existuje řada dosud nezodpovězených otázek, mezi něž patří např. dosud neznámý mechanizmus třídění molekul konjugovaných v endoplazmatickém retikulu na sloučeniny vylučované do žluči a do krve či úloha nosičství patogenních mutací v některé z alel genů *SLCO1B1* či *SLCO1B3* v rozvoji nežádoucích účinků některých běžně používaných léků.

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Bile acids decrease intracellular bilirubin levels in the cholestatic liver: implications for bile acid-mediated oxidative stress

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Abstract

High plasma concentrations of bile acids (BA) and bilirubin are hallmarks of cholestasis. BA are implicated in the pathogenesis of cholestatic liver damage through mechanisms involving oxidative stress, whereas bilirubin is a strong antioxidant. We evaluated the roles of bilirubin and BA on mediating oxidative stress in rats following bile duct ligation (BDL). Adult female Wistar and Gunn rats intraperitoneally anaesthetized with ketamine and xylazine underwent BDL or sham operation. Cholestatic markers, antioxidant capacity, lipid peroxidation and heme oxygenase (HO) activity were determined in plasma and/or liver tissue 5 days after surgery. HepG2-rNtcp cells were used for *in vitro* experiments. Plasma bilirubin levels in control and BDL animals positively correlated with plasma antioxidant capacity. Peroxyl radical scavenging capacity was significantly higher in the plasma of BDL Wistar rats (210 $\pm$ 12%, $P < 0.0001$) compared to controls, but not in the liver tissues. Furthermore after BDL, lipid peroxidation in the livers increased (179 $\pm$ 37%, $P < 0.01$), whereas liver HO activity significantly decreased to 61% of control levels ($P < 0.001$). Addition of taurocholic acid (TCA, $\geq$50 μmol/l) to liver homogenates increased lipid peroxidation ($P < 0.01$) in Wistar, but not in Gunn rats or after the addition of bilirubin. In HepG2-rNtcp cells, TCA decreased both HO activity and intracellular bilirubin levels. We conclude that even though plasma bilirubin is a marker of cholestasis and hepatocyte dysfunction, it is also an endogenous antioxidant, which may counteract the pro-oxidative effects of BA in circulation. However, in an animal model of obstructive cholestasis, we found that BA compromise intracellular bilirubin levels making hepatocytes more susceptible to oxidative damage.

Keywords: taurocholic acid • heme oxygenase • carbon monoxide • lipid peroxidation

Introduction

Obstructive cholestasis, characterized by a failure to secrete bile into the bile duct and intestine, results in the accumulation of bile acids (BA) and bilirubin in circulation. Elevated activities of cholestatic enzymes and plasma levels of bilirubin and BA are used as laboratory markers of cholestasis.

The accumulation of BA inside hepatocytes is the major cause of cholestatic liver damage [1], including structural and functional injuries of hepatocyte membranes [2], cell death [3] and activation of inflammatory and fibrogenic signalling pathways [4]. Several studies have suggested an important role of increased oxidative stress in the pathogenesis of cholestatic injury [5, 6]. Accumulated BA within hepatocytes impair mitochondrial respiration and electron transport and stimulate the generation of reactive oxygen species (ROS) in hepatic mitochondria [7]. Accordingly, mitochondrial free radicals may then modify nucleic acids, proteins and lipids. In fact, an increase in lipid peroxidative products has been observed in cholestatic livers [8].

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The liver possesses a complex defence system including antioxidant enzymes and substrates to control the formation of ROS and repair oxidative damage [9]. Bilirubin, a product of heme catabolism, is a potent antioxidant substance both *in vitro* [10] and *in vivo* [11]. *In vitro* studies with liposomes have shown that both unconjugated (UCB) and conjugated bilirubin (CB) are protective against lipid peroxidation, surpassing that of α-tocopherol, an important lipid-soluble antioxidant [10]. Antioxidant properties of bilirubin were further confirmed by a number of animal and clinical studies demonstrating the protective effects of bilirubin on the development of atherosclerosis [12–14], cancer [15, 16] and other oxidative stress-mediated diseases [17].

The objective of this study was to address the seemingly dichotomous effects of high levels of the antioxidant bilirubin and the pro-oxidant BA in obstructive cholestasis using an animal model.

Materials and methods

Animals

Female Wistar rats obtained from Anlab (Prague, Czech Republic) and hyperbilirubinemic Gunn rats (RHA/jj, in-house colony from 1st Faculty of Medicine, Charles University in Prague) with a congenital deficiency of bilirubin uridine 5′-diphospho (UDP)-glucuronosyltransferase, both weighing from 200 to 280 g, were provided water and food *ad libitum*. All aspects of the animal studies met the accepted criteria for the care and experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University in Prague.

Reagents

L-Ascorbic acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), bovine serum albumin (BSA) 98%, UCB, chloroform (high-performance liquid chromatography [HPLC] grade), hemin, nicotinamide adenine dinucleotide phosphate (NADPH), sulfosalicylic acid, taurocholic acid (TCA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade purchased from Penta (Prague, Czech Republic). UCB was purified and recrystallized according to McDonagh and Assisi [18]. Purified UCB was dissolved in 0.1 M NaOH and immediately neutralized with phosphoric acid. The mixture was subsequently diluted with BSA solution to reach a final concentration of 480 μM UCB and 500 μM BSA in phosphate buffer (25 mM, pH 7.0).

Bile duct ligation (BDL)

Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and biliary trees were exposed through midline abdominal incisions. Microsurgical ligation of bile ducts and resections of extrahepatic biliary tracts were performed as previously described ($n = 7$ in each group) [19]. Sham-operated (SH) rats underwent the same procedure without bile duct resection and ligation ($n = 6$ in each group).

Tissue preparation

After 5 days, all animals were killed and blood (5 ml) was collected from superior vena cava, transferred to tubes containing EDTA, mixed, and placed on ice. An aliquot was centrifuged to separate plasma. Livers were then harvested, thoroughly washed with 10 ml heparinized saline, and rinsed in ice-cold reaction buffer (0.1 M phosphate buffer, pH 7.4). For RNA analysis, 100 mg of tissue was immediately placed in 1.5 ml microfuge tubes containing RNAlater (Qiagen, Valencia, CA, USA). Tubes were stored at −20°C until total RNA isolation.

For HO activity, HO-1 protein, and lipid peroxidation measurements, 100–150 mg tissue was diluted 1:9 (by weight) in reaction buffer, diced, and sonicated with an ultrasonic cell disruptor (Model XL2000, Misonics, Farmingdale, NY, USA). Sonicates were kept on ice and assayed for HO activity or lipid peroxidation within 1 hr or frozen in liquid nitrogen and stored at −80°C until analysis of HO-1 protein.

For liver carbon monoxide (CO) measurements, 150–200 mg tissue was diluted 1:4 in reaction buffer and then sonicated as described above.

For malondialdehyde (MDA) and 4-hydroxyalkenal analysis, 200 mg of tissue was placed in the Eppendorf tube containing 0.1 M PBS, pH 7.4 with 1% BHT, diced and sonicated. Sonicates were stored at −80°C until analysis.

Markers of cholestasis

Plasma biochemical markers (alkaline phosphatase [ALP], albumin) were determined in an automatic analyser (Hitachi, Model 717, Tokyo, Japan), using standard assays. Total plasma BA levels were determined spectrophotometrically using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA).

Liver histology

For histological examination, left lateral lobes of livers were fixed overnight in 10% buffered formalin (pH 7.4) at 4°C followed by a standard procedure for paraffin embedding. Serial sections (6 μm thick) were cut and stained with haematoxylin and eosin, Shikata's orcein method, or elastic-van Gieson stain. Each slide was viewed using standard light microscopy.

Peroxyl radical scavenging capacity

Peroxyl radical scavenging capacity was measured fluorometrically as a proportion of chain-breaking antioxidant consumption present in a biologic sample (plasma, liver homogenate) relative to that of Trolox (a reference and calibration antioxidant compound) as previously described [20].

Bilirubin determination

Plasma and liver CB and UCB levels were determined using an HPLC method as previously described [21]. Briefly, pigments were extracted into chloroform-hexane and subsequently delipidated by second extraction into a minute volume of alkaline aqueous solution. The resulting droplet was separated on HPLC.

Heme oxygenase (HO) activity

Twenty microlitres of 10% liver sonicate (2 mg fresh weight [FW]) was incubated for 15 min. at 37°C in CO-free septum-sealed vials containing 20 μl of 150 μM methemalbumin and 20 μl of 4.5 mM NADPH as previously described [22]. Blank reaction vials contained 0.1 M phosphate buffer, pH 7.4, in place of NADPH. Reactions were terminated by adding 5 μl of 30% (w/v) sulfosalicylic acid. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography (GC) with a reduction gas analyser (Trace Analytical, Menlo Park, CA, USA). HO activity was calculated as pmol CO/hr/mg FW.

Real-time RT-PCR

Total liver RNA was isolated using phenol : chloroform extraction and cDNA was generated using random hexamer primers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed with TaqMan® Gene Expression Assay Kit for HO-1 (Applera, Alameda, CA, USA). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase levels, and then expressed as fold change from control.

Western blots

One hundred micrograms of liver sonicates were mixed with equal volume of loading buffer. Samples were separated on 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in Tween-PBS with 5% milk for at least 1 hr, membranes were incubated with HO-1 antibody (1:666; Stressgen, Victoria, BC, USA), or β-actin (1:8000; Sigma-Aldrich) for 1 hr. After washing, membranes were incubated with anti-mouse IgG-HRP (1:2000; Sigma-Aldrich) for 30 min. After washing, immunocomplexes on the membranes were visualized with ECL Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). HO-1 protein bands were quantified by densitometry, normalized to β-actin, and then expressed as fold change from control.

Liver tissue CO

Forty microlitres of liver sonicate was added to CO-free, septum-sealed vials containing 5 μl of 60% (w/v) sulfosalicylic acid. After 30 min. incubation on ice, CO released into the vial headspace was quantitated by GC as previously described [23].

Carbonylhaemoglobin (COHb) determination

Total haemoglobin was estimated to be 15 g/dl for all the animals. COHb was measured by GC as previously described [24] and expressed as percentage of total haemoglobin.

Lipid peroxidation

Twenty microlitres of liver sonicate was incubated for 30 min. at 37°C with 100 μM ascorbate (80 μl) and 6 μM Fe^{2+} (0.5 μl). BHT (100 μM) was added for the blank reaction. CO produced into vial was quantitated by GC as previously described [25]. The amount of CO produced serves as an index of lipid peroxidation and was expressed as pmol CO/hr/mg FW. Total amounts of lipid peroxidation end-products, MDA and 4-hydroxyalkenals were determined using Bioxytech® LPO-586 Assay (Oxis International, Beverly Hills, CA, USA).

Cell culture

The human hepatoblastoma cell line (HepG2) (purchased from American Type Culture Collection, Manassas, VA, USA) and HepG2 cell line stably transfected with Ntcp transporter (HepG2-rNtcp), kindly provided by Professor Ulrich Beuers (University of Munich, Germany; currently University of Amsterdam, Netherlands), were cultured as described previously [26]. Cells were grown on 10 cm Petri dishes (Orange Scientific, Braine-l'Alleud, Belgium), incubated with TCA for 24 hrs for HO activity and intracellular bilirubin or 4 hrs for mRNA determination. After incubation, cells were quickly washed three times with 10 ml PBS, harvested, centrifuged and pellet was dispersed in 300 μl of 0.1 M phosphate buffer, pH 7.4. For mRNA determination, cell pellets were snap frozen in liquid nitrogen and stored at −80°C until analysis. An aliquot of the cell sonicate was used for protein determination (Bio-Rad DC protein assay, Hercules, CA, USA).

Statistical analyses

Normally distributed data are presented as means ± S.D. and analysed by Student t-test. Non-normally distributed datasets are expressed as medians (25%–75%) and analysed by Mann-Whitney rank sum test. The association between plasma bilirubin levels and antioxidant capacity was tested using Spearman rank-order correlation analysis. Differences were deemed statistically significant when $P < 0.05$.

Results

Markers of cholestasis and liver histology

Significant increases in total BA and ALP were observed in all BDL rats (Table 1). As expected, plasma bilirubin levels were significantly elevated in Wistar rats after BDL. In Gunn rats, which are deficient in bilirubin UDP-glucuronosyltransferase, plasma UCB levels remained unchanged after BDL (Table 2), as expected because most of the bilirubin does not enter the intestinal lumen *via* biliary excretion, but rather *via* direct diffusion across the intestinal mucosa [27].

Histological analysis of liver specimens from BDL rats revealed signs of impaired bile flow, such as large bile duct obstruction with intralobular bilirubinostasis (predominantly in perivenular localisation) and biliary interface activity with portal tract oedema, swelling of periportal hepatocytes and marked ductular proliferation with a disruption of the parenchymal limiting plates, accompanied by polymorphonuclear infiltration. Bile plugs

Table 1 Cholestatic markers and liver and body weights

	Wistar (SH) ($n = 6$)	Wistar (BDL) ($n = 7$)	Gunn (SH) ($n = 6$)	Gunn (BDL) ($n = 7$)
Body weight (g)	235 (233–237)	218 (212–240)	253 (206–280)	220 (200–264)
Liver weight (g)	10.4 (9.8–11.0)	14.0 (13.6–14.6)**	11.0 (10.5–11.5)	12.9 (11.5–13.6)*
TBA (μmol/l)	14.5 (10.3–23.3)	326 (290–404)**	8.5 (8.0–12.8)	288 (248–416)**
ALP (μkat/l)	2.1 (1.9–2.2)	4.4 (4.2–4.6)**	0.9 (0.8–1.0)	5.1 (3.9–5.6)**

Cholestatic markers and liver and body weights in SH and BDL Wistar and Gunn rats 5 days after surgery. Data are presented as median (25–75%).
*$P < 0.05$,**$P < 0.001$ compared to corresponding SH group.
TBA: total plasma bile acids, ALP: alkaline phosphatase.

Table 2 Plasma and liver bilirubin

	SH-plasma (μmol/l) ($n = 6$)	BDL-plasma (μmol/l) ($n = 7$)	SH-liver (nmol/g) ($n = 6$)	BDL-liver (nmol/g) ($n = 7$)
Wistar				
TB	0.32 (0.16–0.37)	193.8 (176.4–195.5)**	2.65 (2.33–3.50)	40.7 (31.5–57.1)**
CB	0	181.4 (164.8–190.2)**	1.73 (1.18–2.39)	39.2 (30.1–54.8)**
UCB	0.32 (0.16–0.37)	8.67 (3.75–13.03)**	1.07 (0.88–1.28)	2.03 (1.75–2.37)*
Gunn				
UCB	137.8 (130.0–145.4)	149.1 (135.4–212.6)	45.2 (40.7–46.1)	24.6 (19.6–28.5)*

Plasma and liver bilirubin in SH and BDL Wistar and Gunn rats 5 days after surgery. Data are presented as medians (25–75%).
*$P < 0.05$, **$P < 0.001$, compared to corresponding SH group.
TB: total bilirubin, UCB: unconjugated bilirubin, CB: conjugated bilirubin.

were present in a few cholangioles and bile infarcts were found in periportal zones.

Bilirubin increases antioxidant capacity in plasma, but not in liver homogenates of BDL rats

Peroxyl radical scavenging capacity was significantly higher in BDL compared to SH Wistar rats (210 ± 13 and 100 ± 30%, respectively $P < 0.001$) (Fig. 1A). We suggest that this increase could be attributed to elevated plasma bilirubin levels. In fact, plasma antioxidant capacity correlated positively with plasma total bilirubin levels (Spearman correlation coefficient = 0.45, $P = 0.027$). Unlike in plasma, we did not find any differences in peroxyl radical scavenging capacity in liver homogenates of BDL and SH Wistar rats (113 ± 17 and 100 ± 17%, respectively, $P = 0.21$) (Fig. 1A).

To further confirm our hypothesis, we investigated the effect of bilirubin and that of TCA on peroxyl radical scavenging capacity in normal rat plasma. Addition of bilirubin resulted in a dose-dependent increase in peroxyl radical scavenging capacity, whereas no effect was observed with TCA (Fig. 1B).

Liver bilirubin levels are relatively decreased compared to plasma in BDL animals

Markedly different antioxidant capacities of plasma and liver homogenates of BDL Wistar rats prompted us to measure bilirubin concentrations in those two compartments. Compared to SH rats, plasma bilirubin levels were 606 times higher in BDL Wistar rats. Surprisingly, in liver sonicates, only a 15-fold increase of bilirubin was observed in BDL rats. In Gunn rat livers, we found a significant decrease of 46% in the liver bilirubin levels in BDL rats compared to SH rats, whereas no significant differences were found in plasma (Table 2).

Bilirubin production is decreased and lipid peroxidation is increased following BDL

To identify the possible underlying mechanism for the relative lack of bilirubin in cholestatic hepatocytes, we investigated the rate of bilirubin production in the liver of Wistar rats. Activity of HO, the rate-limiting enzyme of bilirubin synthetic pathway, was

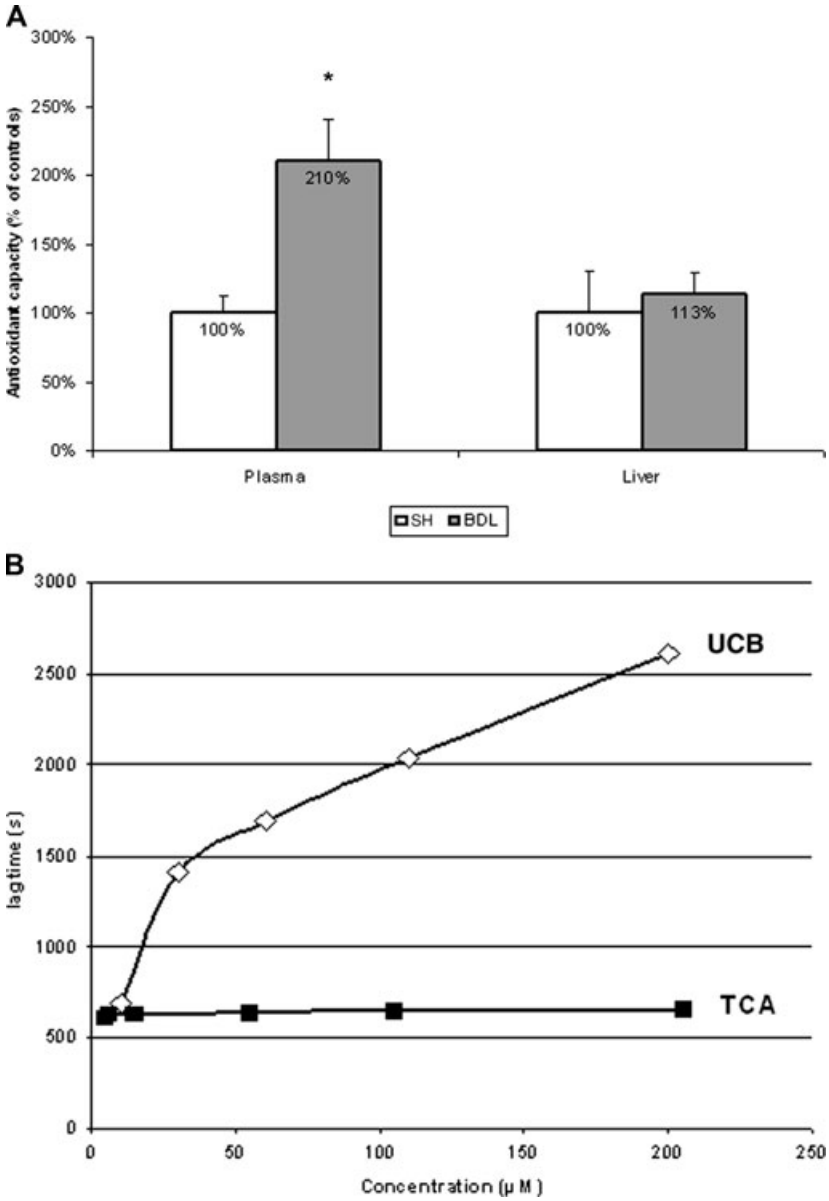

Fig. 1 Antioxidant capacity in plasma and liver homogenates of control (SH) and BDL rats. Effect of bilirubin and TCA. (**A**) Peroxyl radical scavenging capacity of plasma and liver homogenates from SH ($n = 6$) and BDL Wistar rats ($n = 7$). (**B**) Effect of UCB and TCA on peroxyl radical scavenging capacity (lag time) of normal rat plasma.

significantly decreased (54%) in the livers of BDL rats compared to controls (199 ± 36 *versus* 327 ± 48 pmol CO/hr/mg FW, $P = 0.003$). Similarly, HO-1 mRNA expression and HO-1 protein were also significantly down-regulated in BDL livers (Fig. 2A).

Surprisingly, we found significant increases in liver tissue CO and blood COHb levels of cholestatic rats compared to those of controls (7.9 ± 2.4 *versus* 4.0 ± 1.1 pmol CO/mg FW and 0.36 ± 0.04 *versus* 0.18 ± 0.02% total haemoglobin, respectively, $P < 0.05$, Fig. 2B). Because lipid peroxidation represents another source of CO *in vivo* [28], besides HO activity, we analysed the potential for lipid peroxidation. Livers of BDL animals were more susceptible to lipid peroxidation than control livers (85 ± 18 *versus* 47 ± 17 pmol CO/mg FW, respectively, $P = 0.005$). These results were confirmed by direct measurements of liver MDA and 4-hydroxyalkenals, which were significantly higher in BDL compared to SH rats (122 ± 15 and 99 ± 3 μmol/g, respectively, $P = 0.004$).

TCA increases lipid peroxidation in the liver homogenates

To investigate the role of BA in lipid peroxidation, we analysed the effect of increasing concentrations of TCA in normal Wistar

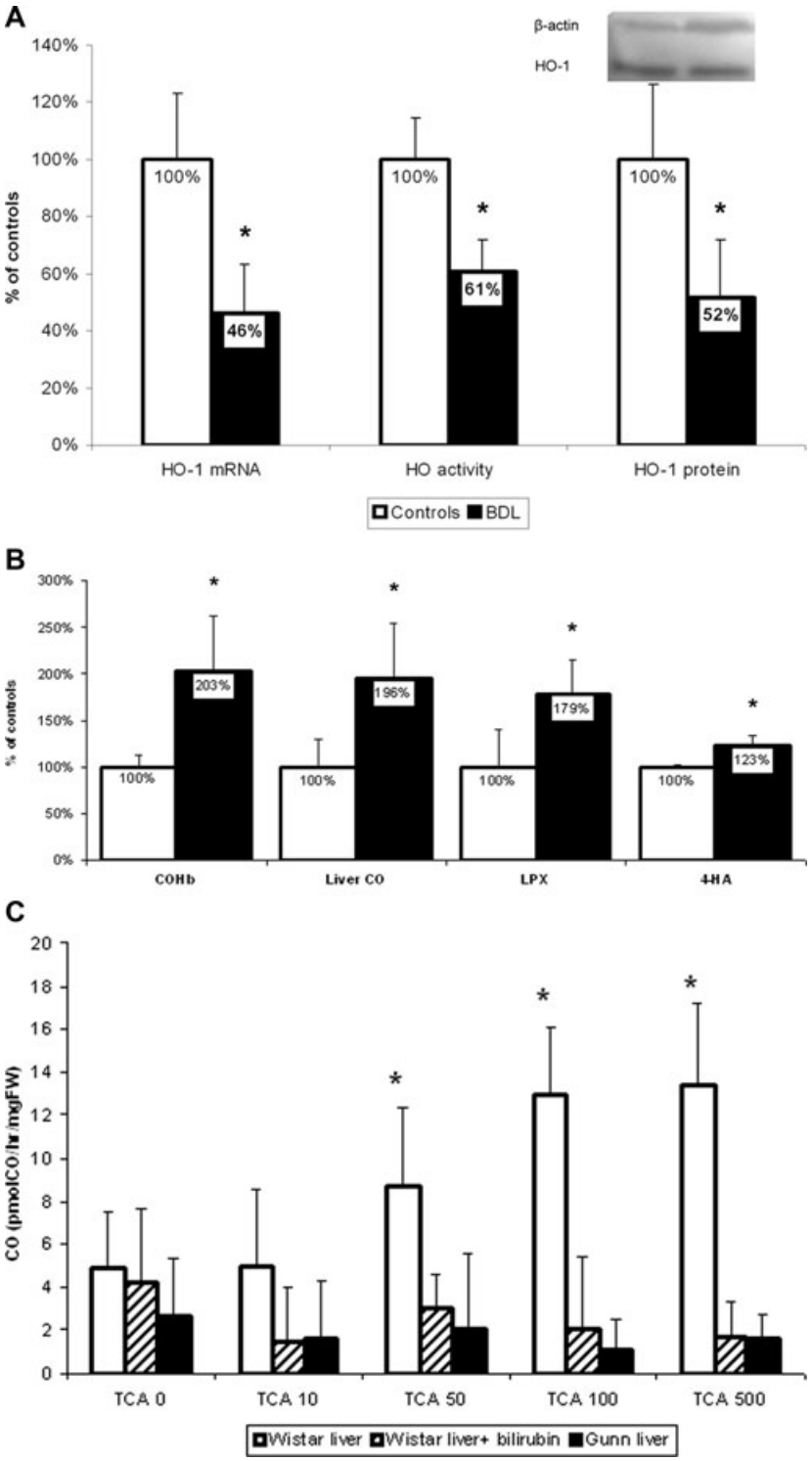

Fig. 2 Bilirubin production and lipid peroxidation following BDL in Wistar rats. Effect of TCA and bilirubin on lipid peroxidation in Wistar and Gunn rat liver homogenates. (**A**) Activity and expression of HO in liver tissue of sham-operated and BDL Wistar rats. Densitometric values of HO-1 protein were normalized to β-actin and all data are expressed as percentage of controls. $^{*}P < 0.05$. (**B**) CO in liver tissue and in the blood (COHb), lipid peroxidation and 4-hydroxyalkenals of cholestatic Wistar rats compared to control animals. Data are expressed as percentage of controls. $^{*}P < 0.05$. (**C**) TCA was added to normal liver homogenates of Wistar and Gunn rats or Wistar rat liver homogenates with 40 μM bilirubin in concentrations of 0, 10, 50, 100 and 500 μM and lipid peroxidation was measured. $^{*}P < 0.05$.

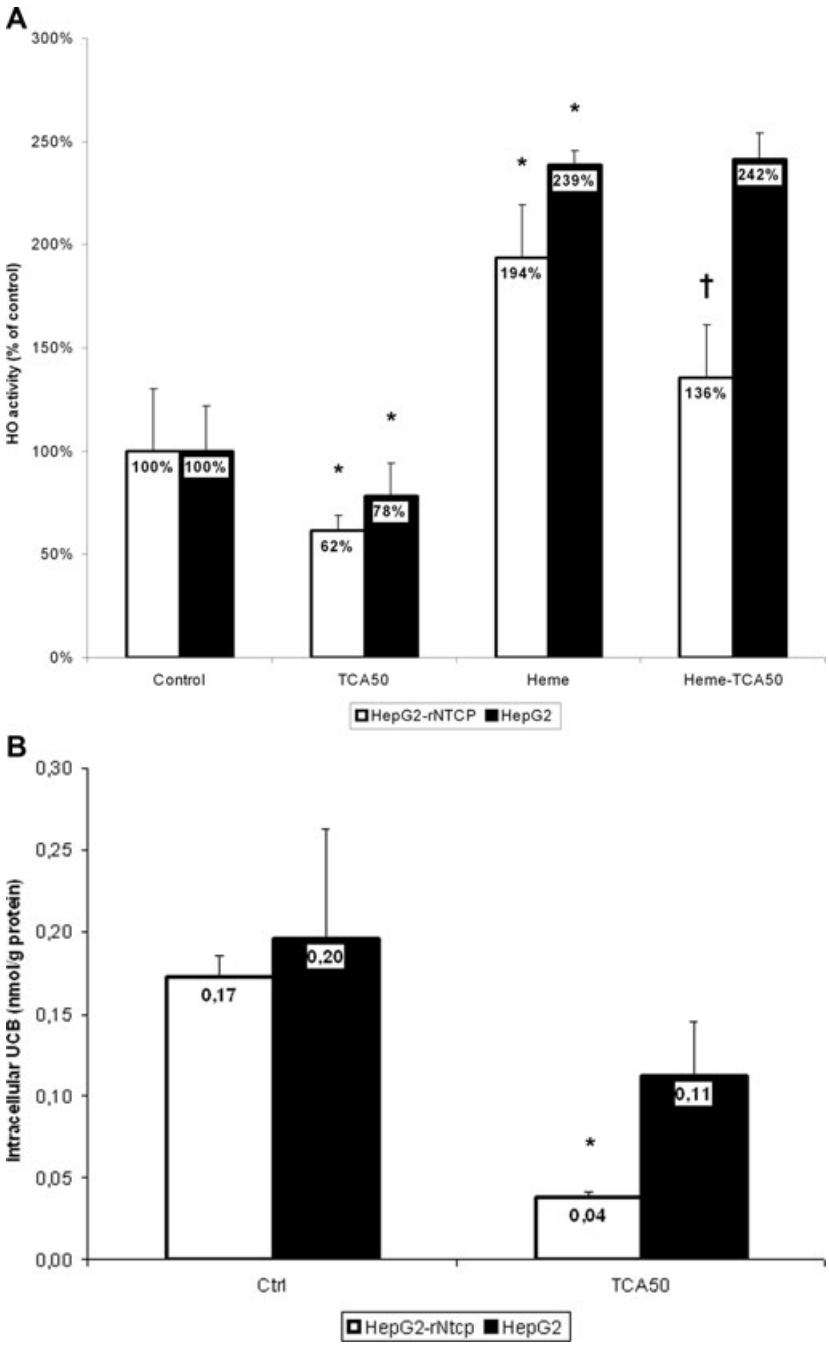

Fig. 3 Effect of TCA on HO activity and intracellular bilirubin *in vitro*. (**A**) HepG2 and HepG2-rNtcp cells (stably transfected with Ntcp transporter) were incubated for 24 hrs with 50 μM TCA, 30 μM heme (HO-1 inducer) or co-incubated with 50 μM TCA and 30 μM heme and HO activity was determined. $^{*}P < 0.05$ compared to controls, $^{\dagger}P < 0.05$ compared to heme-treated cells. (**B**) Intracellular bilirubin levels were measured in HepG2 and HepG2-rNtcp cells 24 hrs after incubation with 50 μM TCA. $^{*}P < 0.05$.

rat and Gunn rat liver homogenates. TCA at concentrations of 50, 100 and 500 μM significantly increased lipid peroxidation in Wistar rat liver homogenates. This effect was completely abolished by addition of 40 μM UCB. In Gunn rats, TCA had no effect on lipid peroxidation within mentioned concentration range (Fig. 2C).

TCA decreases intracellular bilirubin in HepG2-rNtcp cells

Treatment of HepG2 cells stably transfected with the Ntcp transporter with 50 μM TCA resulted in a significant decrease in HO activity (2.43 ± 0.73 nmol CO/hr/mg protein in controls

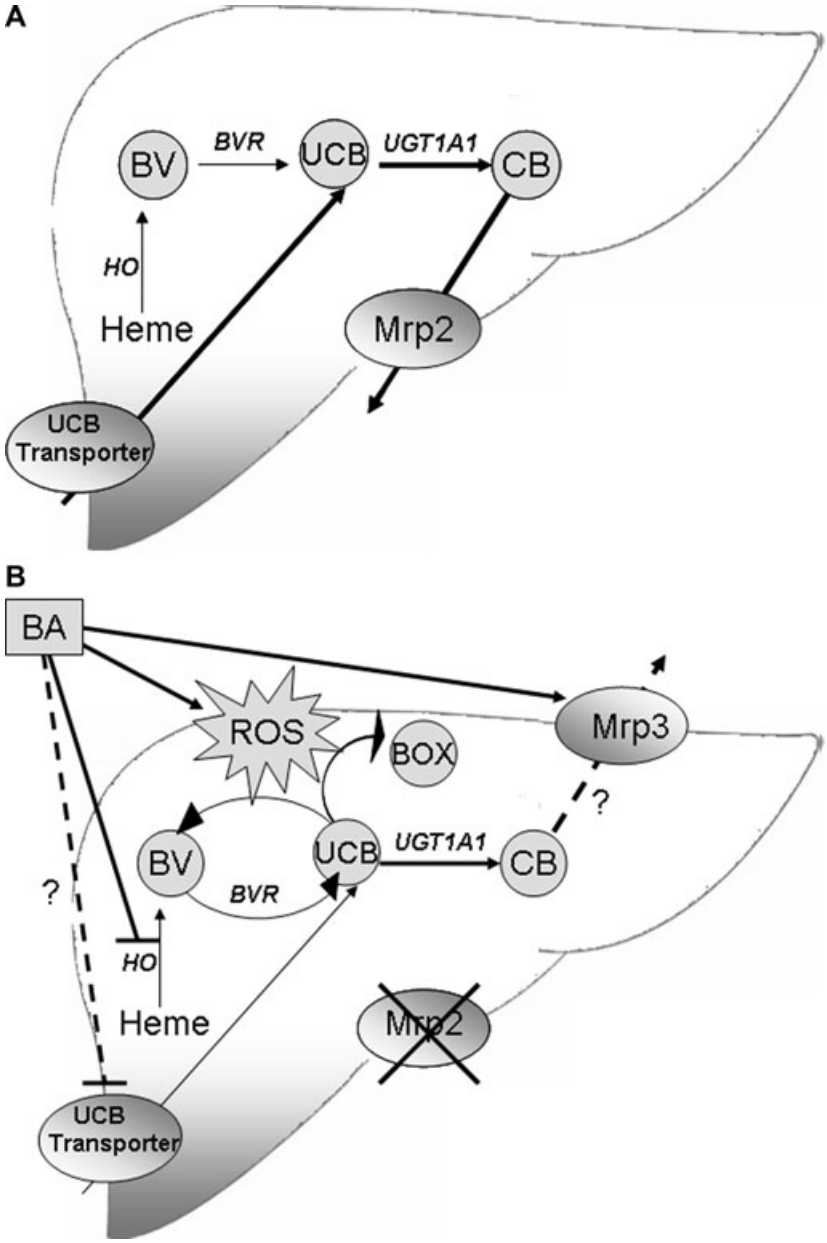

Fig. 4 Proposed bilirubin metabolism in normal (**A**) and cholestatic (**B**) rat liver. (**A**) Under normal conditions, UCB enters hepatocyte *via* carrier mediated mechanism (though the transporter involved still remains to be identified) [36, 37] or is produced intracellulary by oxidative degradation of heme. Intracellular UCB undergoes conjugation catalysed by bilirubin UDP-glucuronosyltransferase (UGT1A1) and CB is eliminated into bile *via* Mrp2 transporter. (**B**) High concentrations of bile acids in cholestatic liver lower bilirubin concentration by (1) triggering oxidative stress which leads to bilirubin consumption through biliverdin reductase (BVR) catalytic cycle [38] and *via* bilirubin oxidation products (BOX) formation (reviewed in [39]), (2) down-regulation of HO resulting in lower bilirubin production and (3) possibly by altering the expression of the basolateral transporters [31]. CB enters systemic circulation across the sinusoidal membrane possibly *via* up-regulated sinusoidal Mrp3 transporter.

versus 1.50 ± 0.11 nmol CO/hr/mg protein in TCA-treated cells, $P = 0.01$, Fig. 3A) and HO-1 mRNA (100% ± 15% *versus* 57% ± 23%, $P = 0.03$). As expected, treatment with 30 μM heme resulted in increase in HO activity though this effect was significantly reduced by co-treatment with TCA. Treatment of HepG2 cells (lacking Ntcp transporter for conjugated BA) with 50 μM TCA resulted in milder decrease in HO activity compared to HepG2-rNtcp cells (1.56 ± 0.34 nmol CO/hr/mg protein in controls *versus* 1.22 ± 0.19 nmol CO/hr/mg protein in TCA-treated cells, $P = 0.03$) and no decrease in HO-1 mRNA (100% ± 10% *versus* 89% ± 13%, $P = 1$). Interestingly, no decrease in HO activity has been observed upon co-treatment with heme plus TCA compared to heme-treated HepG2 cells.

Following treatment with 50 μM TCA, intracellular bilirubin decreased 78% in HepG2-rNtcp cells and only 43% decrease in HepG2 cells compared to control (untreated) cells (Fig. 3B). The decrease of intracellular bilirubin by 78% corresponded to a 31% decrease in HO activity in HepG2-rNtcp cells, suggesting that intracellular bilirubin might be also influenced by other mechanisms (Fig. 4).

Discussion

In this study, we demonstrated that bilirubin is not only a marker of cholestasis and hepatocyte dysfunction; but also, it is an endogenous antioxidant, counteracting the pro-oxidative effects of BA. In addition, we showed that BA lower intrahepatic bilirubin levels and bilirubin production presumably through an interaction between BA and bilirubin.

We found that BDL significantly increases the antioxidative capacity of plasma. Because of the significant positive correlation of plasma antioxidant capacity with bilirubin levels, it appears that bilirubin is the major antioxidant factor. This is supported by the finding that additions of UCB to normal rat plasma increased its antioxidative properties in dose-dependent fashion. These results agree with the data of Granato *et al.* [29] who demonstrated that bilirubin effectively suppresses ROS generation in freshly isolated hepatocytes.

However, a completely different circumstance may exist within liver tissue. BDL did not increase the antioxidant capacity in liver homogenates. We propose that this finding could, at least in part, be explained by the markedly different increases of bilirubin levels in plasma and liver compartments. After BDL, plasma levels of bilirubin increased more than 600-fold; whereas, in the liver only a 15-fold increase was observed. Furthermore, considering the high plasma bilirubin levels, the contamination of the liver with trace amounts of blood could artifactually actually increase liver bilirubin levels. Therefore, liver tissue bilirubin might be even lower in BDL animals compared to controls. Importantly, in Gunn rats, where BDL does not significantly affect plasma bilirubin levels (due to bilirubin elimination across the intestinal mucosa rather than the biliary tract), we observed a marked drop (54%) of liver bilirubin in BDL animals. These findings are of particular importance showing, for the first time, that intracellular bilirubin is actually consumed during cholestasis and that plasma bilirubin concentrations do not necessarily reflect tissue bilirubin metabolism.

To identify possible mechanisms responsible for this lowering of liver bilirubin levels following BDL, we treated HepG2 and HepG2-rNtcp cells with TCA. We found, that TCA down-regulates both the expression and activity of HO (the key enzyme in bilirubin production) and this down-regulation is more pronounced in cells expressing the Ntcp transporter. Accordingly, TCA lowered intracellular bilirubin levels. The markedly higher decrease in intracellular bilirubin compared to that of HO activity suggests that other mechanisms might also be involved. We have previously demonstrated an increased consumption of intracellular bilirubin during oxidative stress [30]. These effects could, together with BA-mediated alteration of bilirubin transport mechanisms [31], account for the relatively low hepatocyte bilirubin levels (Fig. 4).

The present study shows that in obstructive cholestasis, high concentrations of BA are responsible for increased lipid peroxidation in the liver as measured by the accumulation of MDA and 4-hydroxyalkenals, the products of lipid peroxidation. These findings agree with published data showing that MDA levels are increased in the livers of BDL rats [32, 33]. We have also observed increases in liver tissue CO and blood COHb in cholestatic animals. Because HO activity and expression (the main source of CO) are decreased in the livers of BDL animals, our observed elevations of CO concentration could be due to lipid peroxidation [25]. This is supported by our experiments where the addition of TCA to normal liver homogenates increased lipid peroxidation in a dose-dependent manner. However, addition of 40 μM bilirubin to liver homogenate completely abolished this effect. Additionally, no increase in lipid peroxidation was observed following the addition of TCA to liver homogenates from hyperbilirubinemic Gunn rats. All these data further confirm the opposing roles of BA and bilirubin in the development of oxidative stress and support the hypothesis that the higher BA/bilirubin ratio in cholestatic livers could lead to an increased susceptibility of the BDL livers to lipid peroxidation. These observations support also our previous data, showing that treatment of mice with HO inducer, rosuvastatin, led to simultaneous increase in heart HO activity and bilirubin content, but decrease in lipid peroxidation. Pre-treatment with a potent HO inhibitor, tin mesoporphyrin, completely abolished this effect [34].

There are several limitations of our study. We did not measure total BA in cholestatic liver homogenates, however, based on the previous work by Naito *et al.* [35] we can assume that similar concentrations of BA exist both in plasma and the liver. Secondly, only a short-term BDL was performed in our study, therefore, we cannot speculate about the course of chronic cholestasis. To clarify the exact role of HO expression in cholestasis, further studies with HO-1 knockout animals should be performed.

We conclude that high concentrations of BA in cholestasis are responsible for increased lipid peroxidation in the liver. In contrast, bilirubin has an antioxidative effect and is responsible for increased antioxidant capacity of cholestatic plasma. However, in the liver, BA maintain relatively low intracellular bilirubin levels. Therefore, the increase in BA/bilirubin ratio might be implicated in the pathogenesis of oxidative stress-mediated cholestatic liver injury.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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CASE REPORT

Dubin-Johnson syndrome coinciding with colon cancer and atherosclerosis

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Abstract

Hyperbilirubinemia has been presumed to prevent the process of atherogenesis and cancerogenesis mainly by decreasing oxidative stress. Dubin-Johnson syndrome is a rare, autosomal recessive, inherited disorder characterized by biphasic, predominantly conjugated hyperbilirubinemia with no progression to end-stage liver disease. The molecular basis in Dubin-Johnson syndrome is absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT caused by homozygous or compound heterozygous mutation(s) in *ABCC2* located on chromosome 10q24. Clinical onset of the syndrome is most often seen in the late teens or early adulthood. In this report, we describe a case of previously unrecognized Dubin-Johnson syndrome caused by two novel pathogenic mutations (c.2360_2366delCCCTGTC and c.3258+1G>A), coinciding with cholestatic liver disease in an 82-year-old male patient. The patient, suffering from advanced atherosclerosis with serious involvement of coronary arteries, developed colorectal cancer with nodal metastases. The subsequent findings do not support the protective role of Dubin-Johnson type hyperbilirubinemia.

Key words: Dubin-Johnson syndrome; *ABCC2*; Hyperbilirubinemia; Oxidative stress; Atherosclerosis; Cancer

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INTRODUCTION

Recent data have indicated the potent antioxidant properties of mild or moderately elevated serum bilirubin levels with substantial positive clinical consequences and especially their protective effect on atherogenesis and cancerogenesis[1]. A direct link between low serum bilirubin levels and peripheral vascular disease and the protective

effect of mild or moderate unconjugated hyperbilirubinemia on atherosclerosis were confirmed in numerous clinical trials[2-5]. Antiproliferative, cytostatic and pro-apoptotic effects of bilirubin are well-known and have been demonstrated in both *in vitro* and *in vivo* studies[6-10]. However, conflicting data on the possible protective effect of elevated bilirubin levels against sporadic colorectal carcinoma have been reported in the literature[11-13].

Dubin-Johnson syndrome (DJS, OMIM 237500) is a rare, autosomal recessive disorder characterized by non-hemolytic hyperbilirubinemia with no progression to end-stage liver disease[14]. Both the conjugated and unconjugated form of bilirubin can be elevated in DJS subjects, with the former ranging from 17% to 88% of the total bilirubin with a mean value of 60%[14,15]. In addition to the fluctuating jaundice caused by biphasic hyperbilirubinemia due to absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT[16-18], DJS subjects may suffer from non-specific symptoms such as weakness and abdominal discomfort. Urinary coproporphyrin output is normal; however, 80% of the coproporphyrin fraction is represented by coproporhyrin I (normally 25%)[19]. The biliary excretion of anionic dyes including bromosulfophthalein, indocyanine green and cholescintigraphy radiotracers is delayed. Liver histology in DJS shows an accumulation of distinctive melanin-like lysosomal pigment in an otherwise normal liver, which gives the organ a characteristic dark pink or even black colour[14,20].

In this report, we describe the case of unintentionally detected DJS complicating liver injury in an 82-year-old patient who was being followed for previously diagnosed colorectal cancer and ischemic heart disease.

CASE REPORT

An 82-year-old Caucasian male patient, a normotensive non-smoker with a history of right-sided hemicolectomy due to colorectal adenocarcinoma with nodal metastases five years ago and ischemic heart disease, was referred to the hospital due to clinical jaundice with elevated serum bilirubin (total bilirubin 220 μmol/L, direct bilirubin 171 μmol/L), gamma-glutamyltransferase (γGT) activity (4.7 μkat/L) and fluctuating alkaline phosphatase (ALP) activity (1.3-3.6 μkat/L) to exclude bile duct obstruction or metastatic liver disease. The patient complained of slight upper abdominal discomfort without pruritus or nausea. Complete blood count and serum electrolyte levels were normal, as was his serum lipid profile (serum cholesterol 3.5 mmol/L, high-density lipoprotein cholesterol 0.9 mmol/L, low-density lipoprotein cholesterol 2.5 mmol/L, triglycerides 1.3 mmol/L). Abdominal ultrasonography, endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography did not detect any obstacle or dilation of the biliary tree, and only slightly imperfect filling of the ductus choledochus was observed. Serological examinations for viral hepatitis (hepatitis A virus, hepatitis B virus and hepatitis C virus) and autoantibodies were completely negative. During the patient's hospitalization, the activity of γGT and ALP gradually decreased but, due to persistent marked hyperbilirubinemia, a percutaneous liver biopsy was performed.

The liver tissue obtained at biopsy was standardly processed. Sections cut at 4-6 μm were stained with hematoxylin and eosin, periodic acid Schiff, Schmorl's and van Gieson's method. Analysis of the liver biopsy specimen revealed preserved architecture of liver parenchyma with perivenular and perisinusoidal fibrosis, subtle steatosis and only discrete intracellular cholestasis in centrilobular hepatocytes. The most striking feature, however, was an intense parenchymal pigmentation, with centrilobular and midzonal accentuation, consisting of coarse brown black pigment (Figure 1, Panel A-C). Special stainings (Gömöri, silver ammonium complex-Masson's and Perls Prussian blue method) for pigment characterization were added. The pigment was negative in the Perls reaction and displayed rudimentary autofluorescence with gradually increasing intensity, especially in Shandon mounting medium and simultaneously reduced Masson's solution. A retrospective analysis of the patient's medical records back to 1994 revealed long-term, persistent, mixed, predominantly conjugated hyperbilirubinemia (total bilirubin fluctuating within the range 23.0-215.6 μmol/L, conjugated bilirubin 17.7-170 μmol/L). The conjugated-to-total bilirubin ratio was within the range of 40% (total bilirubin 202 μmol/L and conjugated bilirubin 80.6 μmol/L in 2009) to 89% (total bilirubin 41.8 μmol/L and conjugated bilirubin 37.2 μmol/L in 1994). Taking into account the normal activity of aminotransferases, γGT and ALP, DJS was suspected.

For immunohistochemical analysis, 4 to 6 μm-thick sections were incubated with the anti-MRP2 mouse monoclonal antibody (clone M2Ⅲ-6, Kamiya, Seattle, WA). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualization and counterstaining with Harris's hematoxylin was performed. As a positive control, sections of an adult liver without cholestasis were stained, and liver sections incubated without primary antibody were used as negative controls. Immunohistochemical analysis for MRP2 protein was completely negative (Figure 1D).

Ultrastructural analysis was performed on the liver sample fixed with 4% buffered paraformaldehyde, osmicated, dehydrated in ascending ethanol solutions and embedded into Epon-Araldite mixture. Ultrathin sections were double-stained with uranyl acetate and lead nitrate and then examined under a JEM 1200 EX electron microscope. Intralysosomal localization of the pigment was demonstrated.

A mutation analysis of the *ABCC2* gene was indicated to confirm the diagnosis of DJS at the molecular level. Written informed consent was obtained from the patient before the genetic investigation. *ABCC2* was analyzed by direct sequencing of genomic DNA extracted from peripheral leucocytes. All 32 exons, with the adjacent parts of the intronic sequences, were amplified by polymerase chain reaction (PCR) using the intronic oligonucleotide primers listed in Table 1. Amplified fragments were gel-purified, extracted from the gel with QIA quick spin col-

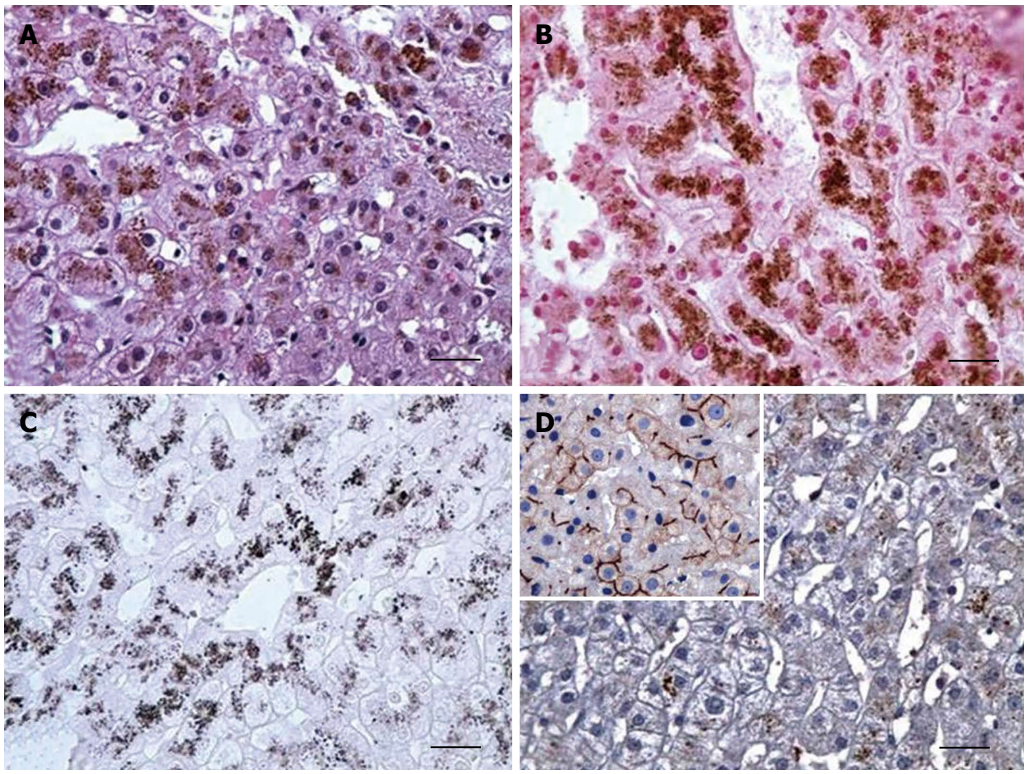

Figure 1 Histochemistry and immunohistology of the liver in Dubin-Johnson syndrome patient. A: Accumulation of distinctive dark brown pigment in hepatocytes was detected in hematoxylin and eosin staining; B: The pigment was negative in Perls reaction; C: The pigment reduced Masson's solution; D: Immunohistochemical analysis for ABCC2/MRP2 protein was negative compared to the positive control (inset). Original magnification ×400 (bars correspond to 100 μm).

Table 1 Primer pairs used for amplification of *ABCC2/MRP2* gene exons and promoters

Pair No.	Sequences of polymerase chain reaction primer pairs used to amplify the *ABCC2/MRP2* gene from genomic DNA		Exon No.
	Forward primer	Reverse primer	
1	5=-TTTACAATGCCTGGCAAAGG-3=	5=-CAGCATGATTCCTGGACTGC-3=	Promoter
2	5=-TCCCACATTCTGGATTTTGAC-3=	5=-ATAAAATAGCTTATCCAGTGTGAGC-3=	Promoter
3	5=-GGTCAGACCAATTTACATTTCCATC-3=	5=-CATAACCACCCATGCAGTATCC-3=	Promoter
4	5=-TACTTTGGGAACTGGTGAGTCT-3=	5=-AGAAGGCAATTTTGCGACTA-3=	1
5	5=-CACAAATAGGAAAATACGGATA-3=	5=-CCTGGGACAGCTGCTTA-3=	2
6	5=-CTGAATCACTGCATACCGCTTTT-3=	5=-CCAACCAAGCTTTGCCTCAC-3=	3
7	5=-CTGAATCACTGCATACCGCTTTT-3=	5=-AATTCGATCCTGGAGCTCAAC-3=	3_4
8	5=-TCATAGTAAATGGCATCAAGT-3=	5=-GGTGGAACATGAGCTTGAGT-3=	5_6
9	5=-AGTGGTGGAGATAGCCTC-3=	5=-GCTATAAAAATGTAAGGACA-3=	7
10	5=-GCCAGGGAGAGATGATCAAA-3=	5=-GGCCAGTCAACATTAAGTG-3=	8_9
11	5=-TGGAGCACATCCTTCCATTG-3=	5=-TTGCCCAAACTCCCATTAAG-3=	10
12	5=-TCACTGGGCACCTCAAGTTC-3=	5=-AGCAGGAATCCATCACCTCT-3=	11
13	5=-ATTTTGGGGACTATATCT-3=	5=-GATGTGATAGCCAGTCATTC-3=	12_13
14	5=-GTTCGGTGGAGATTAGGAG-3=	5=-TCTTATGCAAGCATAGGCTC-3=	14
15	5=-TTCACCTCCTGTTAGCGTA-3=	5=-ACCGAAGACATGCACATAGC-3=	15
16	5=-TTCACCTCCTGTTAGCGTA-3=	5=-CAAGACCTCACCTACTAGCC-3=	15_16
17	5=-ACAAGCACGTGAATACATATCAG-3=	5=-ACCCCTGTGTAGTTCTT-3=	17
18	5=-GTAAGATTTTTAACCCCTTG-3=	5=-GCCCAGGCATAGAGTTTC-3=	18_19
19	5=-GTATGGAGTATTTATGGAGT-3=	5=-TGTAAGTATGCGTTCAAT-3=	20_21
20	5=-GTGGTTGGCATTCTAGGT-3=	5=-CATAATAATTCCTCCCTATCA-3=	22_23
21	5=-CTGGGAACACACAGAATCCAAC-3=	5=-GGCTCCTGGGTATGTCAACA-3=	24
22	5=-GGCTTTTGTCTTGTTCAGACG-3=	5=-CTTGGTAAACGGCAGA-3=	25
23	5=-CCCGATCAAGTCAAAC-3=	5=-GGCATTCATGTCTACTTAGGA-3=	26
24	5=-GGAGGCAAGGATTGTC-3=	5=-TCTGCATACTGTGGACCTTAT-3=	27_28
25	5=-ACAGCTGCCAAGAGAGTCCAT-3=	5=-GCTCAAGTATCCCGAGTAGA-3=	29
26	5=-CCTTGCGGAAGCTCAACC-3=	5=-TGCCAGGCATCACCTAACACG-3=	30
27	5=-GTTTTGAAAGTCTGATCTG-3=	5=-AGGAAGTACGATCGAGGTA-3=	31_32

umns (Qiagene, Hilden, Germany), and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster

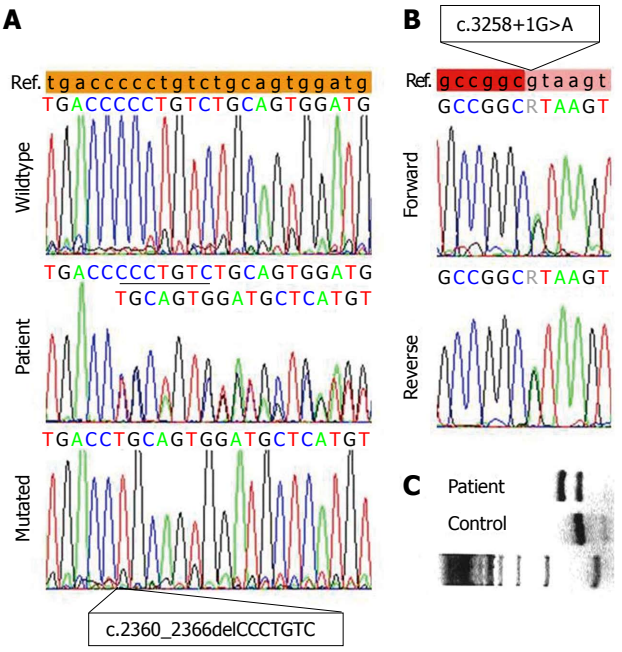

Figure 2 Mutation analysis results. A: Presence of a heterozygous deletion c.2360_2366delCCCTGTC in exon 18 of *ABCC2* (panel A, middle lane) was confirmed by sequence analysis of individual alleles separated by cloning (wildtype allele - panel A, upper lane; mutated allele - panel A, lower lane); B: In addition, a heterozygous splice site mutation c.3258+1G>A was detected in intron 23; C: Presence of the latter mutation was confirmed by polymerase chain reaction-Bsh1236I restriction fragment length polymorphism analysis.

City, CA). The obtained sequence was compared with the reference sequences GenBank NM_000392 (mRNA) and NT_030059 (genomic DNA). An exon with suspected deletion was cloned into a pCR4.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA), and the wild-type and mutated alleles were sequenced separately. Presence of the second mutation was confirmed by PCR-restriction fragment length polymorphism analysis (PCR-Bsh1236I RFLP).

Analysis of the *ABCC2*/*MRP2* gene disclosed two novel mutations: a heterozygous deletion c.2360_2366delCCCTGTC in protein coding exon 18 (Figure 2A), and a heterozygous mutation c.3258+1G>A in intron 23 (Figure 2B and C). The former mutation in exon 18 was predicted to cause a reading frame shift and premature termination of DNA translation at position 803 with protein alteration p.Pro787LeufsX7. The latter mutation affecting the donor splice site of intron 23 was predicted to cause abnormal splicing of mRNA. Therefore, both mutations were considered as pathogenic. Histochemistry and mutation analysis accordingly established the diagnosis of DJS.

The patient suddenly died several months after being released from the hospital. The patient's autopsy revealed no liver metastases and confirmed the diagnosis of advanced atherosclerosis with serious involvement of coronary arteries with signs of chronic myocardial ischemia.

DISCUSSION

The diagnosis of DJS is indicated by the presence of fluctuating predominantly conjugated hyperbilirubinemia, an increased ratio of urinary coproporphyrin I to coproporphyrin Ⅲ, and a unique cholescintigram showing delayed visualization of the liver and biliary tract. Neither a percutaneous liver biopsy with evidence of melanin-like pigment and the absence of MRP2 protein nor mutation analysis are necessary for the diagnosis. Nonetheless, both methods may be helpful in complicated cases to establish the correct diagnosis and rule out more serious liver pathology.

In our patient, neither an examination of urinary coproporphyrin levels nor cholescintigraphy was performed. The diagnosis was based on biochemical evidence of fluctuating biphasic hyperbilirubinemia and characteristic findings in the liver biopsy, and further supported by disclosure of two pathogenic mutations by subsequent mutational analysis. Considering the clinical picture and the results of laboratory tests, the two mutations are supposed to be in transposition. Unfortunately, no living relatives of the patient were found to confirm our presumption.

As the clinical onset of DJS is most often seen during the teenage years or in early adulthood, the first recognition of the disease in old age is highly unusual. To the best of our knowledge, our patient is the oldest reported newly diagnosed DJS patient confirmed by mutation analysis.

The clinical presentation and laboratory findings of our case indicated the coincidence of DJS with mild cholestatic liver damage, the etiology of which remained obscure. The simultaneous occurrence of the syndrome with another hepatobiliary disease is well-known and the coincidence with another disease or pathologic stimulus can modify the clinical picture and results of laboratory tests, including histomorphology[21-24].

A significant fact in our case is the presence of colorectal adenocarcinoma with nodal metastases in a patient with chronic mixed hyperbilirubinemia. Numerous clinical trials have demonstrated the protective effects of hyperbilirubinemia against the development of sporadic colorectal cancer and an association of low bilirubin levels with an increased risk of colon cancer morbidity and mortality[10-12].

In addition, there are reports describing the protective effect of bilirubin on the development of atherosclerosis emphasizing the antioxidative and anti-inflammatory properties of bilirubin[2-5]. Interestingly, our patient, a lifelong normotensive non-smoker with normal lipidogram, suffered from ischemic heart disease and advanced atherosclerosis affecting both large arteries and peripheral circulation, especially coronary arteries.

In conclusion, our case demonstrates that DJS should be included in the differential diagnosis of liver diseases even in atypical age categories. The fact that our patient with DJS developed colorectal adenocarcinoma and clinically significant atherosclerosis indicates that Dubin-Johnson hyperbilirubinemia may not be sufficient to protect from atherogenesis and cancer development, even in the absence of established risk factors.

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Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver

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Bilirubin, a breakdown product of heme, is normally glucuronidated and excreted by the liver into bile. Failure of this system can lead to a buildup of conjugated bilirubin in the blood, resulting in jaundice. The mechanistic basis of bilirubin excretion and hyperbilirubinemia syndromes is largely understood, but that of Rotor syndrome, an autosomal recessive disorder characterized by conjugated hyperbilirubinemia, coproporphyrinuria, and near-absent hepatic uptake of anionic diagnostics, has remained enigmatic. Here, we analyzed 8 Rotor-syndrome families and found that Rotor syndrome was linked to mutations predicted to cause complete and simultaneous deficiencies of the organic anion transporting polypeptides OATP1B1 and OATP1B3. These important detoxification-limiting proteins mediate uptake and clearance of countless drugs and drug conjugates across the sinusoidal hepatocyte membrane. OATP1B1 polymorphisms have previously been linked to drug hypersensitivities. Using mice deficient in Oatp1a/1b and in the multispecific sinusoidal export pump Abcc3, we found that Abcc3 secretes bilirubin conjugates into the blood, while Oatp1a/1b transporters mediate their hepatic reuptake. Transgenic expression of human OATP1B1 or OATP1B3 restored the function of this detoxification-enhancing liver-blood shuttle in Oatp1a/1b-deficient mice. Within liver lobules, this shuttle may allow flexible transfer of bilirubin conjugates (and probably also drug conjugates) formed in upstream hepatocytes to downstream hepatocytes, thereby preventing local saturation of further detoxification processes and hepatocyte toxic injury. Thus, disruption of hepatic reuptake of bilirubin glucuronide due to coexisting OATP1B1 and OATP1B3 deficiencies explains Rotor-type hyperbilirubinemia. Moreover, OATP1B1 and OATP1B3 null mutations may confer substantial drug toxicity risks.

Introduction

Rotor syndrome (RS; OMIM %237450) is a rare, benign hereditary conjugated hyperbilirubinemia, also featuring coproporphyrinuria and strongly reduced liver uptake of many diagnostic compounds, including cholescintigraphic tracers (1–6). RS is an autosomal recessive disorder that clinically resembles another conjugated hyperbilirubinemia, the Dubin-Johnson syndrome (DJS; OMIM #237500) (7, 8). In both RS and DJS, mild jaundice begins shortly after birth or in childhood. There are no signs of hemolysis, and routine hematologic and clinical-biochemistry test results are normal, aside from the primarily conjugated hyperbilirubinemia. RS is, however, distinguishable from DJS by several criteria (1, 2, 9, 10): (a) it lacks the hepatocyte pigment deposits typical of DJS; (b) in RS, but not DJS, there is delayed plasma clearance of unconjugated bromsulphthalein (BSP), an anionic diagnostic dye, and no conjugated BSP appears in plasma (4); (c) the liver in RS is scarcely visualized on ^{99m}Tc-N[2,6-dimethylphenyl-carbamoylmethyl] iminodiacetic acid (^{99m}Tc-HIDA) cholescintigraphy, with slow liver uptake, persistent visualization of the cardiac blood pool, and prominent kidney excretion (5); and (d) total urinary excretion of coproporphyrins is greatly increased in RS, with coproporphyrin I being the predominant isomer (11).

DJS is caused by mutations affecting ABCC2/MRP2, a canalicular bilirubin glucuronide and xenobiotic export pump, thus disrupting bilirubin glucuronide excretion into bile (7, 8). Excretion of bilirubin glucuronides is then redirected into plasma by the action of ABCC3/MRP3, a homolog of ABCC2 that is present in the sinusoidal membrane and is upregulated in DJS (12, 13). The molecular mechanism of DJS is in line with the generally accepted paradigm of normal hepatic bilirubin excretion, according to which a unidirectional elimination pathway is postulated: first, uptake of unconjugated bilirubin (UCB) from blood into hepatocytes; subse-

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Conflict of interest: The research group of Alfred H. Schinkel receives revenues from commercial distribution of some of the mouse strains used in this study.

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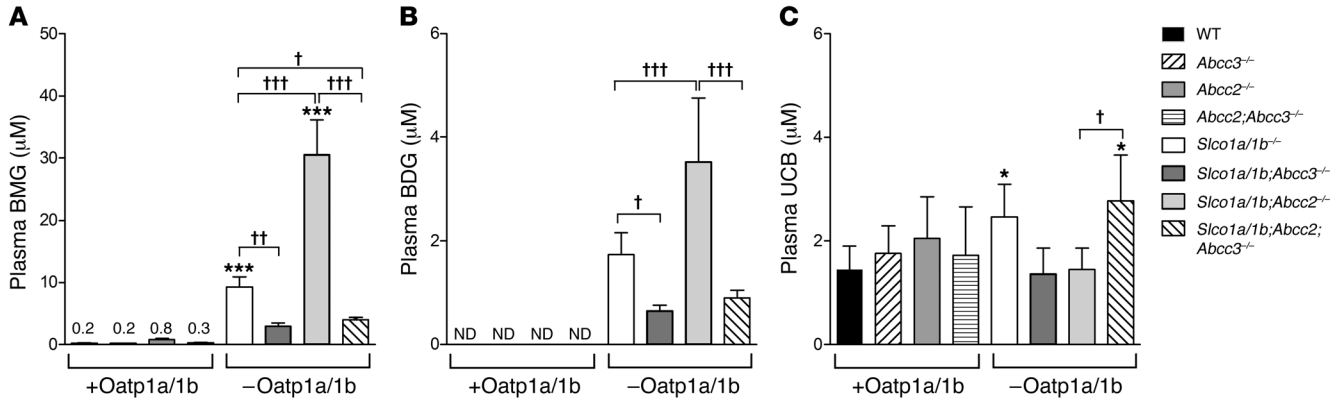

Figure 1

Increased plasma bilirubin glucuronide in *Slco1a/1b*$^{-/-}$ mice is in part dependent on Abcc3. (**A**) BMG, (**B**) BDG, and (**C**) UCB levels in plasma of male wild-type, *Abcc3*$^{-/-}$, *Abcc2*$^{-/-}$, *Abcc2*$^{-/-}$*Abcc3*$^{-/-}$, *Slco1a/1b*$^{-/-}$, *Slco1a/1b;Abcc3*$^{-/-}$, *Slco1a/1b;Abcc2*$^{-/-}$, and *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice (n = 4–7). +Oatp1a/1b denotes strains possessing Oatp1a/1b proteins, and –Oatp1a/1b denotes strains lacking Oatp1a/1b proteins. Data are mean ± SD. *P < 0.05, ***P < 0.001 compared with wild-type mice. Bracketed comparisons: †P < 0.05, ††P < 0.01, †††P < 0.001. ND, not detectable; detection limit was 0.1 μM.

quent glucuronidation; and finally, secretion of bilirubin glucuronide into bile via ABCC2. Individuals with RS, however, lack *ABCC2* mutations (14), and the mechanistic basis of RS is unknown.

Organic anion transporting polypeptides (OATPs, genes: *SLCO*s) contain 12 plasma membrane–spanning domains and mediate sodium-independent cellular uptake of highly diverse compounds, including bilirubin glucuronide, bile acids, steroid and thyroid hormones, and numerous drugs, toxins, and their conjugates (15, 16). Human OATP1B1 and OATP1B3 localize to the sinusoidal membrane of hepatocytes and mediate the liver uptake of, among other compounds, many drugs (15–19). Various SNPs in *SLCO1B1* cause reduced transport activity and altered plasma and tissue levels of statins, methotrexate, and irinotecan in patients, potentially resulting in life-threatening toxicities (20–24).

In a *Slco1a/1b*$^{-/-}$ mouse model recently generated by our group, the importance of Oatp1a/1b proteins in hepatic uptake and clearance of drugs was confirmed, but the mice also displayed marked conjugated hyperbilirubinemia (25). We therefore hypothesized that sinusoidal Oatps in the normal, healthy mouse liver function in tandem with the sinusoidal efflux transporter Abcc3 to mediate substantial hepatic secretion and reuptake of bilirubin glucuronides and other conjugated compounds (25).

Here we describe how a combination of functional studies in mice to address this hypothesis and independent genetic studies in humans has resulted in elucidation of the genetic and mechanistic basis of Rotor syndrome.

Results

To test our hypothesis regarding the involvement of Abcc3 in the sinusoidal cycling of bilirubin glucuronides, and to assess a possible interplay with Abcc2, we generated *Slco1a/1b*$^{-/-}$*Abcc3*$^{-/-}$ (*Slco1a/1b;Abcc3*$^{-/-}$), *Slco1a/1b*$^{-/-}$*Abcc2*$^{-/-}$ (*Slco1a/1b;Abcc2*$^{-/-}$), and *Slco1a/1b*$^{-/-}$*Abcc2*$^{-/-}$*Abcc3*$^{-/-}$ (*Slco1a/1b;Abcc2;Abcc3*$^{-/-}$) mice by crossbreeding of existing strains. All strains were fertile, with normal life spans and body weights. As previously found for *Abcc2*$^{-/-}$ and *Abcc2*$^{-/-}$*Abcc3*$^{-/-}$ mice (26, 27), liver weights of *Slco1a/1b;Abcc2*$^{-/-}$ and *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice were significantly increased (~30% and ~50%, respectively) compared with wild-type mice (data not shown). Quantitative RT-PCR analysis of functionally relevant uptake and efflux transporters in liver, kidney, and intestine of the single and combination knockout strains revealed only some modest expression changes (Supplemental Table 1 and Supplemental Results; supplemental material available online with this article; doi:10.1172/JCI59526DS1). Hepatic UDP-glucuronosyltransferase 1a1 (*Ugt1a1*) expression was not significantly altered in any of the strains.

Importantly, the markedly increased plasma bilirubin monoglucuronide (BMG) and bilirubin diglucuronide (BDG) levels observed in *Slco1a/1b*$^{-/-}$ mice were substantially reduced in *Slco1a/1b;Abcc3*$^{-/-}$ mice, demonstrating that Abcc3 is necessary for most of this increase (Figure 1, A and B). Plasma BMG levels in *Slco1a/1b;Abcc2*$^{-/-}$ mice, even further increased owing to strongly reduced biliary BMG excretion (Figure 2, A and B), were similarly decreased in *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice (Figure 1, A and B). Thus, Abcc3 secretes bilirubin glucuronides back into blood, and Oatp1a/1b proteins mediate their efficient hepatic reuptake, thereby together establishing a sinusoidal liver-blood shuttling loop. The incomplete reversion of plasma bilirubin glucuronide levels in the Oatp1a/1b/Abcc3-deficient strains (Figure 1, A and B) suggests that additional sinusoidal exporter(s), e.g., Abcc4 (28), can partly take over the sinusoidal bilirubin glucuronide extrusion function of Abcc3.

The biliary output of bilirubin glucuronides in the single and combination knockout mice showed that, as long as Oatp1a/1b was functional, Abcc3 improved the efficiency of biliary bilirubin glucuronide excretion, even though it transports its substrates initially from liver to blood, not bile (Figure 2, A and B, strains +Oatp1a/1b). This suggests that, within liver lobules, the bilirubin glucuronide extruded by Abcc3 in upstream hepatocytes is efficiently taken up in downstream hepatocytes via Oatp1a/1b and then excreted into bile. The resulting relief of possible saturation of (or competition for) biliary excretion in the upstream hepatocytes may explain why the overall biliary excretion is enhanced by this transfer to downstream hepatocytes. However, when Oatp1a/1b was absent, Abcc3 instead decreased biliary bilirubin glucuronide excretion (Figure 2, strains –Oatp1a/1b) and

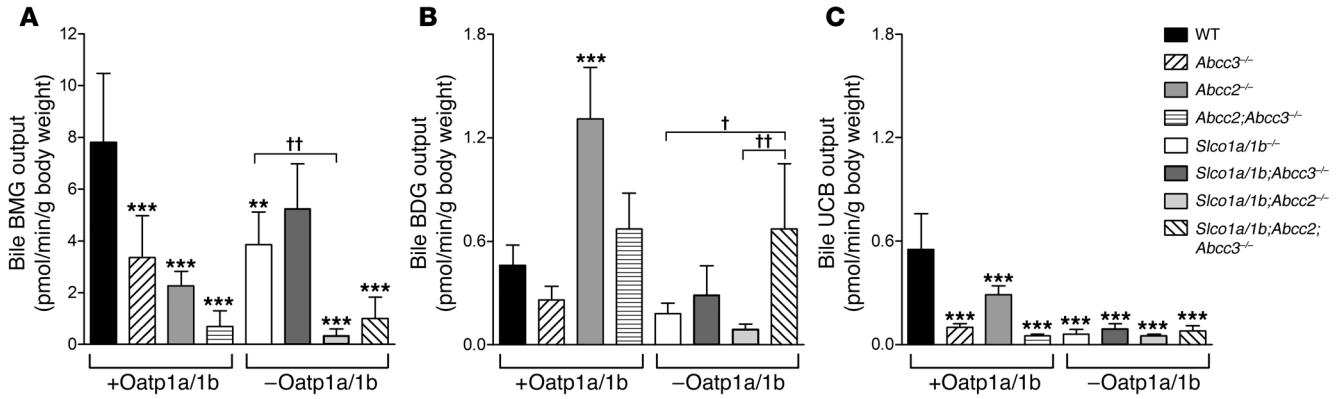

Figure 2

In the presence of Oatp1a/1b, but not in its absence, Abcc3 enhances biliary excretion of bilirubin glucuronides. (**A**) BMG, (**B**) BDG, and (**C**) UCB output in bile of male wild-type, *Abcc3*$^{-/-}$, *Abcc2*$^{-/-}$, *Abcc2*$^{-/-}$*Abcc3*$^{-/-}$, *Slco1a/1b*$^{-/-}$, *Slco1a/1b;Abcc3*$^{-/-}$, *Slco1a/1b;Abcc2*$^{-/-}$, and *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice. Bile collected during the first 15 minutes after gall bladder cannulation was analyzed. +Oatp1a/1b denotes strains possessing Oatp1a/1b proteins, and –Oatp1a/1b denotes strains lacking Oatp1a/1b proteins. Data are shown as mean ± SD (n = 4–7). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with wild-type mice. Bracketed comparisons: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$.

redirected excretion toward urine via the increased plasma bilirubin glucuronide levels (Supplemental Figure 1). Obviously, in the absence of Oatp1a/1b-mediated hepatic reuptake, Abcc3 activity can only decrease hepatocyte levels of bilirubin glucuronide in upstream and downstream hepatocytes alike, and will therefore reduce overall biliary excretion. Thus, both components of the Abcc3 and Oatp1a/1b shuttling loop are necessary to improve hepatobiliary excretion efficiency.

Human hepatocytes express only two OATP1A/1B proteins at the sinusoidal membrane, OATP1B1 and OATP1B3 (15). To test whether these could mediate the identified Oatp1a/1b functions, and in a liver-specific manner, we generated *Slco1a/1b*$^{-/-}$ mice with liver-specific expression of either human OATP1B1 or OATP1B3. Liver-specific expression was obtained using an apoE promoter (29). These strains were viable and fertile, and displayed normal life spans and body weights. Liver levels of transgenic OATP1B1 and OATP1B3 proteins were similar to those seen in pooled human liver samples (data not shown). Both of the transgenic rescue strains displayed a virtually complete reversal of the increases in plasma and urine levels of BMG and BDG seen in *Slco1a/1b*$^{-/-}$ mice (Figure 3, A and B, and Supplemental Figure 2). This indicates that both human OATP1B1 and OATP1B3 effectively reabsorb bilirubin glucuronides from plasma into the liver, in line with their demonstrated in vitro role in bilirubin glucuronide uptake (30). The modest (~1.8-fold) increase in plasma UCB in *Slco1a/1b*$^{-/-}$ mice was also reduced in the rescue strains (Figure 3C), suggesting an ancillary role of these proteins in hepatic UCB uptake.

These findings collectively raised the question as to whether humans with a severe deficiency in OATP1B1 and OATP1B3, possibly leading to a conjugated hyperbilirubinemia, might exist. A literature search suggested RS as a candidate inborn metabolic disorder. A search for RS subjects by part of the present group led to collaboration with another team already working on mapping of the RS gene(s).

In an unbiased approach, scanning the whole genome, we mapped the genomic candidate intervals for RS in 11 RS index subjects from 8 different families, 4 Central European (CE1–CE4), 3 Saudi-Arabian (A1–A3), and 1 Filipino (P1) (Figure 4A and Supplemental Table 2). Homozygosity mapping identified a single genomic region on chromosome 12 for which 8 tested index subjects and no healthy siblings or parents were homozygous (Figure 4B), suggesting inheritance of both alleles from a common ancestor. Three distinct homozygous haplotypes (R1–R3) segregated with RS: R1 in families CE1, CE2, and CE4; R2 in families CE3, A1, A2, and A3; and R3 in family P1 (Figure 4B; for genotyping details, see Methods). Intersection of these haplotypes defined a candidate genomic region spanning the *SLCO1C1*, *SLCO1B3*, *SLCO1B1*, *SLCO1A2*, and *IAPP* genes (Figure 4B). A parallel genome-wide copy number analysis detected a homozygous deletion within the *SLCO1B3* gene in the R1 haplotype and a homozygous approximately 405-kb deletion encompassing *SLCO1B3* and *SLCO1B1* and the *LST-3TM12* pseudogene in the R2 haplotype (Figure 4B and Supplemental Figure 3).

Sequence analysis revealed predictably pathogenic mutations affecting both *SLCO1B3* and *SLCO1B1* in each of the haplotypes (Figure 4, B–D, Table 1, Supplemental Figure 3, and Supplemental Table 3). In the R1 haplotype, a 7.2-kb deletion removes exon 12 of *SLCO1B3*, encoding amino acids 500–560 of OATP1B3 (702 aa long) and introduces a frameshift and premature stop codon, thus removing the C-terminal 3 transmembrane domains. Furthermore, a nonsense mutation in exon 13, c.1738C→T, introduces a premature stop codon (p.R580X) in R1-linked OATP1B1 (691 aa long), removing the C-terminal one-and-a-half transmembrane domains. The 405-kb R2 deletion encompasses exons 3–15 of *SLCO1B3* (sparing only a small N-terminal region) and the whole of *SLCO1B1*, but not *SLCO1A2*. The R3 haplotype harbors a splice donor site mutation, c.1747+1G→A, in intron 13 of *SLCO1B3*. If *SLCO1B3* is still yielding functional mRNA, this would truncate OATP1B3 after amino acid 582, deleting the C-terminal one-and-a-half transmembrane domains. A nonsense mutation, c.757C→T, in exon 8 of R3-linked *SLCO1B1* introduces a premature stop (p.R253X), truncating OATP1B1 before the C-terminal 7 transmembrane domains. All of these mutations would severely disrupt or annihilate proper protein expression and function. Moreover, they all showed consistent autosomal recessive segregation with the RS phenotype in the investigated families (Table 1). No

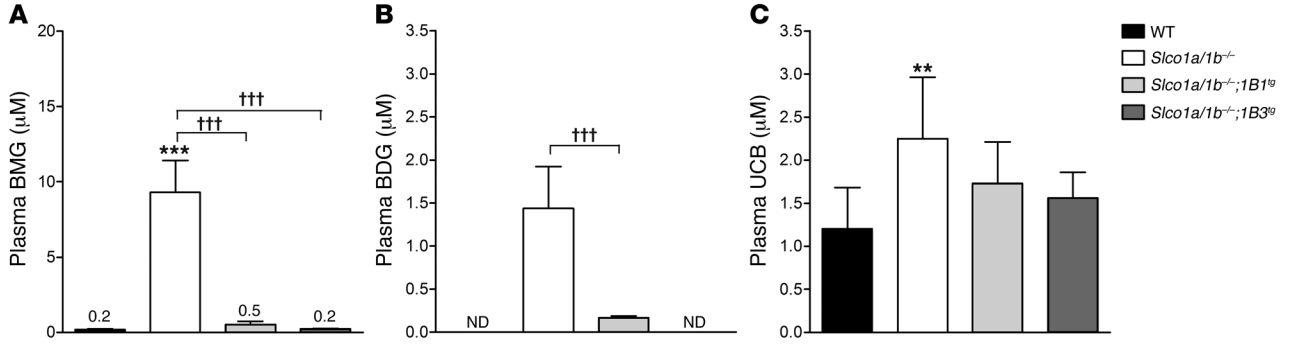

Figure 3

Increased plasma bilirubin glucuronide in *Slco1a/1b*$^{-/-}$ mice is reversed by human OATP1B1 and OATP1B3. (**A**) BMG, (**B**) BDG, and (**C**) UCB levels in plasma of male wild-type and *Slco1a/1b*$^{-/-}$ mice, and of the derived OATP1B1- and OATP1B3-transgenic strains (*Slco1a/1b*$^{-/-}$*;1B1*^{tg} and *Slco1a/1b*$^{-/-}$*1B3*^{tg}, respectively) (n = 5–8). Data are mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with wild-type mice. Bracketed comparisons: $^{†††}P < 0.001$. Detection limit was 0.1 μM.

SLCO1A2 sequence variation was found in probands representing the 3 haplotypes, rendering involvement of OATP1A2 in RS unlikely. The severity of the identified mutations affecting *SLCO1B3* and *SLCO1B1* and their strict cosegregation with the RS phenotype indicate that RS is caused by co-inherited complete functional deficiencies in both OATP1B3 and OATP1B1.

The severity of the mutations was independently supported by immunohistochemical studies of the sparse RS liver biopsy material available. Given their sparseness, immunostaining of these liver biopsies was performed using one antibody recognizing the N terminus of both OATP1B1 and OATP1B3 (31). This revealed absence of detectable staining in probands representing each haplotype (Figure 5). In controls, basolateral membranes of centrilobular hepatocytes stained crisply, as previously reported (31). Thus, the *SLCO1B1* and *SLCO1B3* mutations in each haplotype result in absence of a detectable signal for OATP1B protein in the liver.

In family A2, a heterozygous splice donor site mutation, c.481+1G→T, in intron 5 of *SLCO1B1* would result in dysfunctional RNA or protein. Its co-occurrence with the 405-kb R2 deletion in two asymptomatic family members (Table 1) indicates that a single functional *SLCO1B3* allele can prevent RS.

A search for copy number variations (CNVs) in existing databases and CNV genotyping of more than 2,300 individuals from various populations (see Supplemental Results) revealed additional heterozygous small and large deletions predicted to disrupt *SLCO1B1* or *SLCO1B3* function, including several approximately 400-kb deletions similar or identical to the R2 haplotype–linked deletion. One individual without jaundice, heterozygous for the R1 haplotype–associated c.1738C→T (p.R580X) mutation in *SLCO1B1*, was also homozygous for the R1 haplotype–associated deletion in *SLCO1B3*. Thus, a single functional *SLCO1B1* allele can also prevent RS. Combined with the findings in family A2 described above, this demonstrates that only a complete deficiency of both alleles of *SLCO1B1* and *SLCO1B3* will result in RS.

Discussion

We demonstrate here that RS is an obligate two-gene disorder, caused by a complete deficiency of the major hepatic drug uptake transporters OATP1B1 and OATP1B3. We further identified individuals with a complete deficiency of either OATP1B1 or OATP1B3, which was not recognizable by obvious jaundice. In spite of the documented important functions of especially OATP1B1 in drug detoxification, apparently such deficiencies are compatible with relatively normal life.

Using Oatp1a/1b-knockout mice, which can, retrospectively, be considered to be a partial model for RS, we showed that Abcc3 is an important factor for the RS-like conjugated hyperbilirubinemia. Our data imply that in the normal human liver ABCC3, OATP1B1, and OATP1B3 may form a liver-blood shuttling loop for bilirubin glucuronide, similar to that driven by Oatp1a/1b and Abcc3 in the mouse (Figure 6). A substantial fraction of bilirubin conjugated in hepatocytes is secreted back into the blood by ABCC3 and subsequently reabsorbed in downstream hepatocytes by OATP1B1 and OATP1B3. In RS this reuptake is hampered, causing increased plasma bilirubin glucuronide levels and jaundice. The flexible "hepatocyte hopping" afforded by this loop facilitates efficient detoxification, presumably by circumventing saturation of further detoxification processes in upstream hepatocytes, including excretion into bile. Indeed, we could show that, counterintuitively, but in accordance with the hepatocyte hopping model, loss of Abcc3 in mice resulted in decreased biliary excretion of bilirubin glucuronide, as long as Oatp1a/1b was present (Figure 2). This process likely also enhances hepatic detoxification of numerous drugs and drug conjugates (e.g., glucuronide, sulfate, and glutathione conjugates) transported by OATP1B1/3 and ABCC3. Moreover, this principle may also apply to other saturable hepatocyte detoxifying processes, such as phase I and phase II metabolism, as long as the substrate compounds involved are transported by ABCC3 and OATP1B proteins. Additional sinusoidal efflux and uptake transporters (e.g., ABCC4, OATP2B1, NTCP) will further widen the scope of compounds affected by this hepatocyte hopping process. Results obtained with the *Slco1a/1b;Abcc3*-knockout mice indeed show that in addition to Abcc3 there must be other sinusoidal efflux processes for bilirubin glucuronides. Preventing accumulation of drug glucuronides may be particularly important, since protein adduction by acyl-glucuronides is a well-established cause of drug (hepato)toxicity (32).

One should exercise caution when extrapolating mouse data to humans, and the individual Oatp1a/1b proteins are not straightforward orthologs of human OATP1B1 and OATP1B3. However, there is a strong analogy between the bilirubin phenotypes of Oatp1a/1b-knockout mice and human Rotor subjects. Moreover, the hepatic transgenic expression of human

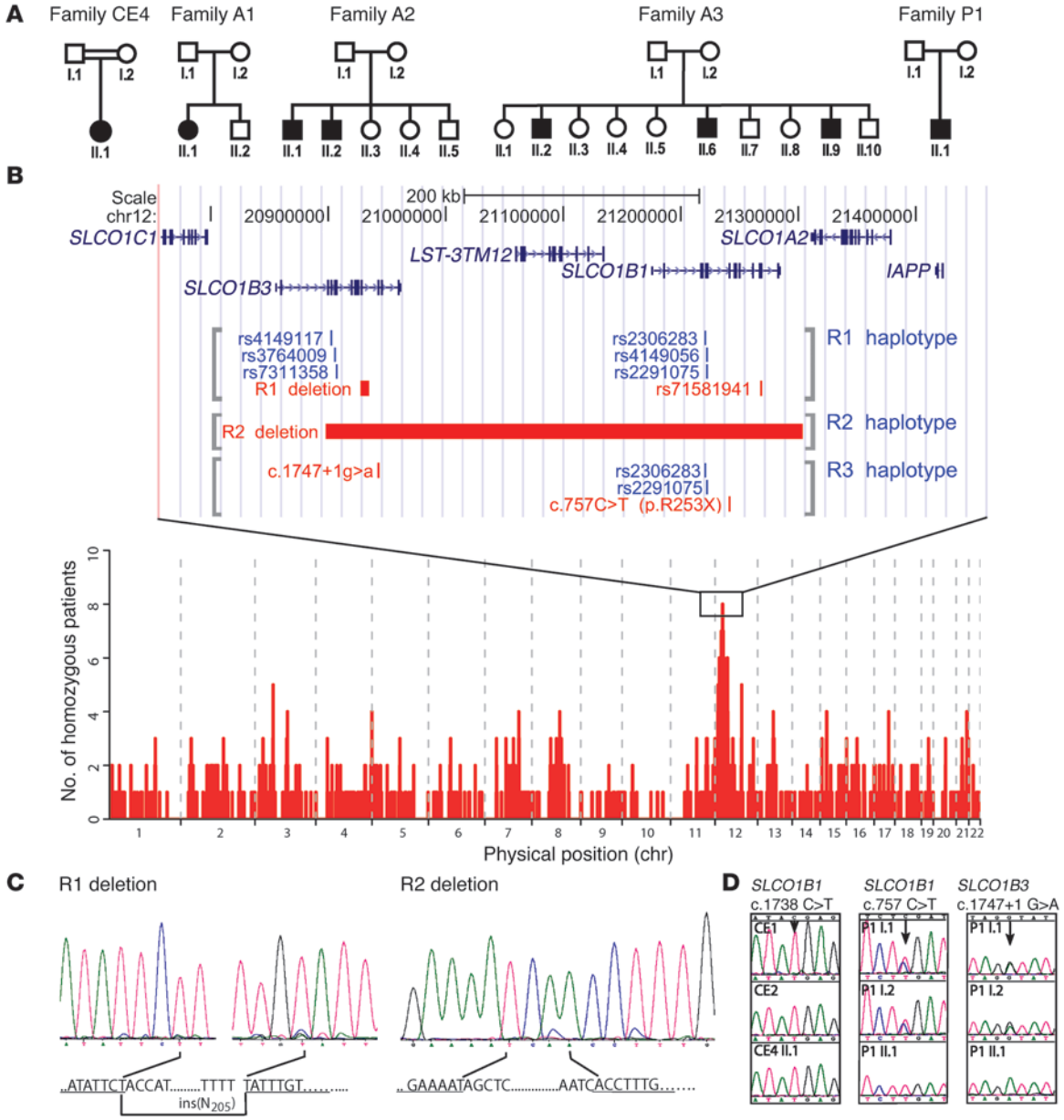

Figure 4

RS families display deficiencies in *SLCO1B1* and *SLCO1B3*. (**A**) Pedigrees of the investigated families. Black symbols denote RS index subjects. Parents in family CE4 had a documented common ancestor. Families CE1–CE3 (only single individuals analyzed) are not shown. (**B**) Homozygosity regions in 8 RS index subjects and overview of detected mutations and polymorphisms. The genome map shows number and location of overlapping homozygosity regions in RS index subjects, gene content of the top candidate region on chromosome 12, and the genotypes forming all 3 identified RS haplotypes. Mutations crucial for RS are shown in red. chr, chromosome. (**C**) Sequences and electropherograms of the R1 and R2 deletion breakpoints. (**D**) Pathogenic point mutations in R1 and R3 haplotypes. Electropherograms indicate the c.1738C→T (p.R580X) mutation in *SLCO1B1* in probands CE1, CE2, and CE4 II.1 and the c.757C→T (p.R253X) and c.1747+1G→A mutations in *SLCO1B1* and *SLCO1B3*, respectively, in family P1.

OATP1B1 or OATP1B3 resulted in virtually complete rescue of the Oatp1a/1b-knockout phenotype for bilirubin handling (Figure 3). This strongly supports that the principles governing bilirubin handling by Oatp1a/1b in mouse liver also apply to OATP1B1 and OATP1B3 in human liver.

Analogous to the mouse data for Oatp1a/1b (25), the extensive glucuronidation of bilirubin in Rotor subjects suggests that OATP1B1 and/or OATP1B3 are not strictly essential for uptake of UCB into the liver. Passive transmembrane diffusion is one likely candidate to take over this process, in hepatocytes and probably many other cell types as well (e.g., ref. 33), but we do not exclude that additional uptake transporters (perhaps OATP2B1) can also contribute to UCB uptake. However, OATP1B1 and/or OATP1B3 probably do contribute to hepatic UCB uptake, since in RS subjects a significant increase in plasma UCB is usually observed and reduced clearance of UCB has been reported (34, 35). Moreover, polymorphisms in *SLCO1B1*

Table 1
Mutations in *SLCO1B* genes detected in RS subjects and their family members

		Haplotype R1-linked mutations		Haplotype R2-linked mutations		Haplotype R3-linked mutations	
Subject	**Family status**	***SLCO1B3* 7.2-kb deletion**	***SLCO1B1* c.1738C→T (p.R580X) rs71581941**	***SLCO1B* locus 405-kb deletion**	***SLCO1B1* c.481+1G→T splice site mutation**	***SLCO1B3* c.1747+1G→A splice site mutation**	***SLCO1B1* c.757C→T (p.R253X)**
CE1	**Proband**	del/del	T/T				
CE2	**Proband**	del/del	T/T				
CE4 I.1	Father	del/WT	T/C				
CE4 I.2	Mother	del/WT	T/C				
CE4 II.1	**Proband**	del/del	T/T				
CE3	**Proband**			del/del	–/–		
A1 I.1	Father			del/WT	–/G		
A1 I.2	Mother			del/WT	–/G		
A1 II.1	**Proband**			del/del	–/–		
A1 II.2	Brother			WT/WT	G/G		
A2 I.1	Father			del/WT	–/G		
A2 I.2	Mother			del/WT	–/T		
A2 II.1	**Brother**			del/del	–/–		
A2 II.2	**Proband**			del/del	–/–		
A2 II.3	Sister			del/WT	–/T		
A2 II.4	Sister			del/WT	–/G		
A2 II.5	Brother			WT/WT	G/T		
A3 I.1	Father			del/WT	–/G		
A3 I.2	Mother			del/WT	–/G		
A3 II.1	Sister			WT/WT	G/G		
A3 II.2	**Proband**			del/del	–/–		
A3 II.3	Sister			del/WT	–/G		
A3 II.4	Sister			del/WT	–/G		
A3 II.5	Sister			del/WT	–/G		
A3 II.6	**Brother**			del/del	–/–		
A3 II.7	Brother			del/WT	–/G		
A3 II.8	Sister			del/WT	–/G		
A3 II.9	**Brother**			del/del	–/–		
A3 II.10	Brother			WT/WT	G/G		
P1 I.1	Father					G/A	C/T
P1 I.2	Mother					G/A	C/T
P1 II.1	**Proband**					A/A	T/T

Boldface indicates index subjects with RS (n = 11; 8 probands, 3 affected siblings); 405-kb deletion (assembly NCBI36/hg18) — g.(20898911)_(21303509)del(CA)ins; 7.2-kb deletion (assembly NCBI36/hg18) — g.(20927077)_(20934292)del(N205)ins. WT, wild-type sequence, i.e., sequence from which all exons of *SLCO1B1* and *SLCO1B3* could be amplified. Genotypes for all empty entries were wild-type in sequence and/or heterozygous or homozygous for the large haplotype R2–linked deletion as predicted.

and *SLCO1B3* have been associated with increased serum UCB levels (36, 37). There was also a significant, nearly 2-fold increase in plasma UCB in the *Slco1a/1b*$^{-/-}$ mice, and this was partially reversed by both human OATP1B1 and OATP1B3 expression (Figure 3C).

It should be noted that UGT1A1-mediated glucuronidation may also occur in extrahepatic tissues, for instance, colon (38), and we cannot exclude that some of the bilirubin glucuronide observed in RS plasma has resulted from such extrahepatic glucuronidation, possibly enhanced by the increased plasma UCB levels. It seems unlikely, however, that all bilirubin glucuronide in RS subjects would derive from extrahepatic glucuronidation. This would require a complete block of hepatic UCB uptake (due to the OATP1B1 and OATP1B3 deficiency), but at the same time require efficient uptake of UCB into UGT1A1-containing extrahepatic cells (e.g., colonocytes) that do not normally express OATP1B1 and OATP1B3, and certainly not in Rotor subjects. If UCB transmembrane diffusion can do this efficiently, it is hard to see why this would not mediate substantial uptake into the liver as well. Only if hepatic diffusion uptake is negligible (which seems physically unlikely) and an unknown efficient UCB uptake system would function in colonocytes (and not in liver), could one envisage such a situation. On balance, this seems rather implausible.

Elucidation of OATP1B1 and OATP1B3 deficiency as the cause of RS can also readily explain the other diagnostic traits of the disorder. Absence of OATP1B1/3-mediated liver uptake would cause the decreased plasma clearance of anionic diagnostic dyes such as indocyanine green and BSP, an excellent substrate of OATP1B1 and OATP1B3 (15), and the greatly reduced or delayed visualization of the liver by anionic cholescintigraphic radiotracers such as ^{99m}Tc-HIDA and ^{99m}Tc-mebrofenin (5, 6). ^{99m}Tc-mebrofenin, for instance, is efficiently transported by both OATP1B1 and OATP1B3 (39).

The markedly increased urinary excretion of coproporphyrins, and the increased preponderance of isomer I over III in urine of RS subjects, could be simply explained by reduced (re)uptake of

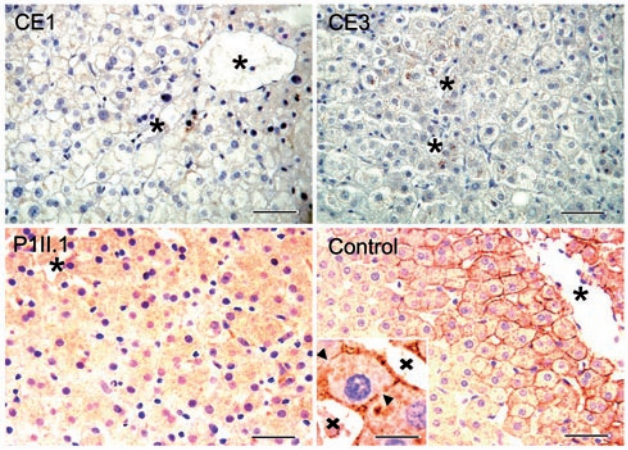

Figure 5
Liver expression of OATP1B proteins in RS subjects and control. With an anti-OATP1B1/3 antibody, basolateral membrane immunostaining of hepatocytes in centrilobular areas was intense in control. Asterisks indicate central veins, arrowheads bile canaliculi, and crosses sinusoids. OATP1B proteins were not detectable in RS subjects CE1 (haplotype R1), CE3 (haplotype R2), and P1 II.1 (haplotype R3). Scale bars: 25 μm (original magnification of CE1 and CE3, ×400; original magnification of P1 II.1 and control, ×200); inset: 5 μm (original magnification, ×1,000).

these compounds into the liver, partly shifting the excretion route from hepatobiliary/fecal to urinary, especially for isomer I. Coproporphyrin I and III thus most likely are transported substrates of OATP1B1 and OATP1B3. Indeed, interaction of several porphyrins with OATP1B1 has recently been demonstrated (40).

Phenotypic abnormalities in RS subjects are surprisingly moderate. Perhaps OATP1B1 and OATP1B3 functions are partly taken over by other sinusoidal uptake transporters, such as OATP2B1. Nevertheless, since even reduced-activity OATP1B1 polymorphisms can result in life-threatening drug toxicities (20–24, 41, 42), such risks are likely increased substantially in RS subjects. Their evident jaundice, however, may have been a warning sign for physicians to prescribe drugs with caution.

The obligatory deficiency in two different, medium-sized genes explains the rarity of RS, with a roughly estimated frequency of about 1 in 10^6, although it might be several-fold lower or higher in different populations. Complete deficiency of either OATP1B1 or OATP1B3 alone will occur much more frequently but will not cause jaundice. For instance, the p.R580X mutation in OATP1B1 occurred at an allele frequency of 0.008 (3 of 354) in a Japanese population (43), suggesting that about 1 in 14,000 individuals in this population would be homozygous for this full-deficiency mutant. Such individuals might demonstrate idiosyncratic hypersensitivity to OATP1B1 substrate drugs, including statins or irinotecan. Similarly, in the present study we identified a non-jaundiced individual homozygously deficient for *SLCO1B3* in our CNV screening of approximately 2,300 individuals, in line with a non-negligible incidence of fully OATP1B3-deficient individuals.

Some drugs, such as high-dose cyclosporine A, can transiently increase plasma levels of conjugated bilirubin without evoking other markers for liver damage (44, 45). Until now, such increases were thought to be primarily mediated by inhibition of ABCC2 as the main biliary excretion factor for bilirubin glucuronide. However, given the insights from the present study, direct inhibition of OATP1B1 and/or OATP1B3 by the applied drug may be an additional or even the main cause of such drug-induced conjugated hyperbilirubinemias. This might for instance apply to cyclosporine A, rifampin, rifamycin SV, or other drugs that are established inhibitors of OATP1B proteins (23). Moreover, heterozygous carriers of the various full-deficiency mutations in OATP1B1/3 might be more susceptible to such inhibitory effects. This also applies to drug-drug interactions mediated through OATP1B1/3 inhibition.

The molecular mechanism we identified in RS may also underlie a similar disorder called hepatic uptake and storage syndrome, or conjugated hyperbilirubinemia type III (OMIM %237550) (46). This hypothesis can now be tested by mutational analysis of OATP1B1 and OATP1B3 in the only reported family to date. Furthermore, a mutant strain of Southdown sheep has also been described as displaying a similar hepatic uptake and storage syndrome (46), and it would not surprise us if these animals would likewise have a deficiency of one or more hepatic sinusoidal OATPs. The observation that mutant Southdown sheep, like the *Slco1a/1b*$^{-/-}$ mice (25), also display strongly reduced clearance of (unconjugated) cholic acid, but not of (conjugated) taurocholic acid (47), further supports this idea.

Collectively, our findings explain the genetic and molecular basis of RS. The demonstration of an Abcc3-, OATP1B1-, and OATP1B3-driven detoxification-enhancing liver-blood shuttling loop in mice and, by implication, most likely also in humans challenges the view of one-way excretion from blood through liver to bile of bilirubin and drugs detoxified by conjugation. Furthermore, the identified full-deficiency alleles of *SLCO1B1* and *SLCO1B3* may contribute to various "idiosyncratic" drug hypersensitivities.

Methods

Mouse strains and conditions. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. *Slco1a/1b*$^{-/-}$, *Abcc2*$^{-/-}$, *Abcc3*$^{-/-}$, and *Abcc2*$^{-/-}$*Abcc3*$^{-/-}$ mice have been described (25–27, 48). Human OATP1B1 transgenic mice have been described (29), and human OATP1B3 transgenic mice were generated in an analogous manner, using an apoE promoter to obtain liver-specific expression of the transgene. Each transgene was crossed back into an *Slco1a/1b*$^{-/-}$ background to obtain the corresponding humanized rescue strains. Routine mouse conditions and analyses of mouse samples are described in Supplemental Methods.

Western blot analysis. Isolation of crude membrane fractions from mouse liver, kidney, and small intestine and Western blotting were as described previously (29). For detection of Abcc2 and Abcc3 primary antibodies, M_2III-5 (dilution 1:1,000) and M_3-18 (dilution 1:25) were used, respectively. For detection of transgenic OATP1B1 and OATP1B3 in mouse liver, the rabbit polyclonal antibodies ESL and SKT, provided by D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany) were used (17, 18).

RNA isolation, cDNA synthesis, and RT-PCR. RNA isolation from mouse liver, kidney, and small intestine and subsequent cDNA synthesis and RT-PCR were as described previously (49). Specific primers (QIAGEN) were used to detect expression levels of *Slco1a1*, *Slco1a4*, *Slco1a6*, *Slco1b2*, *Slco2b1*, *Slc10a1*, *Slc10a2*, *Abcc2–4*, *Abcb1a*, *Abcb1b*, *Abcb11*, *Abcg2*, *Osta*, *Ostb*, and *Ugt1a1*.

Analysis of bilirubin in mouse plasma, bile, and urine. Gallbladder cannulations and collection of bile and urine in male mice of the various strains (n = 4–7) as well as bilirubin detection were as described (25, 50, 51). For details, see Supplemental Methods.

RS families. We examined 11 RS index subjects (8 probands, 3 siblings of probands) of 8 families and 21 clinically healthy members of 5 of these 8 families. Family members of 3 probands (CE1–CE3) were not available.

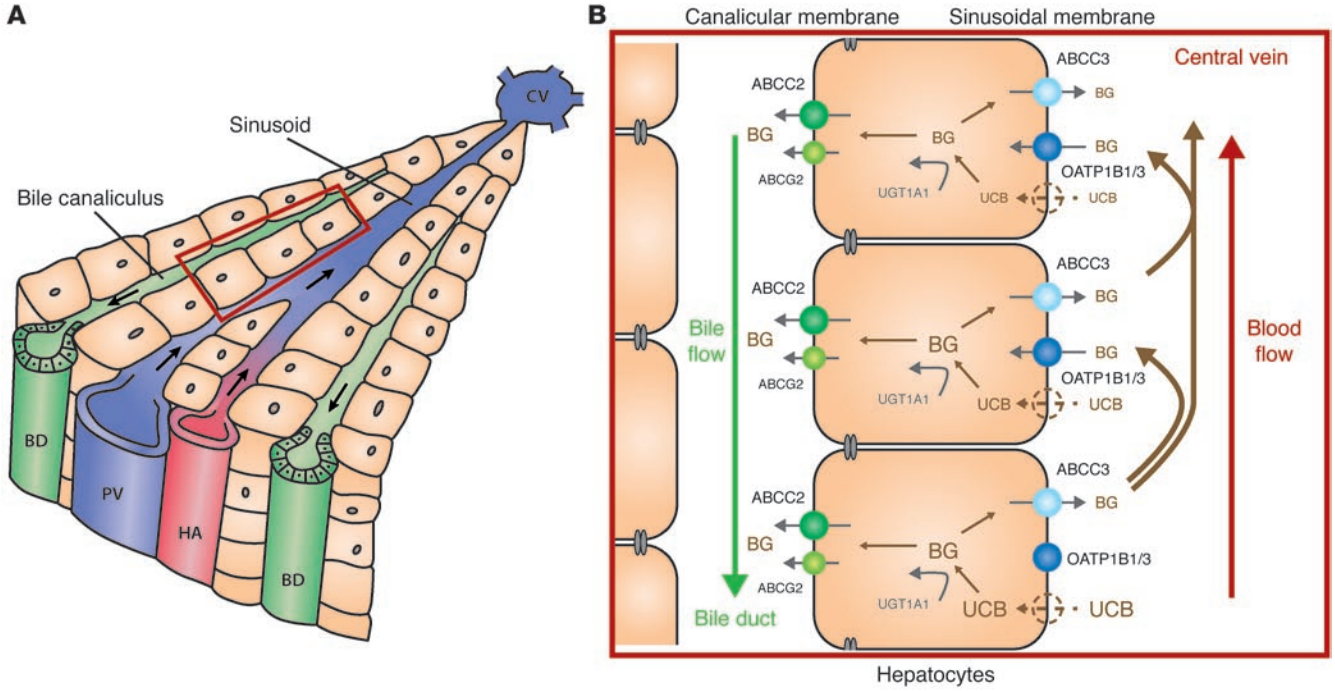

Figure 6

Hepatocyte hopping distributes the biliary excretion load of bilirubin glucuronides across the liver lobule. (**A**) Schematic of liver lobule. Hepatocytes are organized around portal tracts, with branches of the portal vein (PV), hepatic artery (HA), and bile ducts (BD). The PV and HA deliver nutrient- and oxygen-rich blood, respectively, which flows through the sinusoids toward the central vein (CV). Basolateral (sinusoidal) membranes of hepatocytes are flushed with perisinusoidal plasma. Bile flows in the opposite direction toward bile ducts through canaliculi lined by canalicular membranes of hepatocytes. (**B**) Hepatocyte hopping cycle. UCB enters the hepatocytes via passive diffusion and/or transporters, which may include OATP1B1 and/or OATP1B3 in non-Rotor subjects. Conjugation with glucuronic acid by UGT1A1 to bilirubin glucuronides (BG) takes place in endoplasmic reticulum. BG is secreted into bile mainly by ABCC2. ABCG2 also can contribute to this process. Even under physiological conditions, a substantial fraction of the intracellular BG is rerouted by ABCC3 to the blood, from which it can be taken up by downstream hepatocytes via OATP1B1/3 transporters. This flexible off-loading of BG to downstream hepatocytes prevents saturation of biliary excretion capacity in upstream hepatocytes. Relative type sizes of UCB and BG represent local concentrations. Schematic modified, with permission, from ref. 54.

Families CE1–CE4 are of mixed Central European descent by family report. Three families (A1–A3) are Saudi Arabs, and one family (P1) is from the Philippines. Central European families were ascertained at the Institute for Clinical and Experimental Medicine, Prague, and Saudi Arab and Filipino families at the Saudi Aramco Dhahran Health Center. Medical histories were obtained by referring consultants. Subjects CE1 and CE2 were reported as case 1 and case 2, respectively (14).

ABCC2 mutation screening. *ABCC2* mutation screening was performed in 8 probands representing all studied families as described previously (14).

Genotyping. Genotyping was performed using Affymetrix GeneChip Mapping 6.0 Arrays (Affymetrix) according to the manufacturer's protocol. Raw feature intensities were extracted from Affymetrix GeneChip Scanner 3000 7G images using GeneChip Control Console Software 2.01. Individual SNP calls were generated using Affymetrix Genotyping Console Software 3.02. Details of the experiment and individual genotyping data are available at the GEO repository (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE33733.

Multipoint nonparametric and parametric linkage analysis. Multipoint nonparametric and parametric linkage analysis along with determination of the most likely haplotypes was performed with version 1.1.2 of Merlin software (52). Parametric linkage was carried out assuming an autosomal recessive mode of inheritance with a 1.00 constant, age-independent penetrance, 0.00 phenocopy rate, and 0.0001 frequency of disease allele. Results were visualized in version 1.032 of HaploPainter software (53) and in version 2.9.2 of R-project statistical software (http://www.r-project.org/).

Homozygosity mapping. Extended homozygosity regions were identified in Affymetrix Genotyping Console Software version 3.02 using the algorithm comparing values from the user's sample set and SNP-specific distributions derived from a reference set of 200 ethnically diverse individuals. Distribution of extended homozygosity regions in affected and healthy individuals was analyzed and visualized using custom R-script.

Copy number changes. Copy number changes were identified in Affymetrix Genotyping Console Software version 3.02. Data from both SNP and copy number probes were used to identify copy number aberrations compared with built-in reference. Only regions larger than 10 kb containing at least 5 probes were reported.

Quantitative PCR. Quantitative PCR was carried out in duplicate on a LightCycler 480 System (Roche Applied Science). Data were analyzed by LightCycler 480 Software, release 1.5.0. Absolute quantification was used to determine copy number status of a given fragment in analyzed samples. Genomic positions of the analyzed fragments and control genes, corresponding primer sequences, and Universal ProbeLibrary probes used for amplification and quantitation are provided in Supplemental Table 4.

Mutation analysis. Long-range PCR products encompassing the genomic regions of deletion breakpoint boundaries were gel-purified and sequenced using a primer walking approach. DNA sequencing of PCR products and

genomic fragments covering 1 kb of the promoter regions and all of the exons, with their corresponding exon-intron boundaries, of *SLCO1B1*, *SLCO1B3*, and *SLCO1A2* was performed. For details, see Supplemental Methods. Confirmation and segregation of both identified copy number changes and missense mutations in the families, as well as frequency of the mutations in a control population of mixed European descent, were assessed by PCR, PCR-RFLP, and direct sequencing of corresponding genomic DNA fragments. For primer sequences, see Supplemental Table 4.

Histology and immunohistochemistry. Archival liver biopsy specimens were available from 5 unrelated RS index subjects (probands, families CE1, CE2, CE3, and P1; brother [A3 II.9] of proband from family A3). Sections of paraffin-embedded material (formalin or Carnoy solution fixative; 4–6 μm thick) were routinely stained with hematoxylin and eosin and periodic acid–Schiff techniques. For OATP1B1, OATP1B3, and ABCC2 immunostaining, routine techniques were applied (see Supplemental Methods). OATP1B1 and OATP1B3 detection was performed with a primary mouse anti-OATP1B antibody (clone mMDQ, GeneTex; recognizing the N terminus of both OATP1B1 and OATP1B3), 1:100 dilution, overnight at 4°C (31).

Statistics. One-way ANOVA followed by Tukey's multiple comparison test was used to assess statistical significance of differences between data sets. Results are presented as mean ± SD. Differences were considered statistically significant when P was less than 0.05.

Study approval. All mouse studies were ethically reviewed and carried out in accordance with European directive 86/609/EEC and Dutch legislation and the GlaxoSmithKline policy on the Care, Welfare and Treatment of Laboratory Animals. Experiments were approved by the Animal Experimentation Committee (DEC) of the Netherlands Cancer Institute. Investigations involving humans were approved by the Institutional Review Boards of the Institute for Clinical and Experimental Medicine, Prague, Czech Republic, and the Saudi Aramco Dhahran Health Centre, with written informed consent received from participants or their guardians, and conducted according to Declaration of Helsinki principles.

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SUPPLEMENTAL MATERIAL FOR:

Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver

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Containing:

SUPPLEMENTAL RESULTS

Search for mutations associated with Rotor syndrome (RS) in the general population

To assess the general population frequency of RS-causing mutations, we searched the Database of Genomic Variants for genomic copy number variations (CNVs) within the *SLCO1B3* and *SLCO1B1* locus in healthy control samples. This yielded two deletions. Variation_49020, encompassing practically the whole *SLCO1B3* and *SLCO1B1* locus (chr12:20,901,315-21,296,099), was almost identical to the 405 kb deletion present in the R2 haplotype. It was found in 2 of 2026 investigated individuals, as was Variation_49025, which encompasses the first 2 exons of *SLCO1B1* (chr12:21,163,569-21,196,565), including the first coding exon (reference S1). A parallel search for CNVs in the raw data and genotypes generated on HapMap Phase III samples using Affymetrix SNP 6.0 arrays in 1115 individuals from 11 populations, as well as in another 200 DNA samples previously genotyped in our laboratory using the same technology, yielded 2 deletions and a single amplification: A deletion of chr12:20,909,063-20,921,469 encompassing exon 9 of *SLCO1B3*; a deletion of chr12:20,920,041-20,928,922 encompassing exons 9 and 10 of *SLCO1B3*; and an amplification of chr12:20,840,001-21,534,490 encompassing the whole *SLCO1B3* and *SLCO1B1* locus. In these data we also identified 12 individuals homozygous for extended stretches of the R1 haplotype block and 6 individuals homozygous for extended stretches of the R3 haplotype block. Genotyping of genomic DNA of these individuals obtained from Coriell Cell Repositories, did not find the chr12:g.(20,927,077)_(20,934,292)del(N_{205})ins mutation in the R1-haplotype samples, nor the c.1747+1G>A *SLCO1B3* mutation or the c.757C>T (p.R253X) *SLCO1B1* mutation in the R3-haplotype samples, suggesting that these mutations are relatively recent events.

We further genotyped 1,004 control samples of Central European ancestry to assess population frequencies of each of the RS deletions. One of the samples contained the heterozygous 405 kb deletion found in the R2 haplotype. Interestingly, the single identified individual having the R1-haplotype-associated c.1738C>T (p.R580X) mutation in *SLCO1B1* (in heterozygous state), was also homozygous for the R1 haplotype-associated deletion in *SLCO1B3*.

Expression of uptake and efflux transporters in *Slco1a/1b;Abcc2*$^{-/-}$, *Slco1a/1b;Abcc3*$^{-/-}$, and *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice

Quantitative RT-PCR analysis was performed for a range of functionally relevant uptake and efflux transporters in liver, kidney, and intestine of the single and combination knockout strains, as well as UDP-glycuronosyltransferase 1a1 (*Ugt1a1*) in liver (Supplemental Table 1). In the liver, expression of *Abcc2* was somewhat downregulated in *Slco1a/1b*$^{-/-}$ and *Slco1a/1b;Abcc3*$^{-/-}$ mice (1.6- and 1.8-fold, respectively) and expression of *Abcc3* was somewhat downregulated in *Slco1a/1b*$^{-/-}$ mice (1.8-fold). However, these changes in expression were not noticeable on Western blot (data not shown). Hepatic *Abcc3* mRNA was slightly (1.8-fold) upregulated in *Abcc2*$^{-/-}$ mice but not significantly different from wild-type in *Slco1a/1b;Abcc2*$^{-/-}$ mice. *Ugt1a1* expression was not significantly altered in any of the strains.

SUPPLEMENTAL METHODS

Mutation analysis. Long-range PCR encompassing the genomic regions of the deletion breakpoint boundaries was performed using an Expand Long Range dNTPack (Roche Applied Science). Resulting PCR products were gel-purified and sequenced using a primer walking approach. DNA sequencing of PCR products and genomic fragments covering 1 kb of the promoter regions and all of the exons, with their corresponding exon-intron boundaries, of *SLCO1B1*, *SLCO1B3*, and *SLCO1A2* was performed using a version 3.1 Dye Terminator cycle sequencing kit (Applied Biosystems) and electrophoresis on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems). Data were analyzed using SeqScape software.

Histology and immunohistochemistry. Archival liver-biopsy specimens were available from 5 unrelated RS index subjects (probands, Families CE1, CE2, CE3, and P1; brother [A3 II.9] of proband from Family A3). Sections of paraffin-embedded material (formalin or Carnoy-solution fixative; 4-6 μm thick) were routinely stained with hematoxylin/eosin and periodic acid – Schiff techniques. For OATP1B1 and OATP1B3 immunostaining, similar sections, mounted on SuperFrost Plus slides (Dako) were routinely deparaffinized, rinsed in distilled water, and treated in 10 mM sodium citrate buffer, pH 6.0, for 30 min at 96°C. Endogenous peroxidase activity was blocked by 10 min incubation with 1% H_2O_2. After rinsing in distilled water the sections were incubated with primary mouse anti-OATP1B antibody (clone MDQ; recognizing the N-terminus of both OATP1B1 and OATP1B3), 1:100 dilution, overnight at 4°C (31). Bound antibody was visualized with horseradish peroxidase/diaminobenzidine (EnVision), with hematoxylin counterstaining. Adult human livers without cholestasis served as positive controls; for negative

controls, the primary antibody was replaced by buffer. Immunostaining for ABCC2, using primary mouse anti-ABCC2 antibody (clone M2III-6), was performed as described (14).

Mouse strains and conditions. All mice were of identical genetic background (>99% FVB) and between 9 and 14 weeks of age. Mice were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle. Mice received a standard diet (AM-II; Hope Farms) and acidified water *ad libitum*.

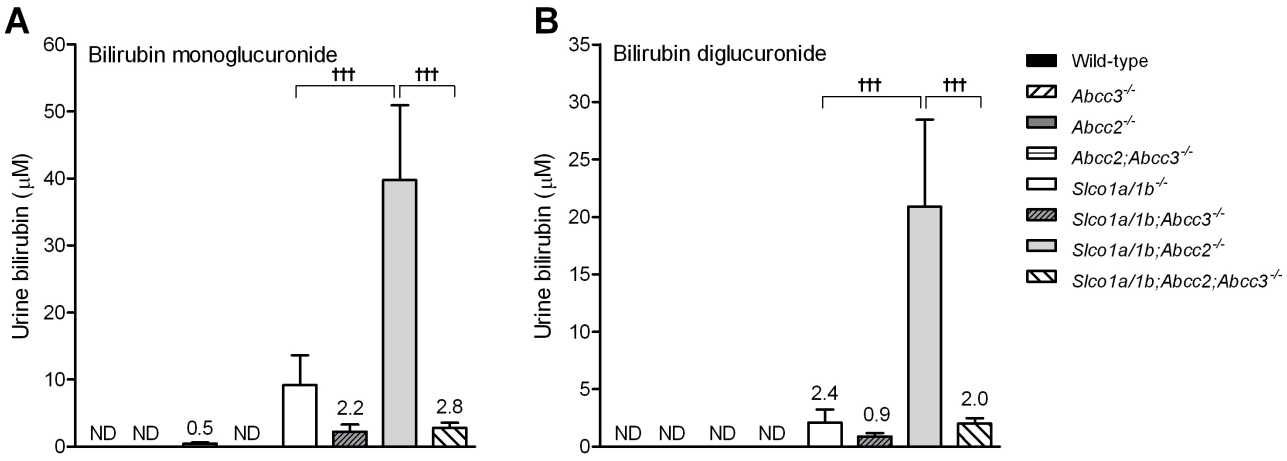
Clinical-chemical analysis of mouse plasma. Blood samples were isolated by cardiac puncture from isoflurane-anesthetized mice. Standard clinical-chemical analyses on EDTA plasma were performed on a Roche Hitachi 917 analyzer to determine levels of total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransaminase, alanine aminotransaminase, γ-glutamyl transferase, lactate dehydrogenase, creatinine, urea, Na^+, K^+, Ca^{2+}, total protein, albumin, uric acid, cholesterol, and triglyceride.

Analysis of bilirubin in mouse plasma, bile and urine. Gallbladder cannulations and collection of bile in male wild-type, *Abcc2*$^{-/-}$, *Abcc3*$^{-/-}$, *Abcc2;Abcc3*$^{-/-}$, *Slco1a/1b*$^{-/-}$, *Slco1a/1b;Abcc2*$^{-/-}$, *Slco1a/1b;Abcc3*$^{-/-}$, and *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice (n = 4-7) were performed as described (25, 50, 51). Bile collected in the first 15 min after gall bladder cannulation was analyzed for bilirubin concentrations. We also collected urine (spot-collection beforehand) and heparin plasma (cardiac puncture afterwards) from these mice. For the detection of bilirubin in *Slco1a/1b*$^{-/-}$*;1B1*^{tg} and *Slco1a/1b*$^{-/-}$*;1B3*^{tg} mice we isolated heparin plasma by cardiac puncture and urine by spot-collection. Ascorbate (100 mg/ml) was added to all plasma (10 µl), urine (10

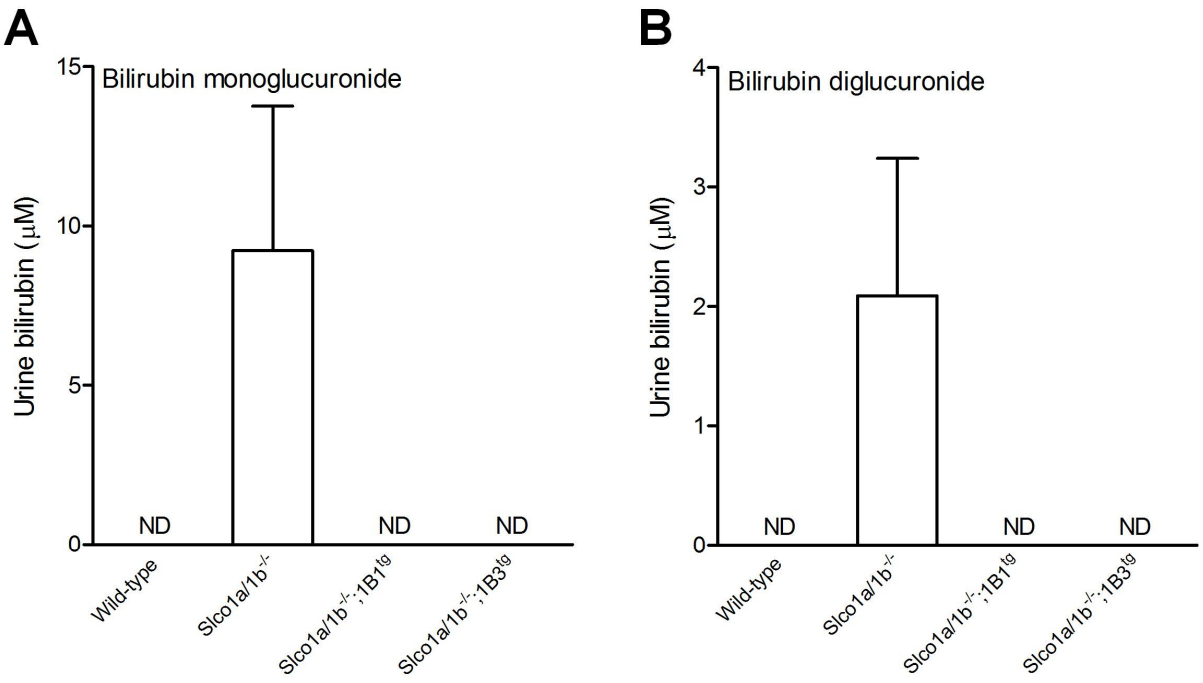
µl), and bile samples (2 µl) in order to prevent oxidation of bilirubin. All samples were immediately protected from the light, snap-frozen, and stored at -80°C until further analysis. Concentrations of bilirubin monoglucuronides (BMG), bilirubin diglucuronide (BDG), and unconjugated bilirubin (UCB) in plasma, bile, and urine were determined as described (51).

SUPPLEMENTAL REFERENCE

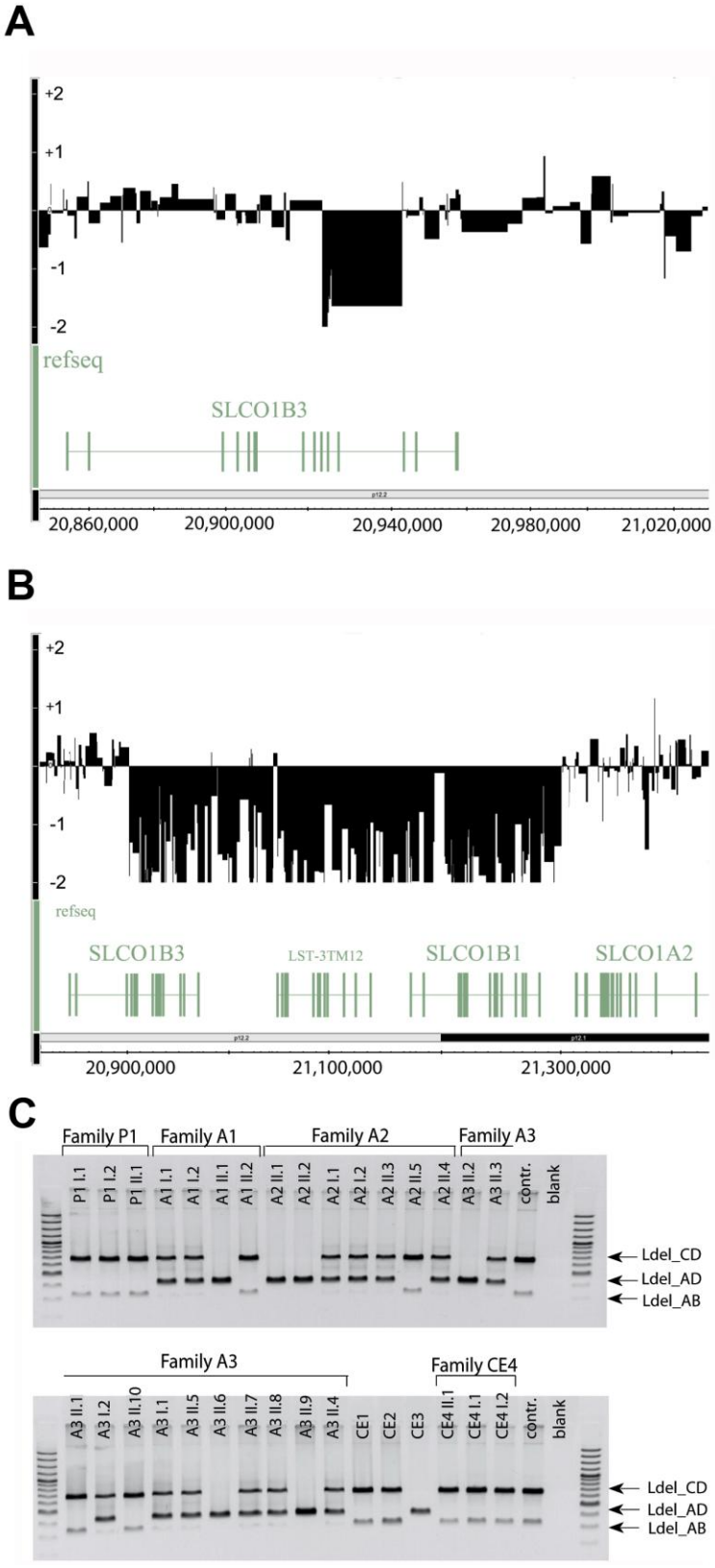
S1. Shaikh TH, et al. High-resolution mapping and analysis of copy number variations in the human genome: a data resource for clinical and research applications. *Genome Res.* 2009;19(9):1682-1690.



Supplemental Figure 1. Urinary bilirubin glucuronide levels in *Slco1a/1b*$^{-/-}$, *Slco1a/1b;Abcc3*$^{-/-}$, *Slco1a/1b;Abcc2*$^{-/-}$ and *Slco1a/1b;Abcc2;Abcc3*$^{-/-}$ mice. A, Bilirubin monoglucuronide and **B**, bilirubin diglucuronide concentrations in urine of male wild-type, *Abcc3*$^{-/-}$, *Abcc2*$^{-/-}$, *Abcc2;Abcc3*$^{-/-}$, *Slco1a/1b*$^{-/-}$, *Slco1a/1b;Abcc3*$^{-/-}$, *Slco1a/1b;Abcc2*$^{-/-}$, and *Slco1a/1b;Abcc2; Abcc3*$^{-/-}$ mice. Data are shown as means ± S.D. (n = 4-7). Urine was collected by spot-sampling. Unconjugated bilirubin concentrations were negligible. Bracketed comparisons: $^{\dagger\dagger\dagger}P < 0.001$. ND, not detectable; detection limit was 0.1 μM. For low bars measured values are presented above the bar.



Supplemental Figure 2. Urinary bilirubin glucuronide levels in *Slco1a/1b*$^{-/-}$, *Slco1a/1b*$^{-/-}$*;1B1*^{tg}, and *Slco1a/1b*$^{-/-}$*;1B3*^{tg} mice. **A**, Bilirubin monoglucuronide and **B**, bilirubin diglucuronide concentrations in urine of male wild-type, *Slco1a/1b*$^{-/-}$, *Slco1a/1b*$^{-/-}$*;1B1*^{tg}, and *Slco1a/1b*$^{-/-}$*;1B3*^{tg} mice. Data are shown as means ± S.D. (n = 4-7). Urine was collected by spot-sampling. Unconjugated bilirubin concentrations were negligible. ND, not detectable; detection limit was 0.1 μM. For low bars measured values are presented above the bar.



Supplemental Figure 3. Mapping of deletions in the *SLCO1B* locus (A and B) and PCR-based genotyping of the R2 haplotype-linked deletion in individuals from RS families (C). Log_2 of fluorescence intensity ratios for microarray probes distributed along the RS candidate locus in approximately 10 kb intervals for haplotypes R1 (**A**), and R2 (**B**), respectively. The value 0 indicates the presence of two copies of the genomic sequence complementary to the probe sequence. Regions with a large rectangular drop (**A**) or with multiple irregular rectangular drops (**B**) to the baseline noise value -2 indicate loss of two copies due to homozygous deletions in probands CE1 (R1 haplotype) and CE3 (R2 haplotype), respectively. The discrepancy between the results of the microarray-based low resolution mapping and sequencing-based exact mapping of the deletion breakpoints, especially for *SLCO1B3* in proband CE1 (Figure 4), is explained by long physical distances between the variations genotyped with the microarray probes. **C**, PCR-based genotyping documents segregation of the R2 haplotype-linked deletion in 4 of 8 investigated RS families. Primer pair Ldel_AD amplifies across the R2 deletion, whereas primer pairs Ldel_AB and Ldel_CD amplify from within the R2 deletion area to just outside the R2 deletion area, each covering one of the two deletion junctions. For primer positions see Supplemental Table 4.

Supplemental Table 1. RT-PCR analysis (ΔCt values) of expression of endogenous uptake and efflux transporters in liver, kidney and small intestine of male wild-type and knockout mice.

A. Liver RT-PCR

	Wild-type	*Abcc2*$^{-/-}$	*Abcc3*$^{-/-}$	*Abcc2/3*$^{-/-}$	*Slco1a/1b*$^{-/-}$	*Slco1a/1b; Abcc2*$^{-/-}$	*Slco1a/1b; Abcc3*$^{-/-}$	*Slco1a/1b; Abcc2/3*$^{-/-}$
Abcc2	-3.30 ± 0.23	-	-3.04 ± 0.40	-	-2.64 ± 0.05*	-	-2.43 ± 0.28*	-
Abcc3	1.82 ± 0.14	0.98 ± 0.20*	-	-	2.68 ± 0.06*	2.21 ± 0.63	-	-
Abcc4	8.67 ± 0.35	6.42 ± 0.78***	8.17 ± 0.46	6.47 ± 0.45***	8.78 ± 0.40	9.28 ± 0.90	8.21 ± 0.49	7.98 ± 0.13
Abcg2	0.31 ± 0.26	0.31 ± 0.28	0.22 ± 0.41	0.18 ± 0.28	0.35 ± 0.05	0.68 ± 0.26	0.14 ± 0.39	0.13 ± 0.45
Abcb1a	5.19 ± 0.33	6.51 ± 0.72	6.63 ± 0.90	7.21 ± 0.34*	6.10 ± 0.86	7.25 ± 0.30*	6.96 ± 1.28	7.21 ± 1.28*
Abcb1b	7.39 ± 0.51	7.81 ± 0.77	6.98 ± 1.05	7.50 ± 0.65	7.95 ± 0.05	8.53 ± 0.43	7.74 ± 0.69	8.11 ± 0.43
Abcb11	-2.53 ± 0.90	-2.95 ± 0.21	-2.84 ± 0.16	-2.82 ± 0.12	-2.76 ± 0.30	-2.53 ± 0.39	-2.57 ± 0.03	-3.20 ± 0.16
Slco1a1	-2.10 ± 0.15	-1.66 ± 0.24	-1.66 ± 0.49	-1.55 ± 0.29	-	-	-	-
Slco1a4	-0.30 ± 0.39	1.13 ± 0.32*	0.90 ± 0.65*	2.36 ± 0.45**	-	-	-	-
Slco1b2	-4.76 ± 0.16	-4.33 ± 0.04	-4.38 ± 0.34	-4.30 ± 0.11	-	-	-	-
Slco2b1	-1.08 ± 0.18	-0.84 ± 0.73	-1.04 ± 0.49	-0.33 ± 0.32	-0.85 ± 0.05	-0.57 ± 0.30	-0.60 ± 0.49	-1.00 ± 0.17
Slc10a1	-4.36 ± 0.10	-3.90 ± 0.12	-3.99 ± 0.28	-3.86 ± 0.07	-4.36 ± 0.13	-3.91 ± 0.57	-4.07 ± 0.37	-4.54 ± 0.21
Ugt1a1	2.28 ± 0.16	2.07 ± 0.14	1.81 ± 0.45	2.34 ± 0.23	2.06 ± 1.18	1.74 ± 0.24	1.87 ± 0.22	2.39 ± 0.04

B. Kidney RT-PCR

	Wild-type	*Abcc2*$^{-/-}$	*Abcc3*$^{-/-}$	*Abcc2/3*$^{-/-}$	*Slco1a/1b*$^{-/-}$	*Slco1a/1b; Abcc2*$^{-/-}$	*Slco1a/1b; Abcc3*$^{-/-}$	*Slco1a/1b; Abcc2/3*$^{-/-}$
Abcc2	-1.74 ± 0.33	-	-1.70 ± 0.14	-	-1.48 ± 0.31	-	-1.58 ± 0.06	-
Abcc3	10.1 ± 1.43	10.7 ± 1.55	-	-	10.2 ± 2.69	8.63 ± 0.43	-	-
Abcc4	3.14 ± 0.13	2.18 ± 0.10**	4.13 ± 0.17**	3.12 ± 0.40	3.65 ± 0.21	2.52 ± 0.25*	4.26 ± 0.09***	2.59 ± 0.41
Abcg2	-1.37 ± 0.12	-0.91 ± 0.15	-0.93 ± 0.16	-1.26 ± 0.55	-1.07 ± 0.28	-0.98 ± 0.14	-0.83 ± 0.16	-1.18 ± 0.12
Abcb1a	4.69 ± 0.20	4.93 ± 0.09	5.95 ± 0.37**	5.51 ± 0.52	5.63 ± 0.69	4.83 ± 0.18	5.64 ± 0.36	4.78 ± 0.44
Abcb1b	4.76 ± 0.09	4.83 ± 0.36	4.41 ± 0.18	4.60 ± 0.47	4.20 ± 0.17	4.89 ± 0.45	4.76 ± 0.74	4.33 ± 0.41
Slco1a1	-1.04 ± 0.15	-0.71 ± 0.16	-0.75 ± 0.14	-1.35 ± 0.14	-	-	-	-
Slco1a6	-3.04 ± 0.21	-2.86 ± 0.14	-2.87 ± 0.34	-3.09 ± 0.43	-	-	-	-
Slco2b1	2.68 ± 0.08	2.91 ± 0.46	2.42 ± 0.13	2.55 ± 0.67	2.64 ± 0.11	2.99 ± 0.05	2.82 ± 0.18	2.54 ± 0.29

C. Small intestine RT-PCR

	Wild-type	***Abcc2*$^{-/-}$**	***Abcc3*$^{-/-}$**	***Abcc2/3*$^{-/-}$**	***Slco1a/1b*$^{-/-}$**	***Slco1a/1b; Abcc2*$^{-/-}$**	***Slco1a/1b; Abcc3*$^{-/-}$**	***Slco1a/1b; Abcc2/3*$^{-/-}$**
Abcc2	0.21 ± 0.32	-	0.17 ± 0.75	-	0.55 ± 0.53	-	0.62 ± 0.45	-
Abcc3	4.85 ± 0.88	4.44 ± 0.93	-	-	5.24 ± 0.65	6.14 ± 0.41	-	-
Abcc4	10.2 ± 0.33	10.2 ± 0.51	9.87 ± 0.32	10.2 ± 0.02	10.6 ± 0.62	10.2 ± 1.06	9.64 ± 0.87	9.65 ± 0.80
Abcg2	3.36 ± 1.02	2.52 ± 1.34	3.24 ± 0.67	3.57 ± 0.30	3.11 ± 0.68	4.28 ± 0.82	3.06 ± 0.65	3.25 ± 0.30
Abcb1a	1.04 ± 0.26	2.37 ± 0.57*	1.90 ± 0.94	0.64 ± 0.35	2.32 ± 0.22*	2.29 ± 0.11*	2.09 ± 0.87	1.36 ± 0.06
Abcb1b	9.71 ± 0.60	9.15 ± 1.26	10.7 ± 0.84	9.66 ± 0.31	10.5 ± 0.72	10.7 ± 6.20	9.68 ± 1.69	10.1 ± 0.13
Slco1a4	5.18 ± 1.40	3.90 ± 1.43	3.96 ± 0.76	4.72 ± 0.91	-	-	-	-
Slco2b1	3.82 ± 0.45	4.37 ± 1.19	4.13 ± 0.20	3.99 ± 0.17	4.37 ± 0.19	4.55 ± 0.60	3.64 ± 0.38	3.60 ± 0.32
Slc10a2	11.3 ± 0.80	10.7 ± 0.61	10.3 ± 0.36	11.1 ± 0.22	12.5 ± 1.65	11.8 ± 1.60	11.0 ± 0.90	10.2 ± 0.59
Ostα	1.52 ± 0.29	1.26 ± 0.66	1.59 ± 0.23	1.57 ± 0.58	1.30 ± 0.23	1.53 ± 0.18	1.06 ± 0.55	1 61 ± 0.63
Ostβ	0.82 ± 0.18	0.63 ± 0.40	1.10 ± 0.46	0.75 ± 0.24	0.79 ± 0.22	0.66 ± 0.42	0.36 ± 0.77	0.14 ± 1.01

Analysis of the results was done by comparative Ct method. Quantification of the target cDNAs in all samples was normalized against the endogenous control β-actin ($Ct_{target} - Ct_{\beta\text{-actin}} = \Delta Ct$). Accordingly, the lower the value, the higher the expression level. Data are presented as means ± S.D. (n = 3); each sample was assayed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with wild-type mice. One-way ANOVA followed by Tukey's multiple comparison test was used to determine statistical significance.

Supplemental Table 2. Laboratory findings in RS index subjects.

RS index subject	Total bilirubin (μM)	Direct bilirubin (%)	Urinary porphyrin output	Coproporphyrin I (% of total coproporphyrin)	Liver uptake of anionic tracers*	Liver on light microscopy	Liver on electron microscopy
Reference	<17 μM	<20%	<200 μg/24 h	<40%	high	normal	normal
CE1 (m)	170	70	80 – 500	57	low	normal***	n.d.
CE2 (m)	41 - 121	53 - 72	n.d.	n.d.	low	normal***	n.d.
CE3 (f)	60 - 90	33	n.d.	n.d.	n.d.	normal***	n.d.
CE4 II.1 (f)	45 - 60	50 - 60	124	n.d.	low	n.d.	n.d.
P1 II.1 (m)	97	77	n.d.	62	low	normal***	normal
A1 II.1 (f)	102	57	206	195 μg/24 h**	low	n.d.	n.d.
A2 II.1 (m)	53	68	n.d.	n.d.	n.d.	n.d.	n.d.
A2 II.2 (m)	68	75	<25	21 μg/24 h**	low	n.d.	n.d.
A3 II.2 (m)	49	66	n.d.	n.d.	low	n.d.	n.d.
A3 II.6 (m)	34	60	n.d.	n.d.	n.d.	n.d.	n.d.
A3 II.9 (m)	53	68	n.d.	n.d.	low	normal***	normal

Boldfaced – probands. m, male; f, female; n.d., not done.

*) Bromosulfophthalein (used only in RS subject CE2) or radiotracer.

**) Total coproporphyrin output, isomers were not fractionated.

***) Immunostaining for ABCC2 was unremarkable in all 5 tested RS subjects. Moreover, no predictably pathogenic ABCC2 mutations have been found in any of the 8 probands.

Supplemental Table 3. Single nucleotide variations in exons and splice sites of *SLCO1B* genes detected in RS index subjects, their parents and selected siblings.

Subject (family identifier, generation number, subject number, family status)		***SLCO1B1***								
		rs2306283 c.388A>G (p.N130D) Exon 5	rs11045818 c.411G>A (p.S137S) Exon 5	rs11045819 c.463C>A (p.P155T) Exon 5	**c.481+1G>T** Exon 5	rs4149056 c.521T>C (p.A174V) Exon 6	rs4149057 c.571T>C (p.L191L) Exon 6	rs2291075 c.597C>T (p.F199F) Exon 6	**c.757C>T (p.R253X)** Exon 8	**rs71581941 c.1738C>T (p.R580X)** Exon 13
CE1	**Proband**	GG	GG	CC	GG	CC	TT	TT	CC	**TT**
CE2	**Proband**	GG	GG	CC	GG	CC	TT	TT	CC	**TT**
CE3	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
CE4 I.1	Father	AG	GG	CC	GG	TC	TC	CT	CC	C**T**
CE4 I.2	Mother	AG	GG	CC	GG	TC	TC	CT	CC	C**T**
CE4 II.1	**Proband**	GG	GG	CC	GG	CC	TT	TT	CC	**TT**
P1 I.1	Father	GG	GG	CC	GG	TT	TT	CT	C**T**	CC
P1 I.2	Mother	GG	GG	CC	GG	TT	TT	CT	C**T**	CC
P1 II.1	**Proband**	GG	GG	CC	GG	TT	TT	TT	**TT**	CC
A1 I.1	Father	A-	G-	C-	G-	C-	C-	C-	C-	C-
A1 I.2	Mother	A-	G-	C-	G-	C-	C-	C-	C-	C-
A1 II.1	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A1 II.2	Brother	AA	GG	CC	GG	CC	CC	CC	CC	CC
A2 I.1	Father	A-	G-	C-	G-	T-	C-	C-	C-	C-
A2 I.2	Mother	A-	G-	C-	**T**-	T-	T-	C-	C-	C-
A2 II.1	**Brother**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A2 II.2	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A2 II.3	Sister	A-	G-	C-	**T**-	T-	T-	C-	C-	C-
A2 II.4	Sister	A-	G-	C-	G-	T-	C-	C-	C-	C-
A2 II.5	Brother	AA	GG	CC	G**T**	TT	TC	CC	CC	CC
A3 I.1	Father	G-	A-	A-	G-	T-	C-	T-	C-	C-
A3 I.2	Mother	G-	G-	C-	G-	T-	T-	T-	C-	C-
A3 II.2	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A3 II.6	**Brother**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A3 II.9	**Brother**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A3 II.10	Brother	GG	GA	CA	GG	TT	TC	TT	CC	CC

Subject (family identifier, generation number, subject number, family status)		***SLCO1B3***						
		rs4149117 c.334T>G (p.S112A) Exon 4	rs3764009 c.360-3C>T Exon 5	rs7311358 c.699G>A (p.M233I) Exon 7	rs60140950 c.767G>C (p.G256A) Exon 8	rs2053098 c.1557A>G (p.A519A) Exon 12	**c.1747+1G>A** Exon 13	rs3764006 c.1833A>G (p.G611G) Exon 14
CE1	**Proband**	GG	TT	AA	GG	**na**	GG	AA
CE2	**Proband**	GG	TT	AA	GG	**na**	GG	AA
CE3	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
CE4 I.1	Father	GG	TT	AA	GG	G-	GG	AA
CE4 I.2	Mother	GG	TT	AA	GG	G-	GG	AA
CE4 II.1	**Proband**	GG	TT	AA	GG	**na**	GG	AA
P1 I.1	Father	TG	CT	GA	GG	AG	G**A**	AA
P1 I.2	Mother	TG	CC	GA	GG	AG	G**A**	AA
P1 II.1	**Proband**	TT	CC	GG	GG	AA	**AA**	AA
A1 I.1	Father	G-	T-	A-	C-	G-	G-	AA
A1 I.2	Mother	G-	T-	A-	G-	G-	G-	AA
A1 II.1	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A1 II.2	Brother	GG	TT	AA	GC	GG	GG	AA
A2 I.1	Father	G-	T-	A-	G-	G-	G-	A-
A2 I.2	Mother	T-	C-	G-	G-	A-	G-	A-
A2 II.1	**Brother**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A2 II.2	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A2 II.3	Sister	T-	C-	G-	G-	A-	G-	A-
A2 II.4	Sister	G-	T-	A-	G-	G-	G-	A-
A2 II.5	Brother	TG	CT	GA	GG	GA	GG	AA
A3 I.1	Father	G-	T-	A-	G-	G-	G-	A-
A3 I.2	Mother	G-	T-	A-	G-	G-	G-	G-
A3 II.2	**Proband**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A3 II.6	**Brother**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A3 II.9	**Brother**	**na**	**na**	**na**	**na**	**na**	**na**	**na**
A3 II.10	Brother	GG	TT	AA	GC	GG	GG	AG

Predictably pathogenic mutations and RS index subjects are in **bold**, **na** – no amplification.

Supplemental Table 4. Primers used for PCR amplification, sequencing, copy number analysis and mapping of the found deletions.

Name	Primer sequence 5´- 3´	Genomic position (NCBI36/hg18)	Amplified region
***SLCO1A2*: PCR & sequencing from genomic DNA**			
SLCO1A2_A1	TCAACACCTGGGAGTGGGTGGT	12:21438995-21439016	Exon 1
SLCO1A2_S1	CAGCTGCGTGCTGGGAGACC	12:21439850-21439869	
SLCO1A2_A2	ACTTTCAGTAGGTAGCATGTATGTCAGG	12:21418296-21418323	Exon 2
SLCO1A2_S2	ACAGTCAGTTCTGAGAAGAACACCC	12:21418796-21418820	
SLCO1A2_A3	TCCTGTGTAGACACACCCTCAGT	12:21378607-21378629	Exon 3
SLCO1A2_S3	TGAAAAGCTTTCTTTTAACCATGTGACCA	12:21379178-21379206	
SLCO1A2_A4	CGGTCAGATTAAATGACCTAAAACAGCA	12:21362876-21362903	Exon 4
SLCO1A2_S4	AGGCATTTTGTCAACAGACGGCA	12:21363178-21363200	
SLCO1A2_A5	AGTGAGGCAGCCAAGAGCACCA	12:21358579-21358600	Exon 5
SLCO1A2_S5	TGCAACCCTAGCACAAATCCAAGT	12:21358994-21359017	
SLCO1A2_A6	GCACCCGGCCCTTTGACTCATT	12:21350818-21350839	Exon 6
SLCO1A2_S6	TGCACATTGCCCACATTGTCCTCA	12:21351232-21351255	
SLCO1A2_A7	AGGGTCACCTCCAGGGGCACTA	12:21348381-21348402	Exon 7
SLCO1A2_S7	TGCACTAGGGGTGCCCTGAGAA	12:21348917-21348938	
SLCO1A2_A8	CAAATTGAAGGTCAAGTAAGGCCATGA	12:21345274-21345300	Exon 8
SLCO1A2_S8	ACCTGTGACCCAGCATGAAAGGGA	12:21345696-21345719	
SLCO1A2_A9	TCTTGACTGGTGACTGTTGATGACA	12:21344418-21344442	Exon 9
SLCO1A2_S9	GCCAATTTTATAGTTGGTTGGGACCCG	12:21344854-21344880	
SLCO1A2_A10	ACACCCGCCATCACACTGTTCG	12:21341539-21341560	Exon 10
SLCO1A2_S10	AGCCAGTCACAAAATGCAAAGCCA	12:21341806-21341829	
SLCO1A2_A11	TGATCTGATCGTGCATTGCCATTGT	12:21339713-21339737	Exon 11
SLCO1A2_S11	CCCTGTGGGTAATGTGTAACTAAGTGGGG	12:21340130-21340158	
SLCO1A2_A12	AGGCCCAGTTCATAGTTGGGAAGT	12:21338079-21338102	Exon 12
SLCO1A2_S12	TGGCTATGTGGTCATCAAAGCAGGT	12:21338479-21338503	
SLCO1A2_A13	AGTCCTGAGTGACTTTCTTAGAACAAGC	12:21336285-21336312	Exon 13
SLCO1A2_S13	TCCATCTTGATCCAACTGGCTGTG	12:21336604-21336627	
SLCO1A2_A14	TGCAGCACACCAACATGGCACA	12:21319419-21319440	Exon 14
SLCO1A2_S14	GGGCTCCTGTGTAGGCTGCCA	12:21319783-21319803	
SLCO1A2_A15	TGGTGCTGCGTTATGCACAGTCT	12:21318608-21318630	Exon 15
SLCO1A2_S15	ACTTGAAAAGGCCCTGTCTGACCT	12:21319148-21319171	
SLCO1A2_A16	GGGGGCTGTTATTGATGTCCCTCC	12:21313537-21313560	Exon 16
SLCO1A2_S16	AGATCAGCATGCAAAACCATCAGC	12:21314067-21314090	
***SLCO1B1*: PCR & sequencing from genomic DNA**			
SLCO1B1P1A1	AGCGTGTGGAAGACACAGAGCA	12:21175537-21175558	Exon 1
SLCO1B1P1S1	AGGTGGTTAATCATCACTGGACTTGT	12:21175339-21175364	
SLCO1B1P2A1	TTGCTTTTCTATACATTAAAGTTCC	12:21185902-21185926	Exon 2
SLCO1B1P2S1	ATTGACCTAGCAGAGTGGTAACG	12:21185567-21185589	
SLCO1B1P3A1	TAAATTTACCCCAGTTGATAACC	12:21217061-21217083	Exon 3
SLCO1B1P3S1	CATGTGCCTATTGACATTATATAGTCC	12:21216782-21216808	

SLCO1B1P4A1	ACAAGGTACTGATAGTGGCACAGAG	12:21218935-21218959	Exon 4
SLCO1B1P4S1	ACGCATGAAGGAGCACCTTACCCT	12:21218456-21218479	
SLCO1B1P5A1	TGCAGTTGGCCCTGTTCAATCCA	12:21221369-21221391	Exon 5
SLCO1B1P5S1	TAACCCACTTAGCCTGGGGTGT	12:21220748-21220769	
SLCO1B1P6A1	ACAGAGATCCCAGGGTAAAGCCA	12:21223160-21223182	Exon 6
SLCO1B1P6S1	CAGCATAAGAATGGACTAATACACC	12:21222600-21222624	
SLCO1B1P7A1	TGCTTAGAAAGACGTTATCATGG	12:21223245-21223267	Exon 7
SLCO1B1P7S1	CATGGTGAATAAGAACCATGC	12:21223062-21223082	
SLCO1B1P8A1	AGTGCAAAAGAAAGCCAACTCCA	12:21241485-21241507	Exon 8
SLCO1B1P8S1	ACTTTCTTCATACCATTATTTCCCTGAACC	12:21241066-21241095	
SLCO1B1P9A1	CAAAATCACTTTCACAATAAAATACC	12:21244938-21244963	Exon 9
SLCO1B1P9S1	GCCTGTGGTATTGCAGGCTATTCTC	12:21244563-21244587	
SLCO1B1P10A1	TGATCCATCCAAGATTACAGTGGTGGT	12:21247266-21247292	Exon 10
SLCO1B1P10S1	ACCGGGGACTGTTGAGGGGT	12:21246396-21246415	
SLCO1B1P11A1	TGTGCTTTTGAATAAGGAGAGG	12:21250256-21250277	Exon 11
SLCO1B1P11S1	TCTCTGCTTTCACTTTACTTCTTCC	12:21249974-21249998	
SLCO1B1P12A1	TCATTAGGTGTGTTTATAGTCTCATGC	12:21261536-21261562	Exon 12
SLCO1B1P12S1	AATGTATTTGCAGCACTGTTAGG	12:21261254-21261276	
SLCO1B1P13A1	TTAACAATCGAATTCTCCTTTAGG	12:21266789-21266812	Exon 13
SLCO1B1P13S1	GGAGAAGGTTTAATGTTGTTTCG	12:21266427-21266449	
SLCO1B1P14A1	GAGATACGAGATTGCTTGATACC	12:21269173-21269195	Exon 14
SLCO1B1P14S1	CATGCAGTTACATTTAAAATATGTTCC	12:21268864-21268890	
SLCO1B1P15A1	CAAAGTCAATTTTTCCTAATACATTACC	12:21284053-21284080	Exon 15
SLCO1B1P15S1	TTTTTCTTTAGGATCTGGATACTGG	12:21283087-21283111	
***SLCO1B3*: PCR & sequencing from genomic DNA**			
SLCO1B3P1A1	AGGGCTCAGAACAAAAGTGTGGAGA	12:20855086-20855110	Exon 1
SLCO1B3P1S1	GGCTTCTGGGGTGAACTCCTAGAATTA	12:20854770-20854796	
SLCO1B3P2A1	TGTTCTTTTTGACAGTTAGTGGCCTTTCT	12:20860081-20860109	Exon 2
SLCO1B3P2S1	CCTGTGGTCAGGAAATAGCAGGCCC	12:20859738-20859762	
SLCO1B3P3A1	TGTTTTTCAACTTATGCAAGTATGG	12:20899398-20899422	Exon 3
SLCO1B3P3S1	AAACTGTTTTAGTTCCATGTACC	12:20899146-20899168	
SLCO1B3P4A1	GCAGCAGGTGAAGTTGTGAAGCC	12:20903107-20903129	Exon 4
SLCO1B3P4S1	AGGGAAGGTACAATGTCTTGGGCA	12:20902532-20902555	
SLCO1B3P5A1	TGTGTTGTTTAAGAATCGACTGC	12:20905493-20905515	Exon 5
SLCO1B3P5S1	TCTGGTAATTTGGAGAAGACAGC	12:20905100-20905122	
SLCO1B3P6A1	TGACATTATTATTTCAAGGGTAGATCC	12:20906801-20906827	Exon 6
SLCO1B3P6S1	TGAATATGAATCACTTGTAATTAGG	12:20906482-20906506	
SLCO1B3P7A1	TTCTTTGGAAGAATGGTGTCC	12:20907103-20907123	Exon 7
SLCO1B3P7S1	TGATTACATTCCCTGGATCTACC	12:20906787-20906809	
SLCO1B3P8A1	AGCAGAAACCTAATCCTCTTCCCCT	12:20919862-20919886	Exon 8
SLCO1B3P8S1	GGTTTACTTTCTTCATCTATGGAGGACTGC	12:20919328-20919357	
SLCO1B3P9A1	CAGCAGTGTTTCATTTATCAAGC	12:20922162-20922184	Exon 9
SLCO1B3P9S1	CAATTTGGTTAATTCACATGTTCC	12:20921805-20921828	
SLCO1B3P10A1	TGCACATAATCTTTTAATTTGATGG	12:20923872-20923896	Exon 10
SLCO1B3P10S1	GAAATAAGAATGGGTGAATTTGG	12:20923526-20923548	
SLCO1B3P11A1	TCTGTGATTTTGATTAAGGAGAGG	12:20925247-20925270	Exon 11
SLCO1B3P11S1	TCTCCTTATCCCCTTGTCTCC	12:20924970-20924990	

SLCO1B3P12A1	TGAGCTCAAAATACAGAAAAATATGC	12:20927864-20927889	Exon 12
SLCO1B3P12S1	ATTCATAGCCCTGTTGTATTTGG	12:20927559-20927581	
SLCO1B3P13A1	TCACAAAATAGAAATGATTCTTACC	12:20942809-20942833	Exon 13
SLCO1B3P13S1	GTATTCATTCTACCAGGGAGAGG	12:20942530-20942552	
SLCO1B3P14A1	CAAAGTAATTGTTCACTAAATGGTAGC	12:20945767-20945793	Exon 14
SLCO1B3P14S1	GATTCCTGGGTGGATGTAAGC	12:20945403-20945423	
SLCO1B3P15A1	ATGGGAGGTTGGAAGAAGATCC	12:20960951-20960972	Exon 15
SLCO1B3P15S1	TTCTTTCTTTTAAGATATGCATACTGG	12:20960109-20960135	Exon 15
Copy number analysis by quantitative PCR			
SLCO1B3_1_L	TTAAAGACCCACATAAATGGAAAAA	12:20894106-20894130	*SLCO1B3* intron 1
SLCO1B3_1_R	GCAGAGAAATCTGGGTAGCACT	12:20894176-20894197	
SLCO1B3_2_L	AGTTAGGCATAGTGGTGCACAG	12:20896533-20896554	*SLCO1B3* intron 1
SLCO1B3_2_R	CTCTTGGGCTCTATCAATCCTC	12:20896588-20896609	
SLCO1B3_3_L	AAAAGCAAAATTTCTTATATCCCTGT	12:20930418-20930443	*SLCO1B3* intron 11
SLCO1B3_3_R	TTCTACATATGGCATTGTTGGTAGA	12:20930467-20930491	
SLCO1B3_4_L	TCCTAGGAACAACAGGCACTC	12:20930615-20930635	*SLCO1B3* intron 11
SLCO1B3_4_R	TCATGGGACCTCCTCAGTTT	12:20930677-20930696	
SLCO1B3_5_L	TGTAATTTGGACATGCAAGACA	12:20960410-20960431	*SLCO1B3* exon 14
SLCO1B3_5_R	CATCTTAATGAATCAATGCAATGTTAG	12:20960445-20960471	
SLCO1B1_1_L	TCCATCATTCATATAGAACGGAGAT	12:21216917-21216941	*SLCO1B1* exon 2
SLCO1B1_1_R	CAATTTCAAAGCTTCCGTCAA	12:21216972-21216992	
SLCO1B1_2_L	AGAGACGAGGTAGAGGCAAAAA	12:21221733-21221754	*SLCO1B1* intron 4
SLCO1B1_2_R	GAATCTCCAGAAAGATTTACAAACG	12:21221791-21221815	
SLCO1B1_3_L	TGGATGAAGCAAACTTAGAATCC	12:21283308-21283330	*SLCO1B1* exon 14
SLCO1B1_3_R	TCCCCTTAACAATGTGTTTCACT	12:21283373-21283395	
SLCO1B1_4_L	TTTGCAATCAATGAAAATAAGAAGA	12:21296103-21296127	Intergenic between *SLCO1B3*&*SLCO1B1*
SLCO1B1_4_R	GAGAGAGACTCGGTTAGTGAGACTG	12:21296222-21296246	
SLCO1B1_5_L	CTTCCCCTGTGCCTATGTCT	12:21302281-21302300	Intergenic between *SLCO1B3*&*SLCO1B1*
SLCO1B1_5_R	CCAAAACCATAGAAACCCTCAA	12:21302325-21302346	
SLCO1B1_6_L	TTGTGCAACTGTTCATAGTACTCTCTT	12:21304533-21304559	Intergenic – between *SLCO1B3*&*SLCO1B1*
SLCO1B1_6_R	AGCAGACTCAGATTGCTAAAATCA	12:21304618-21304641	
Fine resolution mapping of the deletions			
LDEL_A_U	TTGCCTCCACAAAGTTCTATT	12:20898799-20898819	Regions surrounding the large R2 deletion
LDEL_D_L	TAGTGCTGAAAGTTTTAGCCA	12:21303755-21303775	
LDEL_B_L	ATTTTTCCCTATACAAGTTGA	12:20899086-20899106	Regions in the deleted sequence
LDEL_C_U	TGTCACCTGCAAGCGAAGATT	12:21303145-21303165	
ROTOR_934.4_L	AATAGCCTGTTCCTGAACAAT	12:20934470-20934490	Regions surrounding the deletion in *SLCO1B3* linked with the R1 haplotype
ROTOR_926.8_U	ACCACGCCTGGCCAATTCTTT	12:20926892-20926912	

Title

Down-regulation of OATP1B proteins correlates with hyperbilirubinemia in advanced cholestasis

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Running title

OATP1Bs are down-regulated in advanced cholestasis

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Abstract:

Aim: Organic anion-transporting polypeptides OATP1B1 and OATP1B3 are sinusoidal membrane transporters mediating liver uptake of a wide range of substrates including conjugated and unconjugated bilirubin, xenobiotics and drugs. Absence of OATP1Bs in the liver causes Rotor syndrome. Our aim was to correlate OATP1B expression with hyperbilirubinemia in common liver diseases.

Methods: Immunoreactivity of five antibodies against human OATP1Bs was tested on frozen and formalin-fixed paraffin-embedded liver tissue of mouse strains transgenic for *SLCO1B1* or *SLCO1B3* and on human specimens. The proportion of hepatocytes expressing OATP1Bs was then assessed immunohistologically in formalin-fixed paraffin-embedded liver samples obtained from patients with hepatocellular and primary biliary liver diseases. *UGT1A1* promoter TATA-box and *SLCO1B1* rs4149056 genotyping was performed to rule out individuals predisposed to hyperbilirubinemia.

Results: The most specific detection of OATP1B3 was achieved with the H-52 (sc-98981) antibody. OATP1B1 was specifically recognized with the ESL (ab15441) anti-OATP1B1 antibody, but only in frozen sections. The MDQ (ab15442) anti-OATP1B1 antibody cross-reacted with both OATP1B proteins in liver tissue of the transgenic mouse strains. Expression of the OATP1B proteins was decreased in advanced liver diseases and inversely correlated with serum bilirubin levels. The reduction was more pronounced in advanced primary biliary diseases (1.9±1.1 vs. 2.7±0.6; p=0.009).

Conclusions: Down-regulation of OATP1B proteins may contribute to pathogenesis of jaundice accompanying advanced cholestatic liver diseases.

Keywords:

Bilirubin, cholestasis, immunohistochemistry, liver disease, organic anion transporter

Introduction

Organic anion-transporting polypeptides (OATPs in humans, Oatps in rodents) are multispecific transporters expressed in numerous epithelial cells throughout the body, transporting predominantly large and hydrophobic organic anions [1, 2]. OATP1B1 (also termed OATP-C, OATP2, SLC21A6 or LST-1, gene *SLCO1B1*) and OATP1B3 (OATP8, SLC21A8 or LST3, gene *SLCO1B3*) are highly homologous proteins with similar genomic organization into 15 exons. Both proteins are glycosylated and have similar secondary structures with 12 predicted transmembrane helices with both termini located intracellularly [3, 4]. OATP1Bs mediate the Na^+-independent uptake of conjugated and unconjugated bilirubin, unconjugated bile salts and many other organic anions in human hepatocytes [2]. Expression of *SLCO1B1* and *SLCO1B3* is restricted to human hepatocytes and the corresponding protein products are localized to the basolateral (sinusoidal) membrane [3-6]. While OATP1B1 is expressed throughout the lobule, OATP1B3 is predominantly localized to the centrilobular zone [3, 7]. Expression of OATP1B proteins is regulated at the transcriptional [2, 8, 9] and/or protein level [2].

Several polymorphisms in OATP1B1 and OATP1B3 are known to affect kinetics and disposition to transport various OATP1B substrates of either endogenous or exogenous origin [10-13]. The OATP1B1 rs2306283 polymorphism p.N130D is associated with development of severe hyperbilirubinemia in neonates [14], the OATP1B1 rs4149056 polymorphism p.V174A with higher serum bilirubin levels in healthy adults [15,16] and two non-coding variants in *SLCO1B3* may contribute to idiopathic mild unconjugated hyperbilirubinemia [17].

Adaptive changes in expression of liver bilirubin transporters in both hereditary and acquired cholestatic liver diseases - down-regulation of the canalicular multidrug resistance-associated protein MRP2 expression and up-regulation of sinusoidal MRP3 expression - explain the impairment of liver bilirubin uptake and excretion [18-22]. Since complete absence of both OATP1B1 and OATP1B3 results in Rotor-type hereditary jaundice [23,24], down-regulation of OATP1Bs might also contribute to conjugated hyperbilirubinemia in common hepatobiliary diseases.

Our aims were to select antibodies suitable for specific detection of both or either of the OATP1B proteins on formalin-fixed paraffin-embedded liver specimens by testing them in

OATP1B1- and OATP1B3-transgenic mice and to correlate liver expression of OATP1Bs with both forms of plasma bilirubin, cholestatic markers and histological findings in various forms of biliary and parenchymal liver diseases.

Materials and methods

Mouse strains

The human OATP1B1 and OATP1B3 transgenic mice crossed back into a *Slco1a/1b*$^{-/-}$ background to obtain the corresponding humanized rescue strains [23, 25, 26] were used. All animals were between 9 and 14 weeks of age. Mice were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet (AM-II; Hope Farms) and acidified water *ad libitum*. Housing and handling of the animals was in line with the institutional guidelines complying with Dutch legislation.

Patients, biochemistry tests and human liver specimens

Fifty-two patients with end-stage liver disease who underwent orthotopic liver transplantation at the Institute for Clinical and Experimental Medicine between 2008 and 2013 were classified according to their underlying diagnosis. Two groups were constituted. The group of parenchymal liver diseases consisted of patients with alcoholic liver cirrhosis (ALD, n=9), cirrhosis owing to chronic hepatitis C (HCV, n=8), and autoimmune cirrhosis (AIH, n=4). Patients with primary sclerosing cholangitis (PSC, n=11), primary biliary cirrhosis (PBC, n=9), and biliary atresia (BA, n=11) were included in the group of patients suffering from primary biliary diseases. Control liver specimens were obtained from 5 patients who underwent liver resection for metastatic cancer.

Serum samples obtained the day of liver transplantation were analysed for total and conjugated bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyltransferase (GGT) and alkaline phosphatase (ALP) activity by routine clinical biochemistry methods.

Liver specimens were collected from the explanted livers. At least ten samples were obtained from the right and the left lobe and one from the *lobus caudatus*. Not less than two samples of normal liver tissue were taken from patients undergoing resection of liver metastases. All tissue blocks were formalin-fixed immediately after removal and processed for routine histological assessment.

Molecular analysis

Written informed consent was obtained from the patients before their genetic examination. Genomic DNA was extracted from peripheral leukocytes and *UGT1A1* TATA-box promoter polymorphism rs8175347 and the *SLCO1B1* c.521T>C (p.V174A) coding polymorphism rs4149056 were genotyped by direct sequencing on the Applied Biosystems ABI 3130 genetic analyzer (Life Technologies, Prague, Czech Republic).

Primary antibodies

Five antibodies directed against the amino or carboxyl terminus of OATP1B1, Oatp1b2 and OATP1B3 were tested for immunohistochemical detection of human OATP1Bs (Table 1) in frozen and paraffin sections. The MDQ mouse monoclonal anti-OATP2 antibody (ab15442, Abcam, Cambridge, UK) was reported as cross-reacting with both OATP1B1 and OATP1B3 on Western blot, immunoprecipitation and immunocytochemistry [27]. The ESL mouse monoclonal anti-OATP2 antibody (ab15441, Abcam) was declared as specific for OATP1B1 on frozen sections. The third mouse monoclonal antibody Oatp2 A-2 (sc-376424, Santa Cruz Biotechnology, Dallas, TX) should cross-react with human OATP and OATK family members. The rabbit anti-human polyclonal anti-SLCO1B1/OATP2 antibodies LS-C8521 (imunoaffinity purified) and LS-C8522 (unpurified serum), both purchased from LifeSpan Biosciences (Seattle, WA), were raised against a 17 amino acid peptide with identical yet proprietary sequence located near the C-terminus of human OATP1B1. Both LS-C8521 and LS-C8522 should specifically recognize human OATP1B1 in ELISA or on Western blot. The OATP8 H-52 rabbit polyclonal anti-human antibody (sc-98981, Santa Cruz Biotechnology, Dallas, TX) was recommended for detection of OATP1B3 and, to a lesser extent, OATP1B1.

Immunohistochemical staining

Mouse and human liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Four μm thick paraffin sections were cut and deparaffinized. Cryostat sections (8 μm) of frozen liver tissues were fixed in cold acetone for 10 minutes, dried and rinsed in 0.2% Triton X-100 for 5 minutes and in phosphate-buffered saline (PBS). Sections of an adult liver without cholestasis were used as a positive control in human studies and liver sections processed without incubation with primary antibodies served as negative controls in both animal and human studies.

All paraffin sections were pretreated by enzymatic digestion with Proteinase K (Dako, Glostrup, Denmark) or by a heat-induced epitope retrieval (HIER) technique of incubation for 30 minutes at 96°C in citrate buffer - pH 6.0 (Dako), Tris/EDTA buffer pH 8.0 (Leica, Wetzlar, Germany), Tris/EDTA buffer pH 9.0 (Dako) and High pH buffer (Dako). Sections without pretreatment were also used in parallel. Endogenous peroxidase activity was then blocked by 0.3% H_20_2 in 70% methanol for 30 minutes. To prevent non-specific binding, the sections were incubated with the serum from the host of the secondary antibody. Subsequent incubations with primary antibodies (dilution 1:50 and 1:100) were done overnight at +4° C.

For detection of primary antibodies a two-step (Dako, Histofine) or a three-step (Vector, Laboratories, Burlingame, CA) visualization system was used. The two-step detection of primary antibodies was performed using the EnVision + System-HRP Labelled Polymer anti-rabbit or anti-mouse (Dako) or the Simple Stain MAX PO (MULTI) Universal Immuno-peroxidase Polymer anti-mouse, anti-rabbit Histofine (Nichirei Biosciences, Tokyo, Japan) for 30 minutes. Finally, the specimens were stained with the Dako Liquid DAB Substrate-Chromogen System (Dako) for 2 minutes and counterstained with Harris's haematoxylin before they were embedded in Pertex® Mounting Medium (Histolab, Gothenburg, Sweden). The three-step detection of primary antibodies was started by 30 minutes incubation with biotinylated anti-mouse or rabbit IgG (H+L) (Vector) diluted 200x in 1% bovine serum albumin. The sections were incubated with R.T.U. Vectastain Elite ABC Reagent (Vector) for another 30 minutes and then stained with 3,3'-diaminobenzidine (Dako) for 2 minutes. Counterstaining with Harris's haematoxylin was performed at the end.

To minimize the reactivity of the secondary anti-mouse antibody with endogenous immunoglobulin in the mouse tissue, frozen and paraffin sections of mouse livers were stained with the Dako ARK^{TM} (Animal Research Kit) Peroxidase (Dako).

Immunohistological expression of OATP1Bs was evaluated independently by two histopathologists using the following scoring system: 0 - no positivity detected, 1 - positivity in less than 33% hepatocytes, 2 - positivity in 33-66% of hepatocytes, 3 - positivity in more than 66% hepatocytes. Correctness of the immunohistochemical reactions was verified by positive controls on each slide. Histological evaluation was performed in 2-6 slices with sectional area measuring 120-150 mm^2 obtained from different sites of the liver specimen in each case. If no

positivity was detected (e.g. score 0), an additional 2-3 sections from different sites of the explanted liver were stained. Since the architecture of the liver lobules was completely altered in the advanced stages of the liver diseases, the staining patterns of OATP1B proteins in liver parenchyma were irregular and zonal expression of the transporter proteins with centrilobular (perivenular) accentuation previously described in some studies [3, 7, 27] could not be assessed.

Statistical analysis

Results are expressed as the mean ± SD for the two patient groups. To calculate the statistical significance of the differences between the groups, the Mann-Whitney test was used. The relations between the parameters were estimated by the nonparametric Spearman´s correlation coefficient. An exponential model was used for significant correlations. Two-sided $p<0.05$ was considered statistically significant.

Results

Specificity of anti-OATP1B antibodies in frozen and formalin-fixed liver tissue

The anti-OATP2 MDQ antibody detected both OATP1B1 and OATP1B3 polypeptides in frozen and, after HIER pretreatment, in formalin-fixed liver tissue of mice transgenic for either *SLCO1B1* or *SLCO1B3* (Figure 1). Immunopositivity for both OATP1Bs was localized to the basolateral membrane of hepatocytes. Immunostaining in the *SLCO1B1*^{tg} mouse strain was accentuated in periportal areas of the liver lobules whereas the staining pattern in *SLCO1B3*^{tg} mice was irregular with random distribution of positive hepatocytes. The staining in *SLCO1B1* transgenes was weak compared to the *SLCO1B3* animals.

In healthy human liver tissue with preserved lobular architecture the distribution of the anti-OATP2 MDQ immunostaining showed accentuation in centrilobular (perivenular) areas with only weak signal around the portal triads (Figure 2). The polarity of the cell plasma membrane staining of normal hepatocytes localized to the basolateral (sinusoidal) membrane was easily discernible (Figure 2, inset).

The rabbit polyclonal antibody OATP8 H-52 gave a strong positive signal in *SLCO1B3*^{tg} mice (both frozen and paraffin sections) with a pattern similar to that observed with the MDQ antibody. The cross-reactivity with OATP1B1 in *SLCO1B1*^{tg} was also present but weak (Figure 1).

Specific detection of OATP1B1 was obtained with the ESL anti-OATP2 antibody in frozen sections of both *SLCO1B1*^{tg} mouse and human livers with periportal accentuation of staining in mouse tissue and diffuse panlobular staining in human specimens (Figure 2). The antibody did not cross-react with OATP1B3 in *SLCO1B3*^{tg} mice. Unfortunately, the ESL antibody did not recognize specifically OATP1B1 protein in formalin-fixed paraffin-embedded liver tissue.

None of the other tested anti-OATP1B1 antibodies Oatp2 A-2, LS-C8521 and LS-C8522 detected OATP1B1 and/or OATP1B3 either in frozen or in formalin-fixed paraffin-embedded sections of mouse and human liver tissue.

Clinical, laboratory and molecular characteristics of the candidate patients

To rule out individuals genetically predisposed to hyperbilirubinemia, *UGT1A1* promoter TATA-box and *SLCO1B1* rs4149056 genotyping was performed in all patients considered for inclusion in the study. A homozygous genotype $A(TA)_7TAA$ typical for Gilbert syndrome was identified in one patient with PSC and in two patients with hepatitis C. Moreover, three homozygotes for the *SLCO1B1* c.521C allele were identified in the group of primary biliary diseases (BA, PSC and PBC) and one patient with alcoholic cirrhosis. All these seven patients were excluded from further statistical evaluations.

Clinical and laboratory characteristics of the remaining 45 enrolled patients are presented in Table 2. As expected, total and conjugated serum bilirubin levels and GGT and ALP activities were higher in the patients suffering from primary biliary disorders compared to the individuals with primary hepatocellular diseases (Table 2) and the differences were statistically significant (Table 3). However, no difference between the serum unconjugated bilirubin levels was detected (Table 3).

Expression of OATP1Bs in advanced liver diseases

Immunohistological expression of the OATP1B proteins detected by the MDQ antibody (ab15442) in paraffin sections was irregular in advanced liver diseases with variable intensity of positive staining ranging from none or only small groups of positive cells to diffuse strong positivity. Moreover, polarity of the cell staining localized to the basolateral (sinusoidal) membrane of hepatocytes in the normal liver tissue with preserved lobular architecture was retained only partially in the setting of cirrhosis. The immunohistological OATP1B expression was decreased in advanced stages of both groups of patients with significantly lower values in the group of primary biliary disorders (1.9±1.1 vs. 2.7±0.6; p=0.009; Table 3).

Inverse correlations between the immunohistological OATP1Bs expression score and serum total, conjugated and unconjugated bilirubin levels were observed in the advanced stages of primary biliary diseases. By contrast, no statistically significant correlation was found between the same parameters in the group of primary hepatocellular (parenchymal) diseases (Figure 3). Moreover, expression of OATP1Bs did not correlate with the activity of cholestatic enzymes in both groups of diseases (data not shown).

Discussion

Despite extensive efforts, we did not achieve specific immunostaining of either OATP1B1 or OATP1B3 in formalin-fixed paraffin-embedded liver specimens with any of the 5 tested antibodies. The only two effective antibodies, MDQ and H-52, cross-reacted with both OATP1Bs whereas the anti-OATP1B1 antibodies ESL, Oatp2 A-2, LS-C8521 and LS-C8522 did not provide specific reactions with the membrane antigens on paraffin specimens. Cross-reactivity of the mouse monoclonal anti-OATP2 antibody [MDQ] (ab15442) with both human OATP1B1 and OATP1B3 proteins in Western blot, immunoprecipitation and immunocytochemistry of transfected cells has already been described by Cui et al. [27]. In our study we proved that the same antibody also recognizes antigenic determinants of both OATP1B proteins after immunohistochemical processing of formalin-fixed paraffin-embedded tissue sections and can serve as a useful tool in the diagnosis of Rotor syndrome caused by simultaneous absence of both OATP1B transporters [23, 24]. Our results obtained with the MDQ antibody in transgenic mouse and normal human liver tissue sections are consistent with previous reports [6, 25, 27]. It should be noted though, that polarity and zonal accentuation of the OATP1B transporters distribution is substantially altered in the advanced stages of liver diseases characterized by complete parenchymal architectural disturbance and vascular reorganization.

In the second part of the study, based on the immunodetection of both OATP1Bs with the MDQ antibody, we observed lower immunohistological expression of OATP1B in end-stage liver diseases. The decrease was more marked in the group of primary biliary disorders characterized by predominantly obstructive type of cholestasis compared to the primary non-cholestatic parenchymal diseases. Our observations are well in accordance with the previously published studies demonstrating down-regulation of OATP1B mRNA levels and/or protein products in patients suffering from PSC, PBC, and biliary atresia [20, 21, 28- 30]. Substantial differences between biliary and parenchymal diseases at the same stage (e.g. cirrhosis) strongly indicate presence of distinct mechanisms resulting in decreased numbers of the OATP1B-expressing cells and/or reduced density of OATP1B transporters at the basolateral membrane of hepatocytes.

Alteration of hepatobiliary transporters in hereditary and acquired liver diseases explains impaired hepatic (re)uptake and excretion of both forms of bilirubin, bile salts, and other biliary constituents resulting in cholestasis and jaundice [18, 19]. Cholestasis with blockade of MRP2-mediated transport is followed by up-regulation of the basolateral homologue MRP3 at the basolateral (sinusoidal) membrane of hepatocytes and conjugated bilirubin is secreted into sinusoidal blood via MRP3 with consequent urinary excretion [31, 32]. This MRP3 induction in cholestatic conditions, mediated by transcriptional pathways associated with bile acids, is supposed to protect cholestatic hepatocytes from glucuronides [33-35]. A substantial fraction of bilirubin conjugated in the liver and splanchnic organs secreted into portal and sinusoidal blood via MRP3, is subsequently taken up by hepatocytes via OATP1B1 and OATP1B3 for final biliary excretion [23, 36]. Except for up-regulation of canalicular and basolateral efflux pumps, elevation of serum bilirubin levels in advanced stages of biliary diseases may also be, at least in part, a consequence of the decreased basolateral bilirubin uptake which is supposed to represent a part of an adaptive process protecting hepatocytes against accumulation of toxic biliary constituents during chronic cholestasis [20, 22, 36].

Since the human material has been collected retrospectively in this study, only formalin-fixed and paraffin-embedded tissue was available in most of the patients. Considering the fact that immunohistochemistry combined with calculation of the OATP1B-expressing cell rate is a semiquantitative method, quantification of OATP1B protein expression should be performed in prospectively collected fresh liver tissue.

We conclude that the MDQ antibody can serve as a tool in histopathological differential diagnosis of hyperbilirubinemia syndromes and may be helpful in identification of Rotor subjects. Down-regulation of both OATP1B proteins altering bilirubin re-uptake at the basolateral membrane of cholestatic hepatocytes may, apart from impaired MRP2 and MRP3 expression, contribute to molecular pathogenesis of predominantly conjugated hyperbilirubinemia accompanying advanced liver diseases with predominantly obstructive type of cholestasis.

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Abbreviations

AIH	autoimmune hepatitis
ALD	alcoholic liver disease
ALT	alanine transaminase
ALP	alkaline phosphatase
AST	aspartate transaminase
BA	biliary atresia
GGT	γ-glutamyltransferase
HCV	hepatitis C virus
HIER	heat-induced epitope retrieval
HNF1α	hepatocyte nuclear factor 1α
HNF3β	hepatocyte nuclear factor 3β
MRP2/ABCC2	multidrug resistance-associated protein 2/ ATP-Binding Cassette Sub- Family C Member 2
MRP3/ABCC3	multidrug resistance-associated protein 3/ ATP-Binding Cassette Sub-Family C Member 3
OATP	organic anion-transporting polypeptide
OATP1B1	organic anion-transporting polypeptide 1B1
OATP1B3	organic anion-transporting polypeptide 1B3
PBC	primary biliary cirrhosis
PSC	primary sclerosing cholangitis
SLCO1B1	solute carrier organic anion transporter family member 1B1
SLCO1B3	solute carrier organic anion transporter family member 1B3
UGT1A1	uridine diphosphate glucuronosyltransferase 1A

Table 1: **Primary antibodies**

Primary antibody	Immunogen amino acid positions	Immunogen sequence	Recommended applications	Known cross-reactivity
OATP 2 MDQ	OATP1B1, 1-24	MDQNQHLNKTAEAQPS ENKKTRYC	WB, IP, IHC-Fr, ICC/IF	OATP1B3 on WB
OATP2 ESL	OATP1B1 671-691	ESLNKNKHFVPSAGADS ETHC	WB, IP, IHC-Fr, ICC/IF,	no
Oatp2 A-2	Oatp1b2, 611-660	ASFLPALFILILMRKFQFP GDIDSSDTDPAEMKLTA KESKCTNVHRSPTM	WB, IP, ICC/IF and ELISA	OATPs and OATKs
OATP2 LS-C8521	OATP1B1, C-term*	17 aa, sequence not provided	WB and ELISA	no
OATP2 LS-C8522	OATP1B1, C-term*	17 aa, sequence not provided	WB and ELISA	no
OATP8 H-52	OATP1B3, 651-702	FQGKDTKASDNERKKV MDEANLEFLNNGEHFVP SAGTDSKTCNLDM QDNAAAN	WB, IP, IHC-Fr, IHC-P, ICC/IF and ELISA	OATP1B1

Legend: †amino acid positions not provided; *Abbreviations:* WB - Western blot, IP - immunoprecipitation, IHC - immunohistochemistry, Fr - frozen, P - paraffin, ICC - immunocytochemistry, IF - immunofluorescence.

Table 2: **Clinical and laboratory characteristics of the patient groups**

Diag.	N	Sex M/F	Stage (fibrosis)	Child-Pugh score	Bili T (μmol/L)	Bili C (μmol/L)	ALP (μkat/L)	GGT (μkat/L)	OATP1B score
PSC	9	7/2	4	7.7±3.3	107±181	82±144	7.0±4.0	5.7±5.1	2.2±0.7
PBC	8	0/8	4	8.0±3.2	388±326	284±239	5.4±4.0	4.0±2.7	1.7±1.3
BA	10	6/4	4	9.3±1.6	282±277	183±193	5.9±5.0	1.9±1.2	1.7±1.3
ALD	8	6/2	4	8.3±1.4	48±25	21±10	3.4±2.0	1.7±1.0	2.8±0.5
HCV	6	5/1	4	8.0±1.7	46±39	27±24	2.4±1.1	2.0±1.3	2.7±0.8
AIH	4	2/2	4	7.5±2.4	66±56	26±14	2.5±1.0	2.0±1.6	2.5±0.6

Legend: Bili T - total bilirubin, Bili C - conjugated bilirubin, ALP - alkaline phosphatase, GGT - γ-glutamyltransferase, OATP1B score - immunohistological expression score of OATP1B proteins, PSC - primary sclerosing cholangitis, PBC - primary biliary cirrhosis, BA - biliary atresia, ALD - alcoholic liver disease, HCV - hepatitis C virus, AIH - autoimmune hepatitis.

Table 3: **Comparison of biochemical values and immunohistological OATP1B expression in the group of primary biliary and parenchymal diseases**

	Biliary (n=27)		Parenchymal (n=18)		
	mean	SD	mean	SD	p
Bili T (μmol/L)	255	280	51	36	**0.005**
Bili C (μmol/L)	179	203	24	16	**0.001**
Bili U (μmol/L)	76	85	27	24	0.093
ALP (μkat/L)	6.1	4.3	2.9	1.6	**0.005**
GGT (μkat/L)	4	3.9	1.9	1.2	**0.019**
OATP1B score	1.9	1.1	2.7	0.6	**0.009**

Legend: Bili U - unconjugated bilirubin, Bili T - total bilirubin, Bili C - conjugated bilirubin, ALP - alkaline phosphatase, GGT - γ-glutamyltransferase, OATP1B score - immunohistological expression score of OATP1B proteins.

Figure 1 **Immunohistological expression of OATP1B proteins in the formalin-fixed liver specimens of transgenic mice.** MDQ and H-52 antibody positively stained the basolateral membrane of hepatocytes in both transgenic mouse strains. No specific positivity was observed with the ESL antibody in paraffin sections. Control: *Slco1a/1b*$^{-/-}$ [35]. Asterisks mark portal triads. Paraffin sections, HIER pretreatment at pH 6.0. Bar corresponds to 50μm. Original magnification x200.

Figure 2 **Immunohistological expression of OATP1B proteins in frozen human liver tissue.** MDQ and H-52 immunostaining showed accentuation in centrilobular area with only weak signal around the portal triads. ESL antibody gave strong panlobular signal. Positivity was localized to the basolateral membrane of hepatocytes (inset). Asterisks mark central vein, triangles mark portal triads. Bar corresponds to 50μm, in inset 20 μm. Original magnification x200, inset x600.

Figure 3 **Correlation between the expression score of OATP1Bs and serum bilirubin levels.** The OATP1Bs expression score correlates inversely with serum conjugated (C), unconjugated (U) and total (T) bilirubin level in the group of primary biliary disorders (blue), but not in primary parenchymal diseases (red). The p values indicate statistical significance of correlation expressed as Pearson correlation coefficient r.

Figure 1

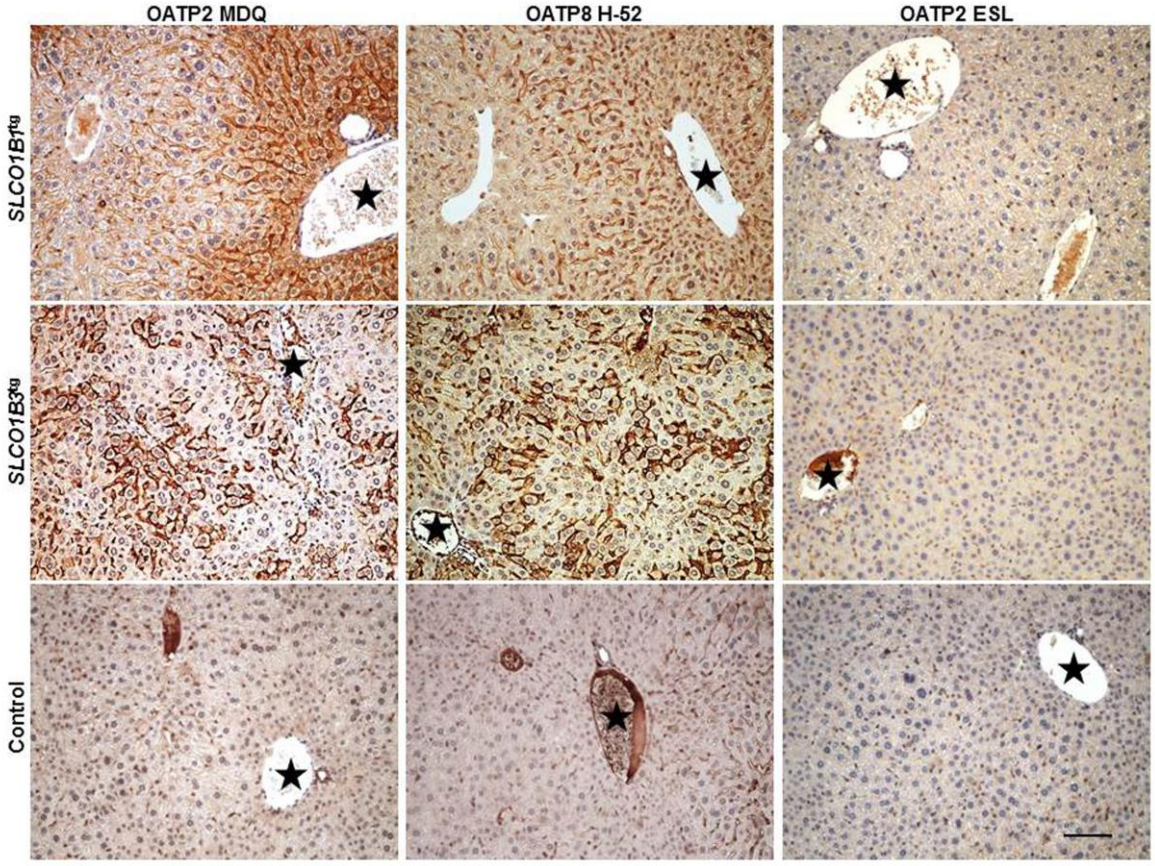


Figure 2

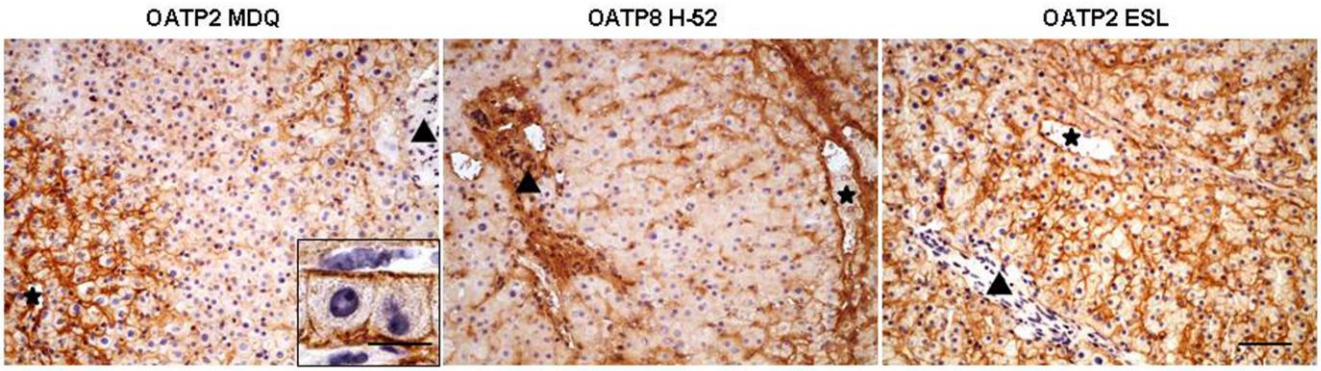
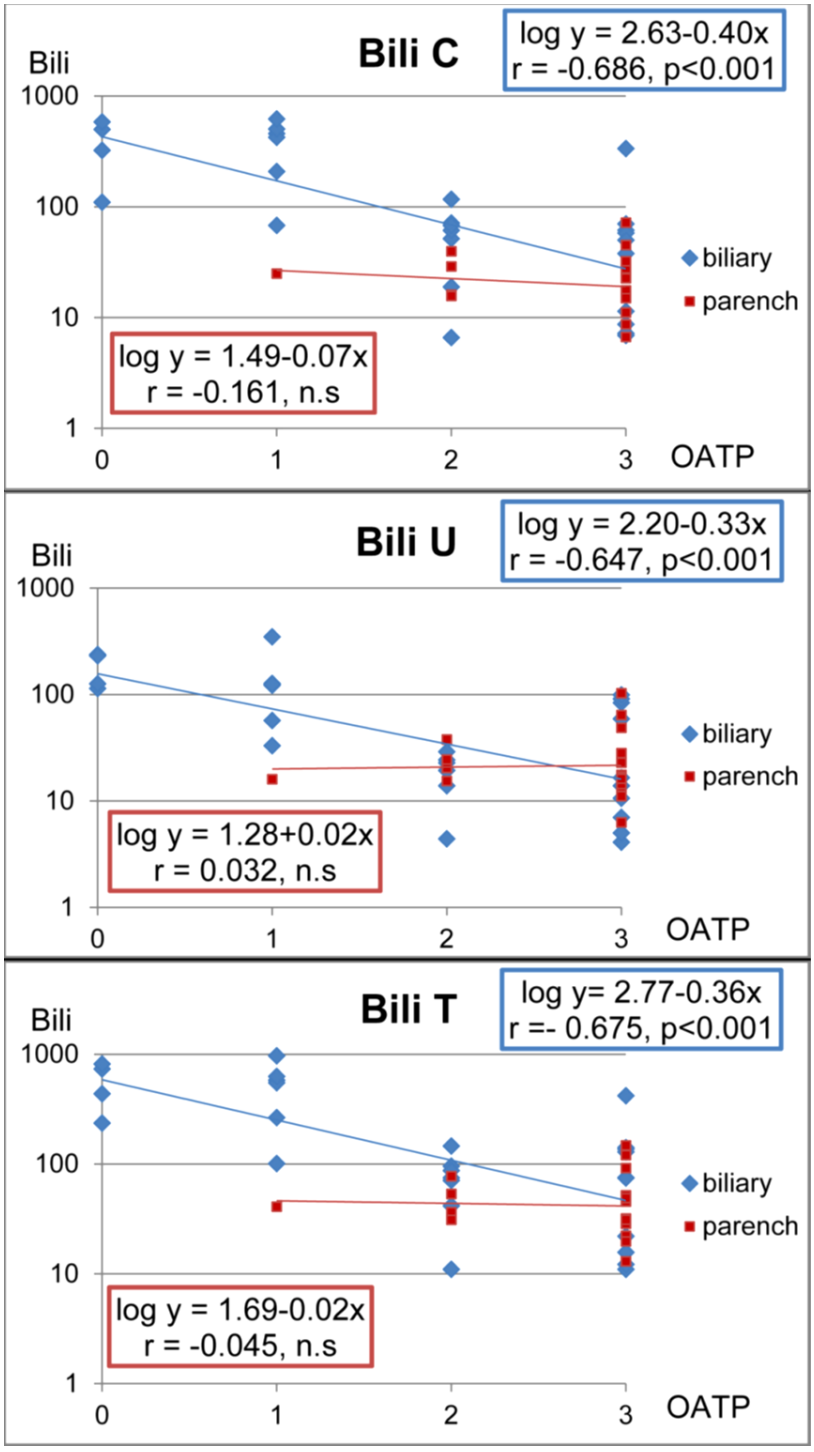


Figure 3


Bili C
log y = 2.63-0.40x
r = -0.686, p<0.001
Bili
1000
100
10
1
0
1
2
3
OATP
biliary
parench
log y = 1.49-0.07x
r = -0.161, n.s
Bili U
log y = 2.20-0.33x
r = -0.647, p<0.001
Bili
1000
100
10
1
0
1
2
3
OATP
biliary
parench
log y = 1.28+0.02x
r = 0.032, n.s
Bili T
log y= 2.77-0.36x
r =- 0.675, p<0.001
Bili
1000
100
10
1
0
1
2
3
OATP
biliary
parench
log y = 1.69-0.02x
r = -0.045, n.s