Charles University in Prague

First Faculty of Medicine

Study program: Biomedicine

Study field: Biochemisty and pathobiochemistry

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Molekulární patologie vybraných dědičných hyperbilirubinémií

Molecular pathology of selected inherited hyperbilirubinemias

PhD thesis

Supervisor: Prof. MUDr. Pavel Martásek, DrSc.

Prague, 2015

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In Prague, June 9th 2015

Identification record

ŠLACHTOVÁ, Lenka. Molekulární patologie vybraných dědičných hyperbilirubinémií *[Molecular pathology of selected inherited hyperbilirubinemias].* **Prague, 2015, 110 pages. PhD thesis**. Charles University in Prague, First Faculty of Medicine, Thesis supervisor: Martásek, Pavel.

Acknowledgements

I would like to thank following people for their support and understanding during the years I have conducted my research.

First, I would like to thank my supervisor, Prof. MUDr. Pavel Martásek, DrSc. for his guidance, interest, encouragement, advices, and continuous support during my studies.

Then I would like to thank my colleagues from the Department of Pediatrics, 1st Faculty of Medicine, Charles University in Prague and the collaborators from the Departments of Genetics from Kosice and Spisska Nova Ves. I would also like to thank all my colleagues from Laboratory of the Study of Mitochondrial Disorders, who had inspired me and Doc. MUDr. Onřej Šeda, PhD for his amazing advices during the preparation of the manuscript.

The great thanks belong to my family, and especially to my husband, for his patience, love, encouragements and understanding.

CONTENT

SUMMARY

Inherited hyperbilirubinemias are a group of metabolic disorders, characterized by increased levels of total serum bilirubin or its conjugated fraction. Most of these hyperbilirubinemias are inherited autosomal recessively and are manifested in young age. Increased bilirubin reflects the genetic disturbances in one of the enzymes of heme degradation pathway, the defect of bilirubin conjugation (*UGT1A1 gene*) or its transport (*ABCC2, OATP1B1, OATP1B3*). All of these proteins are involved not only in elimination of bilirubin, but various substrates; therefore the performed studies have a great pharmacogenomics impact. We have studied the molecular pathology of hereditary hyperbilirubinemias in Caucasian and Roma population and to compare the clinical and biochemical results with the molecular genetic data. We described the impact of compound defect of c.-3279T>G and g.175492_175493insTA on total serum bilirubin and calculated the linkage disequlibrium of these two variants in promoter region of *UGT1A1* gene. We also verified, that the population distribution of both variants is in concordance with the literature.

In our second study, we have described the rare conjugated hyperbilirubinemia Dubin-Johnson type among 7 Roma families. We have found a novel variant NG_011798.1:c.[1013_1014delTG] together with the dual genetic defect – a combination of Dubin-Johnson and Gilbert`s syndrome. We have described a founder effect of the mutation in *ABCC2* and found a common haplotype of 86 bp encompassing the gene. We have also characterized the excretion of coproporphyrin isomers in patients with DJS and compared it with those with dual hereditary jaundice.

KEY WORDS

Bilirubin, jaundice, hyperbilirubinemia, *UGT1A1, ABCC2*, coproporphyrin isomers

SOUHRN

Dědičné hyperbilirubinémie představují skupinu metabolických onemocnění, charakterizovaných zvýšenou hladinu celkového bilirubinu nebo jeho konjugované frakce v séru. Většina těchto hyperbilirubinémií je děděná autosomálně recesivně a jsou manifestovány zejména v mladém věku. Zvýšený bilirubin je zapříčiněn poruchami v některém z enzymů metabolické dráhy hemové degradace, ať už jeho konjugace (*UGT1A1*) nebo transportu (*ABCC2, OATP1B1, OATP1B3*). Všechny proteinové produkty těchto genů se účastní nejen eliminace bilirubinu, ale take různých dalších substrátů. Studie těchto protein mají tedy široký farmakogenomický vliv. Tato studie popisuje molekulární podstatu dědičných hyperbilirubinémií u kavkazské a romské populace, srovnává klinická a biochemická data s výsledky molekulární genetiky. Dále popisujeme vliv současného výskytu variant c.-3279T>G a g.175492_175493insTA na hladinu celkového sérového bilirubinu . Stanovili jsme vazebnou nerovnováhu obou variant lokalizovaných v promotorové oblasti genu UGT1A1. Zároveň jsme ověřili konzistenci populačních dat s výsledky v literature.

Ve druhé studie jsme popsali vzácnou konjugovanou hyperbilirubinémii Dubin-Johnsonova typu u 7 romských rodin. Našli jsme novou variantu v *ABCC2* genu NG_011798.1:c.[1013_1014delTG] a take dvojitý genetický defect – kombinaci Dubin-Johnsonova a Gilbertova szndromu. Popsali jsme novou mutaci v genu ABCC2 a našli společný haplotyp o velikosti 86 bp v blízkosti genu. Dále jsme charakterizovali exkreci izomerů koproporpfyrinu a srovnali data pacientů s DJS a dvojitou hereditární žloutenkou.

KLÍČOVÁ SLOVA

bilirubin, žloutenka, hyperbilirubinemie, *UGT1A1, ABCC2*, izomery koproporfyrinu,

ABBREVIATIONS

ABCC2 – *gene*/protein ABCC2, ABC cassette transporter 2

bp – base pair

cBi – conjugated bilirubin

cMOAT – canalicular multiorganic anion transporter

CNJ – Crigler-Najjar syndrome

CO - carbon monoxide

DJS – Dubin-Johnson syndrome

GS – Gilbert` s syndrome

HO – heme oxygenase

MRP2 – multidrug resistance associate protein 2

NADPH - nicotin amide dinucleotide phosphate cofactor

OR – ODDs ratio

PBREM – phenobarbital responsive enhancer element

RS – Rotor syndrome

Tbi – total serum bilirubin

UGT1A1 - *gene*/protein UGT1A1, uridine diphosphate glucuronosyltransferase 1A1

I. INTRODUCTION

I. I.HISTORY

From ancient Greek, a jaundice, a yellowish pigmentation of skin or sclerae, has been observed as an unhealthy or diseased condition. Since that time, humans have come a long way to identify its causes and find a desired cure. Depending on the development of investigation techniques, various causes of jaundice have been discovered, including those caused by genetic defects. The first comment on jaundice was made by the French physician Jean Baptiste Thimotee Baumes (Baumes, 1806) in his book Traite de L'amaigrissement des enfans. In 1922, the ages before the identifying genes or DNA, Meulegracht hypothesized, that some of the hyperbilirubinemias might be inherited (Meulengracht, 1992).

Bilirubin and inherited hyperbilirubinemias became a popular topic. The key molecule of UDP glucuronosyl-tranferase was experimentally proven to play a significant role in inherited hyperbilirubinemia, yet not in all of them. Later, the scientific world was shaken by the exciting epoch of molecular biology and this progress added a new layer in the understanding of hyperbilirubinemias. The milestone was firmly set by the revelation of *UGT1A1* gene and its defects, responsible for the majority of inherited defects of bilirubin metabolism. The key offender was identified, but still many pieces were left to complete the puzzle and the causes of conjugated hyperbilirubinemias still remained unknown. The expectation of genetic defects in bilirubin transporter ABCC2 was confirmed in 1998 by the work of Wada et al (Wada et al, 1998). The final puzzle piece remained.

Identification of the last inherited hyperbilirubinemia, Rotor syndrome, examined and challenged researchers for several years. Finally, in 2012, a digenic disturbance of bilirubin transport was uncovered by team of Netherland and Czech authors (Steeg et al, 2012).

Therapy of jaundice wasn`t quite following the path of discoveries, and as in most of the findings, serendipity played a significant role. In the mid 1950`s, a nurse from England noticed that newborns exposed to sunlight by windows showed less jaundice and lower bilirubin levels than others. This observation led to the development of the biggest tool of jaundice treatment up to today – a phototherapy.

Today`s knowledge of inherited hyperbilirubinemia answered many questions and saved many lives. And as in almost every field of science, more questions were put on the table.

I.II. BILIRUBIN

Heme is the essential compound of the eukaryotic cells. As a part of hemoproteins, it is involved in cellular respiration, oxidative responses and signaling. On the other side, its excessive amount is toxic, therefore intracellular heme pool and heme catabolism is tightly regulated. Heme is degraded by heme oxygenase and the resulting product of heme degradation is biliverdin Ixα, which is further transformed to bilirubin Ixα, carbon monoxide and iron. In humans, a major source of bilirubin is heme, derived from hemoglobin released from senescent erythrocytes (~ 80%) (London et al, 1950). Other sources of heme are hemoproteins such as peroxidases, catalases; or myoglobin. Erythrocytes lifespan is about 120 days and a daily production of bilirubin is 250 – 400 mg. Even though every eukaryotic cell is able to produce bilirubin, the majority of heme degradation occurs in the reticuloendothelial system of spleen and liver.

I.II.I. Biochemistry of heme degradation

1. Heme oxidation

Once released from the hemoglobin molecule, heme is available for the heme degradation. The breakdown reaction starts with the oxidative cleavage of the α-methylene bridge of heme by the enzyme heme oxygenase (HO) (Tenhunen 1968). As a product, equimolar amounts of CO, biliverdin and iron is made (Tenhunen et al., 1968). Reaction needs reduced form of nicotin amide dinucleotide phosphate cofactor (NADPH) and 3 molecules of oxygen.

The reaction occurs in three mono-oxygenation cycles. In each of the cycle iron binds oxygen which accepts the second electron from NADPH (Yoshida et al). Such an activated oxygen complex cleaves the heme ring. In the first cycle the hydroxyl-heme is formed, in the second verdoheme is formed, and in the third reaction ferribiliverdin IXa is formed. Additional reduction of ferribiliverdin by NADPH is followed by release of ferrous iron from biliverdin (Yoshida 1978).

Heme b + $3O_2$ + 3½NADPH + 3½H^+ → biliverdin + Fe^{2+} + CO + 3½$NADP^+$ + $3H_2O$

Heme oxygenase is a ubiquitous highly inducible enzyme from a family of heat shock proteins. There are three isoforms of the enzyme (HO-1, HO-2, HO3). The protein is anchored in the membrane of the endoplasmic reticulum, a place of the heme oxidation. It is also present in the other membrane compartments. The oxidation of heme by HO is very important as it is the only rate limiting step in bilirubin formation. Before the oxidation step, it is unclear how the hydrophobic molecule of heme is transported from phagosome where it is generated through the cytosol up to the endoplasmic reticulum.

The most important in heme degradation is the inducible form of heme oxygenase, HO-1, highly expressed in liver cells. HO-1 inducibility is highly responsive to various stimuli, including physical, chemical, or biological impacts. Compared to the HO-1, HO-2 expression is constitutive and it is limited to certain tissues including testes or neuronal cells. Third isoform HO-3 is not catalytically active. In vitro, the HO activity can be induced adding NADH or NADPH. On the other side, HO is inhibited by its reaction product – biliverdin. This step is final for heme degradation in reptiles and amphibians who are able to excrete biliverdin.

Aside of the main purpose to create biliverdin and furtherly bilirubin, the reaction of heme breakdown has significant effects. Iron cleaved off the heme is toxic for the cell, therefore it has to be transported or bound to the protein for its reutilization. CO, the other by-product of heme degradation is important on both cellular and tissue levels for its antiinflamatory and antiapoptotic effects, and for the regulation of the vascular tone through cGMP or by activating MAPK.

2. Reduction of biliverdin to bilirubin

The next step of bilirubin formation is the reduction of biliverdin IXa by biliverdin reductase (Tenhunen et al, 1970). The reaction requires the presence of NADPH as a cofactor. Biliverdin reductase is a cytosolic enzyme of great importance. Next to its ability to convert biliverdin to bilirubin, biliverdin reductase is a regulator of insulin/IGF -1/IRK/PI3K/MAPK pathways, suggesting that it can be a potential regulator of cell proliferation and growth with the implications in a cancer therapy. Another effect of biliverdin reductase is antiinflamatory, antiapoptotic and oxidative stress prevention (Kim et al, 2015). However, the step of the

reduction of biliverdin to bilirubin is interesting. The biliverdin itself is water soluble already and by the reduction by biliverdin reductase, the product becomes insoluble. The step occurs in the cytosol.

3. Conjugation of bilirubin with glucuronic acid

Insoluble 4Z, 15Z-bilirubin IXa even when highly antioxidant, is toxic for the cells. Therefore it has to be converted to a water soluble product to be easily eliminated. This is mediated by the conjugation of bilirubin with glucuronic acid by enzyme UDP- glucuronosyl transferase 1A1, the only enzyme catalyzing bilirubin glucuronidation in humans (Blanckaert, Bock). Reaction results in the esterification of carboxyle groups of bilirubin by the glucuronic acid and releasing of hydrogen bonds in the molecule. The products are in great majority bilirubin diglucuronides (~ 80%), also monoglucuronides are formed (Onishi, 1980, Chowdhury 1982). The process occurs in endoplasmic reticulum of hepatocytes.

The role of UGT1A1 is to metabolize not only bilirubin, but a myriad of toxic endogenous xenobiotics. UGT1A1 is a member of an extensive UGT family with a large substrate affinity, playing the most important role as a phase 2 drug metabolizing enzymes. UGT is present in bacteria, plants, animals, as well as in humans. According to a modeled structure, UGT1A1 has a deep pocket formed by N-terminal domain next to the donor binding site of the molecule. According to the characteristics, the pocket is probably the acceptor binding site of the enzyme (Figure 1). The enzymatic activity of UGT1a1 can be stimulated by various chemicals, including the most known stimulant, the phenobarbital.

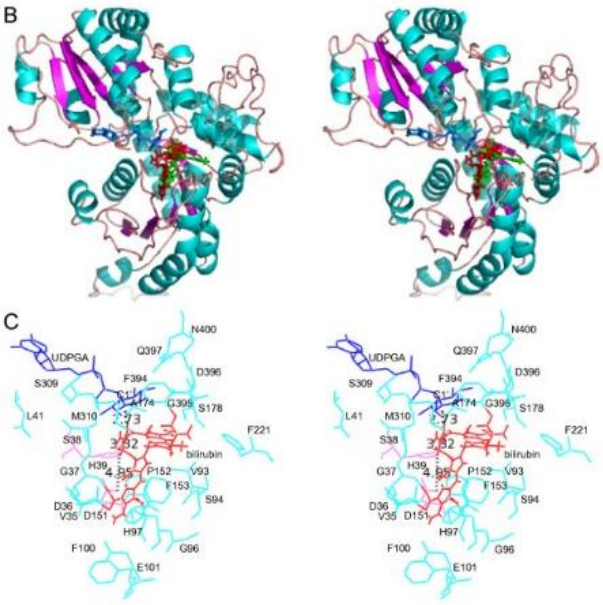


*Figure 1: Molecular docking of bilirubin into the human UGT1A1 protein. (**B**) Diagram showing positions of the modeled substrates. The UGT1A1 is shown in a ribbon diagram with the secondary structure highlighted. The donor UDP glucuronic acid (UDPGA) is shown in a dark blue. The two docked conformations of bilirubin are shown as red and green sticks. (**C**) Diagram showing bilirubin (red conformation) docked into the acceptor-binding pocket of UGT1A1. UDPGA (blue) and bilirubin (red) are shown as lines. Some residues in the acceptor-binding pocket are labeled and shown in cyan. Modified according to Li and Wu*

4. Bilirubin transport

Bilirubin conjugation with glucuronic acid forming mostly bilirubin diglucuronides makes the bilirubin water soluble and accessible for its transport into the bile. In humans, this step is mediated by multidrug resistance associated protein 2 (MRP2), an ATP dependent transport pump localized at the canalicular membrane of hepatocytes. By MRP2, the bilirubin diglucuronide (bilirubin conjugate) is transported from hepatocytes to bile canaliculi and to the bile. Mutations in MRP2 cause Dubin Johnson syndrome, the inherited conjugated hyperbilirubinemia (Dubin and Johnson 1954, Wada, 1998).

5. Bilirubin catabolism

Conjugated bilirubin, transported from hepatocytes into the bile, is as a part of bile directed into the duodenum and then to large intestine. In a large intestine it is degraded by the bacterial enzymes glucuronidases, cleavaging the glucuronide residues and forming colorless urobilinogens. Certain amount of bilirubin is excreted by alternative pathway via kidneys (Watson 1969, Vitek et al 2005, Vitek et al 2006, Chowdhury et al 1983).

I.II.II. Bilirubin structure

Bilirubin was a compound of a great interest in the first half of the 20th century and the most significant findings were made in the period of World War II. Originally, bilirubin IXα has been isolated by Virchow in 1847 and named by Stadeler. The first synthesis was made, and structure of bilirubin was solved in 1942 by a German chemist Fisher and his group, who despite the struggling environment of World War II made indisputably one of the biggest progressions in bilirubin research (462, Fisher H, Plieningher H, 1942. Synthese des biliverdins (uteroverdins) und bilirubins. Naturwissenschaften 30:382????). After a remarkable effort, the Fisher`s research methods reached their limits. Few questions remained about tautomerism, stereochemistry and conformation: a) The presence of a lactam or a lactim ring at the A and D ends of the bilirubin; b) the geometry of 5 and 15 bridges forming E or Z conformation (Pigm of life, 434). c) double or single bonds of C4-C5 and C15-C16

The cutting edge work answering these questions was Bonnett`s work who used X-ray diffraction crystallography. His work confirmed the final structure of bilirubin as Ixα, 4Z,15Z and formation of double bonds between C4-C5 and C15-C16 and single bonds between C5-C6 and C14-C15.

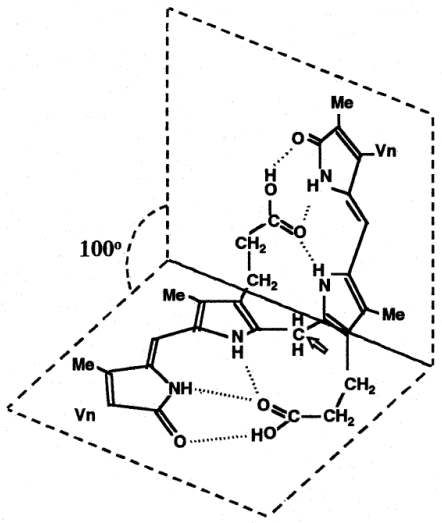


Figure 2 – Bilirubin structure, according to Chowdhury et al, in Disorders of bilirubin metabolism

I.II.III. Chemical characteristics of bilirubin

Chemical name of bilirubin is 1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid. The molecule has a form of "ridge tile" which means that two from four pyrrolic rings lie in a different plane with the angle 98-100` in between these two planes (Figure 2,3). In both of the pyrroles, hydrogen bonds are between carboxylic and amino groups and lactam oxygens, connecting the propionic acid side chains to the opposite pyrrolic and lactam side of the molecule. These intramolecular bonds prevent bilirubin from interacting with polar solvents and are responsible for a major characteristic of bilirubin – water insolubility. This characteristic is a key issue in bilirubin elimination in vivo. As none of the organisms want to store its toxic products, the solution is in making bilirubin soluble by its conjugation with glucuronic acid. In this reaction, carboxyl group of propionic acid is esterified and hydrogen bonds are disrupted. This mechanism is used in Van den Bergh reaction, a method using standard diazo-reaction to estimate a conjugated fraction of bilirubin. Bilirubin is weakly acidic.

Unconjugated bilirubin can form various isomers. In human body, the most abundant is the isomer Ixα, 4Z,15Z. Other isomers are produced as a minority. The ability of bilirubin to create different isomers comes from the tetrapyrrol structure of the original compound heme. The

isomers are formed according to the place of the cleavage of methionine bonds (Aziz, Blanckaert); this characteristic of bilirubin applies to the therapeutic purposes as a treatment of jaundice.

I.II.IV. Bilirubin and light

The effect of light on bilirubin is widely used in therapy of newborns` jaundice. Bilirubin undergoes photo-oxidation to form a singlet oxygen (McDonagh 1971). The light absorption is at 450 – 474 nm. Bilirubin itself is not fluorescent, however it becomes fluorescent at 510 – 530 nm if dissolved in albumin solution or detergents. The principle of a phototherapy using blue light is a change of bilirubin conformation from Z (trans) to E (cis) with resulted bilirubin isomers in ZE, EE or EZ conformation. These isomers lack internal hydrogen bonds, therefore making bilirubin more polar and unnecessary to be conjugated with glucuronic acid. (Mc Donagh et al 1972, Itoh et al 1985; Onishi et al 1980, Yokoyama et al 1984).

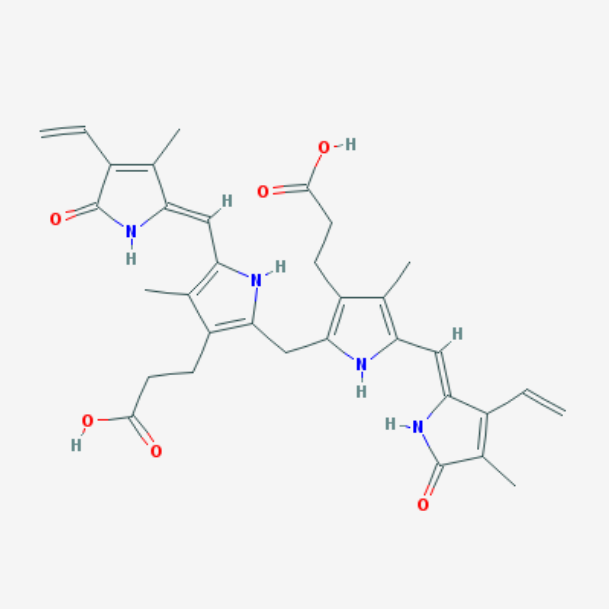


Figure 3. Bilirubin 2D structure – according to http://pubchem.ncbi.nlm.nih.gov/compound/bilirubin

I. II. V. Bilirubin as an antioxidant

Bilirubin antioxidant properties have been discovered in 1987 (Stocker et al, 1987). Originally, the biggest bilirubin aware was because of its neurotoxic effects in newborns with non-mature

blood brain barrier (Criegler and Najjar 1952). This is a risk especially in infants with high bilirubin levels, caused by increased turnover of red blood cells as well as non-matured liver system. However, it was found and confirmed by numerous studies, that bilirubin is one of the strongest antioxidants presented in a human body. It has a scavenger effect against the free radicals protecting vascular cells. This means, that Gilbert`s syndrome in adults, characterized by elevated levels of total serum bilirubin, has a protective impact on patients with cardiovascular disorders such as hypertension and coronary heart disease (Wang et al, 2015). In perspective of high incidence of Gilbert`s syndrome in Caucasians and current increasing incidence of lifestyle disorder, bilirubin plays still important role in human body homeostasis.

I.III. Jaundice and its therapy

Jaundice appears as a clinical sign of elevated bilirubin in adults when total bilirubin > 50 and mostly around 80 umol/L, therefore it is a clinical sign of several inherited hyperbilirubinemias (Silbertnagl et al, 2009). It is presented as a yellowish pigmentation of skin and/or sclerae. The most severe jaundice occurs in patients with Crigler-Najjar syndrome type I, which, without the early treatment, can be fatal. On the other side, in patients with Crigler-Najjar syndrome type II, the hyperbilirubinemia and jaundice are milder. Occasionally, jaundice is presented in Dubin-Johnson, Rotor or Gilbert` s syndrome.

The therapy of jaundice is well developed because of abundant incidence of jaundice in newborns. The pathophysiology of jaundice in newborns is different compared to inherited hyperbilirubinemias; it results from the rapid turnover of erythrocytes and also from the non-mature hepatobiliary system. However, implications from the newborns therapy of jaundice have been adopted also for the treatment of inherited hyperbilirubinemias. As inherited hyperbilirubinemias are mostly mild disorders not associated with the life-threating conditions, the therapy is often based on regime measurements and on avoiding certain pharmacotherapies (Cisplatin, Irinotecan, contraceptives). Lifestyle measurements are based on sufficient amount of sleep and avoiding alcohol and drugs. Dietary guidelines support healthy balanced regular diet. Abnormal intake of fat or imbalanced irregular sugar intake is not recommended, especially

because of the sugar importance in bilirubin conjugation. Also the natural-based supplements can have certain effect thanks to their content of flavonoids or other active substances.

This implies to the mildest types of hereditary hyperbilirubinemia – Gilbert`s syndrome, Crigler-Najjar syndrome II, Dubin-Johnson or Rotor syndrome. The most severe inherited hyperbilirubinemia – Crigler-Najjar syndrome I demands different therapy. Both, Crigler-Najjar syndrome I and II are mostly diagnosed within first days of newborns life (CNJ I) or during the childhood. The cause of both Crigler-Najjar syndromes lies in the defect of UGT1A1 gene. In case of CNJ II, a trace activity of the enzyme still remains and it is inducible by phenobarbital. Not so in CNJ I, which is a severe life-threatening condition with almost no activity of UGT1A1. CNJ I is characterized by excessive amount of bilirubin circulating in blood, unable to conjugate and to be excreted. Without therapy, the disorder has a fatal consequences. The early diagnosis is very critical. The blood-brain barrier of newborns is not fully maturated yet and bilirubin can cross from blood to the brain and because its neurotoxicity it causes kernicterus (Criegler and Najjar 1952). Kernicterus is serious a neonatal condition when the circulating bilirubin enters the brain and causes encephalopathy. It is based on bilirubin lipid solubility, able to penetrate neuronal and glial membranes in brain (Figure 4). Shevell et al reviews the pathologic effects of bilirubin penetrated to brain tissues in the most severe hyperbilirubinemia - Crigler-Najjar syndrome I.

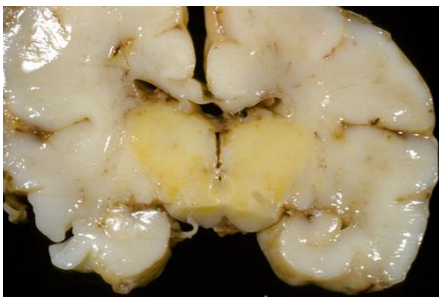


Figure 4 – kernicterus in the brain, according to

http://neuropathology-web.org/chapter3/chapter3eBilirubinencephalopathy.html

I.III.I. Phenobarbital therapy

Phenobarbital has been used as a diagnostic test of Gilbert`s syndrome few years ago and it is not currently used in a developed countries. Phenobarbital effect on bilirubin metabolism was

described in seventies by several authors (Crigler et al, Catz et al). It is an inducer of hepatic cytochrome P450 enzyme providing faster clearance of drug metabolized by cytochrome P450. It is also an inducer of UGT1A1, responsible for bilirubin conjugation with glucuronic acid. Therefore, phenobarbital intake decreases the time necessary for bilirubin excretion in vivo (Crigler, Arias). The induction of UGT1A1 is currently used as a parameter of differential diagnostics between Crigler-Najjar syndrome I and II. Crigler-Najjar syndrome I. Therapeutic use of phenobarbital have been replaced by light therapy, mainly because of the lack of its side effects.

I.III.II. Light therapy

The effect of light on bilirubin has been described in detail in previous chapter. The mechanism of the therapy is in transformation of non-soluble unconjugated bilirubin unable to excrete, to its soluble form. As in most individuals with hyperbilirubinemia, the origin of the defect is in incapability to conjugate bilirubin in sufficient amount, light therapy enables to create easily soluble bilirubin isomers. (Mc Donagh 1972, Itoh 1985; Onishi 1980, Yokoyama 1984).

The phototherapy as a treatment of jaundice is used since 1958. Special lamps are used to emit the blue visible light with the wave lengths range of 400 – 500 nm. The light cause bilirubin isomerization from bilirubin to lumirubin and it is further easily excreted by urine. During the procedure, the eyes of patients are protected by pads or by mask. Intensity of the phototherapy is measured by radiometer. The American Academy of Paediatrics recommends the average light intensity of 10 - 30m W/cm2/nm to the possible area of radiation in the infants (Bhutani et al, 2011).

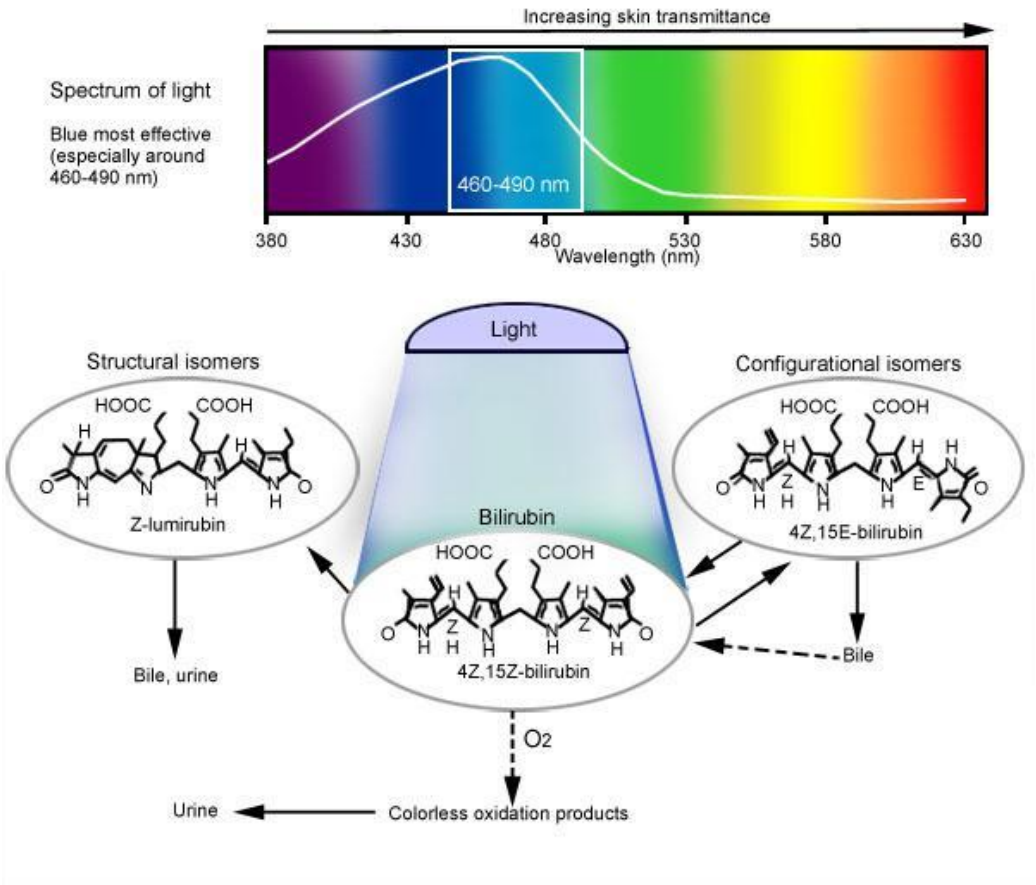


Figure 5. Principle of a phototherapy. According to Maisel MJ and McDonagh AD, 2008

Several light devices are used as the light source:

- Micro-Lite White Halogen lights
- Fluoro-Lite - 2 Blue and 2 White Fluorescent lights
- Fluoro-Lite - 2 Blue and 2 White Fluorescent lights
- Medela Bilibed Blue Fluorescent light

I.IV. Excretion of coproporphyrin isomers in hereditary hyperbilirubinemia

The heterocyclic compounds coproporphyrins are intermediates of the heme synthethic pathway in hematopoetic cells in liver and bone marrow. Normally, coproporphyrins are excreted as a products of bilirubin breakdown into the bile, feces and urine. The unusual distribution of coproporphyrin isomers in urine of patients with Dubin-Johnson and Rotor syndrome became one of the hallmark of these disorders (Koskelo et al, 1967, Mor-Cohen 2001). Yet porphyrins and their presence in body fluids is known for a long time, the exact mechanism of their excretion has not been described.

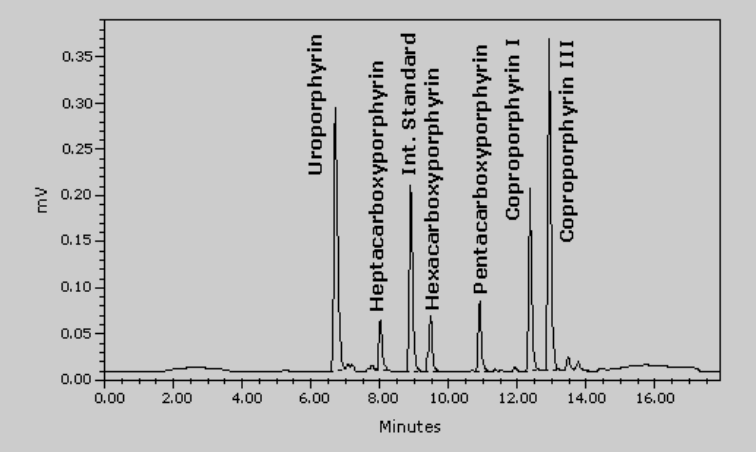


Figure 6. Chromatograph of a normal excretion profile in healthy individual, Chromsystems

I.V. *UGT1A1* gene

UGT1A1 gene (uridine diphosphate glucuronosyltransferase) is a member of a broad enzymatic family of uridine diphosphate glucuronosyltransferases, strongly involved in metabolism of xenobiotics (Bosma, 1995). These enzymes protect organism from its toxic compounds by glucuronidation – the addition of the glycosyl residue to the small molecule. Resulting product is water soluble and able to be eliminated by kidneys or by intestine. UGT1A1 mediates the conjugation of numerous substrates with glucuronic acid; it is the second phase of biotransformation of the molecules. Reaction occurs at the endoplasmic reticulum of hepatocytes. Following table surveys the substrates, inducers and inhibitors of UGT1A1 (adjusted according to www.pharmacologyweekly.com)

Table 1 – Substrates, inducers and inhibitors of UGT1A1

Substrates	**Inducers**	**Inhibitors**
Acetaminophen	Phenobarbital	Atazanavir
Atorvastatin	Carbamazepine	Gemfibrozil
Buprenorphine	Nicotine	Indinavir
Carvedilol		Ketoconazole
Estradiol		
Etoposide		
Ezetimibe		
Febuxostat		
Fluvastatin		
Gemfibrozil		
Irinotecan		
Levothyroxine		
Losartan		
Lovastatin		
Ketoconazole		
Morphine		
Naltrexone		
Naphthol		
Paracetamol		
Raloxifene		
Raltegravir		
Rosuvastatin		
Simvastatin		
SN-38		

Genetic defects in UGT1A1 result in impaired glucuronidation and unconjugated hyperbilirubinemia. The most prevalent defects in a promoter region of the gene are associated with Gilbert`s syndrome, the rare defects in a structural part of the gene result in Crigler-Najjar syndrome type II or in a most severe hereditary hyperbilirubinemia – Crigler-Najjar syndrome type I.

I.VI. Gilbert`s syndrome

The defect in *UGT1A1* gene results in inherited hyperbilirubinemia, Gilbert´s syndrome (GS) (OMIM ID:143 500). GS is a hereditary, chronic, mild, benign, unconjugated hyperbilirubinemia resulting from impaired bilirubin clearance with otherwise normal liver function. The degree of hyperbilirubinemia is relatively mild, mostly less than 80 µmol/L (4.7 mg/dL) in adults (Bosma et al, 1995). Considerable daily and seasonal variations are observed, and bilirubin levels occasionally may be normal in as many as one third of patients. Icterus is mild, usually limited to sclerae and mucosae. Typically, bilirubinemia is enhanced following hunger, strenuous physical exercise, surgery, stress, or fever. Patients may report general discomfort or fatigue. The animal model to study Gilbert`s syndrome is Gunn rat.

Genetic variants associated with GS

Gilbert´s syndrome is linked to the mutations in uridine diphosphate glucuronosyltransferase (UGT) 1A1 gene located on chromosome 2q37 with autosomal recessive inheritance. In Caucasians, the frequency of GS is 5–10% (Owens and Evans, 1975). The most common variant is the TA insertion in the TATAA box of the UGT1A1 called UGT1A*28 or NG_002601.2:g.[175492_175493insTA]. Wild type A(TA)6TAA is replaced by A(TA)7TAA allele; the variant is prevalent in a Caucasians with the frequency of 11-16% in homozygous state (Bosma et al, 1995, Owens and Evans 1975). The other non-pathogenic variant, also presented in promoter region of UGT1A1, is c.-3279T>G (rs4124874), located in phenobarbital enhancer responsive module upstream of TATAA box of the gene. This variant also results in decreased glucuronidation and impaired excretion of bilirubin. Both variants, c.-3279T>G and UGT1A*28 are tightly linked (Maruo et al, 2004, Jirsa et al, 2006) Other types of mutations in Gilbert´s syndrome are primarily missense mutations, which are rare in Caucasians but common in Asians. UGT1A1 protein preferentially binds and conjugates bilirubin in hepatocytes thereby eliminating

products of heme breakdown. The activity of the enzyme is about 30% of the normal level in patients with Gilbert´s syndrome (Bosma et al, 1995, Takeuchi et al, 2004).

I.VII. Crigler-Najjar syndrome I

The most severe inherited hyperbilirubinemia is Crigler-Najjar syndrome I (CNJ I) (OMIM 218 800) with the total serum bilirubin around 340-685 μmol/L or higher. The syndrome was firstly described by Crigler and Najjar in 1952. Hyperbilirubinemia is obvious since early days of newborn life with no significant decrease after phenobarbital administration. This is caused by the mutations in the structural part of *UGT1A1* gene, leading to almost absent activity of UGT1A1 enzyme. Since the UGT1A1 is the only enzyme catalyzing conjugation of glucuronic acid with bilirubin, the defect has serious life threating consequences and immediate treatment is necessary. The only long-term treatment is in liver transplant (Ciotti et al, 1997). The inheritance of CNJ I is autosomal recessive (Chowdhury et al, 2001). Interestingly, four cases of a rare CNJ I were found among Slovakian Roma population (Zmetakova et al, 2007).

I.VIII. Crigler-Najjar syndrome II

As well as Crigler-Najjar syndrome type I, CNJ II is also caused by the genetic defects in the structural part of UGT1A1 gene. Unlike CNJ I, in CNJ II the activity of mutated UGT1A1 is > 10 %, which enables its inducibility. The hyperbilirubinemia is from 60-340 μmol/L (Crigler and Najjar, 1952, Arias et al, 1969). Also phototherapy has a better perspective. Most of the defects are substitutions in *UGT1A1* gene, inherited autosomal recessively, however also autosomal dominant model of CNJ II was detected (Koiwai et al, 1996, Bosma et al, 1995) The differentiation between CNJ I and CNJ II is possible by phenobarbital test, molecular genetic analyses and clinical history.

I. IX. *ABCC2* gene

The link between the *ABCC2* gene and hereditary jaundice was discovered by Paulusma and Elferink group in 1996, the scientists exploring the congenital jaundice in TR- rats. Previously, *ABCC2,* furtherly named *cMOAT*, was discovered at cisplatin-resistant tumor cells and cloned by

Taniguchi and by Buchler the same year. (Taniguchi et al 1996, Buchler et al, 1996). The gene is located at human chromosome 10q24 chr10:99782732-99852192 (hg19). The Genomic structure of *ABCC2* was described by Toh et al (Toh et al, 1999). ABCC2 spans 32 exons and exhibits high similarity to *ABCC1* and *ABCC3.*

Although the association of *ABCC2* and Dubin-Johnson syndrome was predicted by Zimniak already in 1993, the first mutations were found by Wada`s team five years later. Up to today, more than 40 mutations in *ABCC2* causing Dubin-Johnson syndrome have been discovered (see table). Moreover, a rare dual hereditary hyperbilirubinemia was found as a result of defect in two genes - *ABCC2* and *UGT1A1* (Cebecauerova et al, Slachtova et al 2015). However, more attention than mutations in *ABCC2* attracted the often prevalent SNPs in *ABCC2*, known as the significant influencers of drug elimination.

Table 2. Mutations found in *ABCC2* gene, May 2015

Mutation	**Effect**	**Reference**
c.821_822delCT		Devgun (2012) Ann Clin Biochem 49, 609
c.974C>G	p.S325*	Corpechot (2006) Am J Gastroenterol 101,2427
c.998A>G	p.D333G	Arlanov (2012) Hum Mutat 33, 750
c.1013_1014delTG	p.V338Efs14*	Slachtova (2015) Eur J Hum Genet
c.1031+4A>G		Mor Cohen (2005) Hepatol Res 31, 104
c.1135C>A	p.Q379K	Devgun (2012) Ann Clin Biochem 49, 609
c.1177C>T	p.R393W	Machida (2005) J Gastroenterol 40, 366, Lee (2006) Pediatr Res 59: 584
c.1234A>G	p.R412G	Hulot (2005) Pharmacogenet Genomics 15,277
c.1249G>A	p.V417I	Meyer zu Schwabedissen (2005) Drug Metab Dispos 33, 896, Megaraj (2011) Pharmacogenet Genomics 21: 506 Deo (2012) Drug Metab Dispos 40: 852
c.1256_1272delins17		Cebecauerova (2005) Gastroenterology 129, 315
c.1321C>A	p.L441M	Lee (2006) Pediatr Res 59, 584
c.1815+2T>A		Wada (1998) Hum Mol Genet 7, 203
c.1967+2T>C		Kajihara (1998) Biochem Biophys Res Commun 253, 454
c.2026G>C	p.G676R	Wakusawa (2003) J Hum Genet 48, 425
c.2125T>C	p.W709R	Machida (2004) Hepatol Res 30, 86, Uchiumi (2012) Hepatol Res
c.2302C>T	p.R768W	Wada (1998) Hum Mol Genet 7, 203, Hashimoto (2002) Hepatology 36: 1236
c.2360_2366delCCCTGTC		Sticova (2013) World J Gastroenterol 19, 946
c.2366C>T	p.S789F	Hirouchi (2004) Pharm Res 21, 742, Megaraj (2011) Pharmacogenet Genomics 21: 506

		Wada (1998) Hum Mol Genet 7, 203,
c.2439+2T>C		Toh (1999) Am J Hum Genet 64: 739
c.298C>T	p.R100*	Shoda (2003) Hepatol Res 27, 322
c.3196C>T	p.R1066*	Paulusma (1997) Hepatology 25, 1539
c.3258+1G>A		Sticova (2013) World J Gastroenterol 19, 946
c.3399_3400delTT		Lee (2006) Pediatr Res 59, 584
c.3449G>A	p.R1150H	Mor-Cohen (2001) J Biol Chem 276, 36923
c.3517A>T	p.I1173F	Mor-Cohen (2001) J Biol Chem 276, 36923
c.3521G>A	p.R1174H	Arlanov (2012) Hum Mutat 33, 750
c.3542G>T	p.R1181L	Arlanov (2012) Hum Mutat 33, 750
		Floreani (2006) Hepatology 43, 1152,
c.3563T>A	p.V1188E	Sookoian (2009) J Nutr Biochem 20: 765
c.3732T>G	p.N1244K	Arlanov (2012) Hum Mutat 33, 750
		Corpechot (2006) Am J Gastroenterol 101,2427,
c.3817A>G	p.T1273A	1000 Genomes Project (2010) Nature 467: 1061
c.3825C>G	p.Y1275*	Lee (2006) Pediatr Res 59, 584
c.3872C>T	p.P1291L	Arlanov (2012) Hum Mutat 33, 750
c.3928C>T	p.R1310*	Tate (2002) Genes Genet Syst 77, 117
		Sookoian (2008) J sHepatol 48, 125,
c.3972C>T	p.I1324I	Benz-de Bretagne (2011) J Biomed Biotechnol 2011: 498757
c.4054G>C	p.E1352Q	Lee (2006) Pediatr Res 59, 584
		Toh (1999) Am J Hum Genet 64, 739,
c.4145A>G	p.Q1382R	Hashimoto (2002) Hepatology 36: 1236
c.4175_4180delGGATGA		Tsujii (1999) Gastroenterology 117, 653
c.4292_4293delCA		Cebecauerova (2005) Gastroenterology 129, 315
		Hirouchi (2004) Pharm Res 21, 742,
c.4348G>A	p.A1450T	Megaraj (2011) Pharmacogenet Genomics 21: 506
c.4430C>T	p.T1477M	Megaraj (2011) Pharmacogenet Genomics 21,506
		Elens (2011) Pharmacogenet Genomics 21,884,
c.4544G>A	p.C1515Y	Sookoian (2009) J Nutr Biochem 20: 765

I.IX.I. Non-pathologenic variants in *ABCC2* gene

The biggest interest in *ABCC2* and its variants is related to its drug-metabolizing function. Functional SNPs in *ABCC2* as well as in other transporters (*ABCB1, ABCB2*) have been investigated for their modulatory effect on mrp2 expression or other association with anti-cancer therapy such as tamoxifen, cisplatin etc (Schwabedisser et al 2005, Dai et al,2008, Kiyotani et al 2010) or with antiepileptic drugs, carbamazepine or valproate (Hung et al 2012)

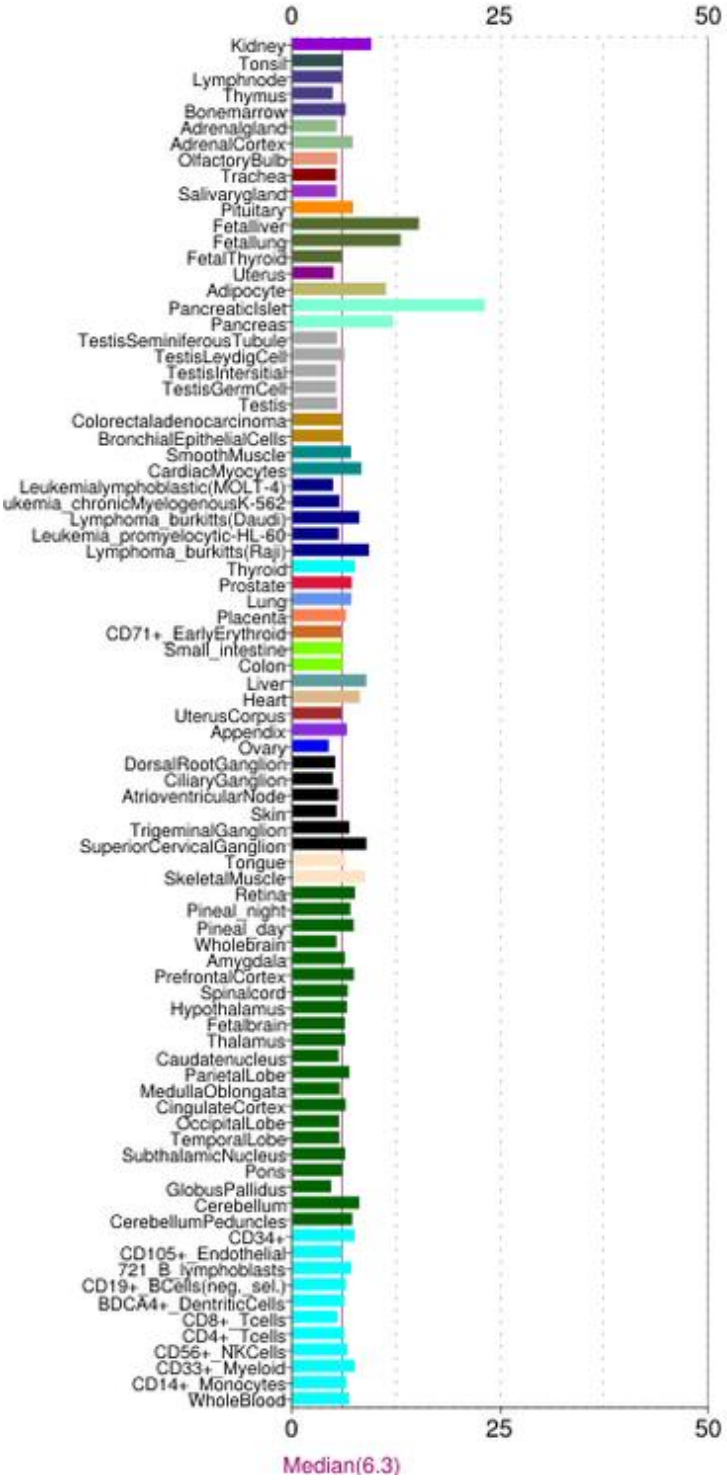
I.IX.II. Expression of *ABCC2*

ABC transporters play an important role in biotransformation of various organic anions and elimination of toxic compounds from the organism. The largest, ABCC subfamily has 13

transporters, most of them involved in active transport of xenobiotics and other metabolic products. ABCC2 or MRP2 is a 190 kDa transmembrane protein expressed on many tissue barriers, apical membrane of polarized cells. In vertebrates it is hepatocytes, enterocytes, renal proximal tubules, placenta, or in endothelial cells of blood-brain barrier. The expression is regulated by several chemical or biological agents such as drugs (phenobarbital, glucocorticoids), by transcription regulators (Nrf2), or by disease conditions such as cholestasis or in the presence of a tumor (Wang et al, 2014, Payen et al, 2002).

MRP2 protein is highly conserved and occurs in protozoa, bacteria, plants or, vertebrates. AA conservation within the species (orthologs). The amino acid identity between MRP2 and its paralog MRP1 is not high ~ 48%, but the substrate specificity is surprisingly similar for both of the transporters (Jedlitschky 2006).

Figure 7 - Expression of *ABCC2* in various tissues, according to NCBI



I.IX.II. Molecular characteristics of MRP2

Cellular localization of MRP2 corresponds to its function as the ATP transporter. Achored in cytoplasmic membrane, MRP2 exports and imports various substrates using energy from ATP hydrolysis. The protein has 1545 amino acids, three membrane spanning domains, a linker and two nucleotide binding domains (NBD). In nucleotide binding domain, ABC transporterss have

the specific motifs with a consensus sequence is called Walker motifs, specifically Walker motif A, B, and C. These motifs are highly conserved and MRP2 has all of them (Taniguchi et al,1996).

Transmembrane domains MSD1 and 2 consists of six transmembrane helixes. Compared to the other ABC proteins forming only two membrane spanning domains (MSD1, MSD2), MRP2 has one more MSD located at NH2- end of the protein. This MSD0 is important for targeting and anchoring to the apical membrane (Hipfner 1997). The other end of the protein, the C-terminal part, contains a PDZ-binding domain, critical for the apical sorting of the protein (Nies et al, 2002).

The mechanism of substrate transport suppose the existence of two binding sites – one for the substrate and the other one for regulating the affinity by allosteric regulation (Zelceret al, 2003). To enable transport, tight cooperation of both units is necessary.

Crystal structure of ABC transporters, thanks to their high impact on human life, has been studied with a tremendous effort and interest. Since the transporters have several domains, and are anchored in a plasma membrane, it was not easy to obtain the structural information at all. Up until today, the only crystal structure of MRP2 was obtained from trypanosoma brucei in a complex with MRP1 and guide RNA by Schumacher et al (Schumacher, et al 2006).

I. IX.III. Post translational modification of MRP2

Post translational modifications of proteins (PTM) are reactions based on the covalent addition of functional group to the proteins after translation and they play an important role in cell signaling. Several PTMs are known for MRP2 – glycosylation and ubiquitination. Cells are using the ubiquitination in a process of elimination of disrupted proteins by marking the protein by ubiquitin and by then adding ubiquitins next. Ubiquitinated protein is recognized and targeted to

proteasome for further destruction. Ubiquitination in MRP2 has been described in rats and also in cell culture by Hayashi and Aido.

I.IX.IV. Mrp2 and drug resistance

The first instance of multidrug resistance of ABC protein was made already in 1976 by Juliano, observing P-glycoprotein abundantly expressed in Chinese hamster ovary drug-resistant cells. The exo- and endogenous ATP-dependent anionic pump protects organisms from the toxic compounds of their catabolism across the cell membrane, transporting mainly conjugates with glucoronate, glutathione, sulphate or amphiphilic anions. This life-protecting function of the cell and organism on the other hand has become the serious obstacle in pharmacotherapy. Multidrug resistance is implicated in numerous failures of cancer treatment, in resistance to antibiotics or herbicide resistance in plants (Holland et al).

Due to the severe consequences of multidrug resistance, a great effort has been developed to eliminate MRPs function during cancer treatment; and for such a purpose, four generations of MRP modulators have already been created (Coley et al). MRP2 modulators supposedly work by stimulating or inhibiting two MRP2 binding sites – ones the substrate binding site or the other – affinity modulating site. However, each modulator interacts with MRP2 and substrate specifically depending on the substrate (Zelcer et al, 2003).

I.IX.V. Animal models

The first animal model considered as the alternative of Dubin-Johnson syndrome in humans was Corriedale sheep, characterized by the pigmented liver and disturbed excretion of coproporphyrins (Cornelius et al, 1965). TR- rat strain, also called Groningen Yellow, was developed as the next animal model and led to the discovery of the connection between Dubin-Johnson and MRP2 and further enabled functional characteristic of MRP2. TR- rats have a deletion in g.1179.Also Eisai rats, the other model of DJS, derived from Sprague-Dawley and Wistar rats, are still used these days. Eisai rats have a mutation in ABCC2 resulting in a stop codon. A rare model of DJS, a golden lion tamarin monkey is characterized by conjugated hyperbilirubinemia and bromosulfophthalein retention; it is not currently used for DJS studies (Schulman et al).

I.IX.VI. In vitro system

Systems in vitro has been developed mainly to study and characterize the transporter`s function and substrate binding on cellular level. Compared to animal models, in vitro experiments use transport vesicles as well as cell cultures; these models enable fast and affordable experiments. In early studies of bile and glucuronide transport, the liver plasma membrane vesicles were used, but for the studying the substrate and sole transporters itself the model wasn`t sufficient. Liver plasma membrane vesicles contain a lot of ABC transporters, therefore a more specific system was necessary. Cell lines provided the adequate technology for this purpose and they are used for studying MRP2 up until today – HEK 293, MDCK II. Another favourable model to study drug elimination, transporters and substrate specificity is primary hepatocytes, which –compared to transport vesicles and cell lines – provides the natural system for ABC transporters, preserving their natural environment (Guofeng).

I.X. Dubin-Johnson syndrome

As the inherited hyperbilirubinemia, Dubin-Johnson syndrome (OMIM 237 500) was firstly described by Dubin and Johnson and coincidentally by Sprinz and Nelson in 1954. Thanks to the similarity of disturbances in coproporphyrin isomers excretion, one period of time it was thought that DJS is one of the other metabolic disorders – porphyrias (Kadish, 1999), resulting from the defect of uroporphyrin co-synthetase. However, this enzyme has been found linked to congenital erythropoetic porphyria and the pathways of both proteins were found totally separate.

DJS is a rare recessively inherited form of conjugated hyperbilirubinemia. It is caused by the homozygous or compound heterozygous mutations in ABCC2 gene. Genetic disturbance results in a defected protein responsible for mediating the transport of bilirubin glucuronides and other endogenous substances out of the hepatocytes.

I.X.I. Clinical and laboratory characteristics of DJS

Laboratory tests do not show elevated transferases or alkaline phosphatase in DJS patients. Clinically, several patients have vague symptoms of abdominal pain or fatigue, and depending on

the bilirubin level, jaundice can occur. There is no pharmacotherapy necessary. There are several clinical signs of DJS with no other liver pathology:

1/ Increased conjugated bilirubin in blood and a mild elevation of total bilirubin (Kadish for total) The reference range for conjugated bilirubin in blood of healthy individuals is < 4 umol/L, patients with DJS show cBi > 4 umol/L. This effect is caused by inability of hepatocytes to transfer conjugated bilirubin into the bile, normally mediated by MRP2. When MRP2 is mutated, bilirubin, instead of going to the bile to be further metabolised in the intestine, mostly returns back to the blood stream.

2/Disturbed pattern of coproporphyrin isomers excretion in urine with predominance of coproporphyrin I (Koskelo 1967, Ben-Ezzer 1971). Physiologically, there are 2 isomers of coproporphyrin excreted to the urine of healthy adults: coproporphyrin I and coproporphyrin III. In humans, Isomer III occurs as the product of the heme degradation. In healthy adults, the ratio is 70-80% of coproporphyrin III compared to 20-30% of excreted coproporphyrin III in patients with DJS. Heterozygotes carrying one mutated copy of *ABCC2* and one healthy allele are excreting mostly around 40-60% of coproporphyrin III. The excretion of coproporphyrins also varies in newborns, where the hepatobiliary system is not yet matured. The mechanism of inverted excretion of coproporphyrin isomers has not been described yet, however it is clear that there is some connection with MRP2, which is also expressed at epithelial cells of kidneys proximal tubuli. Before the era of molecular genetics the analysis of coproporphyrin isomers have been the major laboratory tool to distinguish between homozygotes DJS and heterozygous carriers.

3/ Delayed excretion of sulfobromophtalein (BSP) or 99mTc-HIDA in bile tract.

Shani et al described delayed retention of sulfobromopthalein in DJS patients. Plasma BSP has been observed to be higher in 90 minutes after injection compared to 45 minutes. Again, this effect of delayed excretion of BSP organic anion is caused by the defect or absent MRP2, so the conjugates are returned to the blood stream. The same principle has also been documented on the administration of bilirubin IXa to the patients with DJS or, with the excretion of (64)Cu-5 in TR- rats (Yoo). The experiment using BSP is not used anymore however for the visualization of gallbladder and bile ductus is mostly used 99mTc-HIDA, developed by Bar-Meir et al. *transitions*

4/pigmentation of liver

A pigment presented in lysosomes of hepatocytes in patients with DJS is found by a liver histology. The pigment has been characterised as a melanin like pigment containing lipofuscin, also presented in DJS animal model – a Corriedale sheep (Cornelius C, 1965). Experiments with diet of TR- rats, the other model of DJS, showed, that liver pigmentation develops when diet is rich on aromatic amino acids, suggesting the clearance of their metabolites by MRP2 (Kitamura T) . Except the pigmentation, liver physiology remains intact. However, pigmentation hallmark of DJS is not found in all of the patients with DJS.

I.X.II. Population genetics of DJS

As all hyperbilirubinemias except Gilbert`s syndrome, Dubin-Johnson syndrome is a rare disorder. The higher prevalence of recessively inherited Dubin-Johnson syndrome is linked to the isolated populations. Such populations are often characterized by the common place of origin, cultural, historical, religion or social attributes. The impact resulting from the enclosed populations is loss of natural genetic variability within the generations and higher occurrence of recessively inherited disorders, such as Dubin-Johnson or Rotor syndrome. According to literature, DJS is highly prevalent in Iranian (1:20, mutation I1173), Moroccan (frequency of carriers 1:100, R1150H) or Ashkenazi Jews (Mor Cohen et al, 2005). Founder effect was discovered in a population of Slovakian Roma according our study and confirmed by haplotyping (Slachtova et al, 2015).

I.XI. Rotor syndrome

Recently discovered Rotor syndrome (RS, OMIM 237 450) is a conjugated type of inherited hyperbiblirubinemia, caused by a defects in two genes encoding transport proteins. The association of OATP1B1 and OATP1B3 with Rotor syndrome was found by Steeg et al in 2012 (Steeg et al, 2012). Both proteins are ATP dependent pumps, responsible for transport of organic anions on the membrane of hepatocytes. Conjugated hyperbilirubinemia is mild, the excretion of coproporphyrin isomers is switched as well as in Dubin-Johnson syndrome. However, the excretion of coproporphyrin isomer I is ~ 60% compared to 80 – 90 % as it is in DJS. The bile tract cannot be visualised at all (Wolkoff et al, 1976).

II. AIMS OF THE STUDY

The aims of the study were to characterize the molecular pathology of hereditary hyperbilirubinemias in Caucasian and Roma population and to compare the clinical and biochemical results with the molecular genetic data.

1. To find the coincidence of c.-3279T>G variant in PBREM of *UGT1A1* and UGT1A*28 allele with TA insertion in TATAA box and its impact on serum bilirubin levels in probands with Gilbert`s syndrome and in healthy controls

2. To characterize the Dubin-Johnson syndrome on both molecular genetic and biochemical levels; To perform a mutation analysis of *ABCC2* in patients with DJS and its effect on bilirubin elimination

3. To study the excretion of coproporphyrin isomers in patients with DJS and its comparison with the genetic defect in *ABCC2*

4. To describe genetic and biochemical characteristics of Dual hereditary jaundice

III. HYPOTHESES

We tested following hypotheses:

1. Coincidence of c.-3279T>G variant in PBREM of *UGT1A1* and UGT1A*28 allele with TA insertion in TATAA box impact on serum bilirubin levels in probands with Gilbert`s syndrome. Based on the functional studies of c.-3279T>G variant we suppose that the presence of both genetic defects in UGT1A1 results in increased level of total serum bilirubin.

2. Patients with Dubin-Johnson syndrome have elevated conjugated bilirubin in blood; hyperbilirubinemia depends on the size of damage of ABCC2 protein.

3. Excretion of coproporphyrin isomers reflects the genetic disturbances in ABCC2, resulting in elevated isomer I in urine samples of the patients with DJS. Excretion is dependent on genetic background of the defect and on environmental factors of xenobiotic elimination.

4. Patients with Dual hereditary jaundice, a coincidental defect in both *ABCC2* and *UGT1A1* genes have different patterns of bilirubin elimination compared to a single defect in patients with DJS. We expect that patients with Dual hereditary jaundice have decreased availability of conjugated bilirubin for its transport, compared to patients with only DJS.

IV. MATERIAL AND METHODS

IV.I. Study ethics and informed consent

According to Czech law, all genetic studies carried out have to be approved by responding Ethic and Medical Committee. Informed consent, provided by the subjects included to the studies, has two main purposes. Once, it is in both patient`s interest to obtain sufficient information in advance to any medical procedure, including the blood testing and more importantly, DNA analyses. Secondly, the act is legally documented as the act of the free will by the assignment of three participating subjects (individual or his/her legal representative, the healthcare professional and a witness). All the studies hereto described were held after the proper education of investigated subjects, and the studies were approved by the Committee of Medical Ethics at the First Faculty of Medicine, Charles University and General Faculty Hospital in Prague.

IV.II. Patients

All individuals enrolled to the studies were Caucasians from Czech or Slovak Republic. In the first study, the patients and controls from the study #1 were adolescents from Department of Pediatrics, First School of Medicine, Charles University and General Faculty Hospital in Prague. In the second study, patients and their family members were enrolled by clinical genetics based on the signs of hereditary hyperbilirubinemia. All patients and family members from study #2 are Roma origin living in Slovak Republic.

IV.III. Measurement of bilirubin

In all patients and in most of the family members the standard biochemical liver tests (ALT, AST, total and conjugated serum bilirubin) were performed from 7ml anticolaguant blood sample. Bilirubin was measured by a photometry based on diazo reaction developed by van den Bergh using Ehrlich` s reagent. The measurement was carried on automatized analyzers (Beckman Coulter) as a part of routine biochemical laboratory testing.

IV.IV. Coproporphyrin isomers` analysis

Coproporphyrine isomers were measured from the urine samples of 8 patients with hereditary hyperbilirubinemia (study #2). For each measurement, 100 mL of urine per patient was taken and stored in a dark at -80C and further separated by HPLC. HPLC instrument (GE Healthcare) was equipped by a fluorescence detector, allowing excitation at 405 nm and emission at 620 nm. The column and precolumn cartridge used for separation of was from Chromsystems (44100, 18044). Buffers used for the analysis were Mobile Phase A and B from the same manufacturer (#44001, #44002). Coproporphyrin isomers were separated using commercially available kit Porphyrins in Urine (# 44000, Chromsystems).

IV.V. Molecular genetic analyses

For DNA analyses, 2,5 mL (for infants, children) or 7 mL of whole blood in EDTA were taken. DNA was extracted from peripheral leukocytes using standard desalting method or Qiagen QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany), following the manufacturer`s protocol. DNA was eluted in water and stored in 4°C for a long term use. For DNA analyses, DNA was further diluted for working concentration 1-20 ng/uL. Primers used for amplification were designed, with exception of primers for c.-3279T>G, described previously (Sugatani et al, 2002).

IV.V.I Gene UGT1A1 and surrounding region

TA insertion in UGT1A1 gene

HGVS names: NG_6002601.2:g.175492_93 [insTA]

rs8175347

UGT1A*28

We have investigated the most important variant causing GS in UGT1A1, UGT1A*28. Initially, the key region - the TATA box of UGT1A1 gene was sequenced (Slachtova et al, 2009). Even when we`ved experienced high quality results by sequencing, we`ve switched to a less time consuming and cheaper method using fluorescent labeled primers – a fragment analysis. For the sequencing

analysis we used the primer with the same sequence but no labeling. For fragment analysis, the primers were labeled using 6FAM dye.

TATAA box of *UGT1A1*

Primer	Sequence	Product	Tm
TATA F	6FAM-AACTCCCTGCTACCTTTGTGG	231/243	60˚C
TATA R	TCCAACAGTATCTTCCCAGCATGGG		

PCR MIX

	uL
PP Mix	6,25
H2O	4,95
Primer forward + reverse 2,5 pmoL	0,4 + 0,4
DNA 50 ng/ul	0,5
Total	12,2

PCR Program

1. 95˚C/2 min
2. 95˚C/30 sec
3. 62˚C/40 sec
4. 72˚C/30 sec
5. Go to step 2, repeat steps 2-4/25x
6. 72˚C/30 sec

Variant in phenobarbital enhancer module c.-3279T>G

HGVS names: chr2:hg19:g172270T>G

rs4124874

PBREM enhancer variant was assessed with RFLP described previously (Sugatani et al, 2002) The method use DNA amplification by PCR and following digestion using restriction enzyme. After the PCR product is digested, the products with a different length are separated on 2% agar gel electrophoresis (BioRad), stained with Ethidium Bromide and visualized by Gel Reader.

IV.V.II. *ABCC2* Gene

In second study, all 56 individuals were investigated for a genetic defect in ABCC2 gene. In probands, all 32 exons and adjacent intron regions of ABCC2 gene were investigated. Exons were numbered according to Ref. Seq. NG_011798.1. Proband`s DNA was amplified using PCR, PP Master Mix polymerase with buffer (Top-Bio) and 2.5 pM of primer (see table). Amplified products were purified using Promega A9282 kit and sequenced with 3.2 pM primer by ABI PRISM 3100 AVANT (Applied Biosystems, Big Dye Terminator 3.1 Sequencing Kit). Unlike probands, all healthy family members or carriers were investigated only for a causal disease-causing variant in exon eight.

PCR MIX

	uL
PP Mix	6,25
H2O	4,95
Primer forward + reverse 2,5 pmoL	0,4 + 0,4
DNA 50 ng/ul	0,5
Total	12,2

PCR Program

1. 95˚C/2 min
2. 95˚C/30 sec
3. Tm˚C/40 sec
4. 72˚C/30 sec
5. Go to step 2, repeat steps 2-4/30x
6. 72˚C/30 sec

Primers

Exon	Primer	Sequence 5´-3´	Product Size	Tm
1	DJ1F	aggtcatcctttacggagaaca	209	60
	DJ1R	tgagtctgagaagagtcaatatgaaga		
2	DJ2F	aaagcagtgggatgtgctg	309	60
	DJ2R	tgtctctactgtgcaccaagg		
3	DJ3F	tatccatcaccggaaaccat	310	60
	DJ3R	atttgtttcaccccattcca		
4+5	DJ4-5F	catgttctctgacatccttctcc	311	60

	DJ4-5R	ccgtgagacccagacatctt		
6	DJ6F	cccatgaagttcctgtctcc	312	60
	DJ6R	ggcaacaagagcaaaactcc		
7	DJ7F	gtcccattcttctgtccctct	313	60
	DJ7R	atcgccatgatgctgatgta		
8	DJ8F	gagctgctcaggccagtaac	314	61
	DJ8R	aaaggagggtggcagagga		
9	DJ9F	gtctagctggctgtgcatga	315	60
	DJ9R	tgaggggattttctttggtg		
10	DJ10F	tagtatccttggctttgtcc	316	55
	DJ10R	gggtttacactttaaagaaagc		
11	DJ11F	ccctctctcatggaagcgta	317	60
	DJ11R	gagagccactgcttctgtcc		
12	DJ12F	atacacctggtgccctttca	318	60
	DJ12R	Cctcttcaggaagctgatgc		
13	DJ13F	tagcacaatgctgcttggtc	319	60
	DJ13R	ctggactccaaggcttttaacc		
14	DJ14F	tctctctgcttgtgctcgtt	320	59
	DJ14R	gcgaataagtttgggaagca		
15	DJ15F	gtcacgtggggacctacatt	321	60
	DJ15R	aataggccaggcagtgagaa		
16	DJ16F	tcaatacccaacccctgcta	322	60
	DJ16R	attcgggagtcagaggcttt		
17	DJ17F	tccttcaaccctgcgtttct	323	61
	DJ17R	cttcaatatgccttcacccttg		
18+19	DJ18-19F	tgtgaaggtggatctagggagt	324	60
	DJ18-19R	acccatggcccaagttctat		
20+21	DJ20-21F	gggatctatgcagctctttcc	325	60

	DJ20-21R	tcaaatgctacttttctgtgtgg		
22+23	DJ22-23F	ggttggcattctaggtgattc	326	59
	DJ22-23R	cagtgttgtctaggggggaca		
24	DJ24F	cctcagtgatggtgtatctctcc	327	60
	DJ24R	ggaaagaggctgggctttta		
25	DJ25F	agaaaggaggaagatggtgga	328	60
	DJ25R	aaccccaaagtacacacatgg		
26	DJ26F	gctggttaagatgaggacgtg	329	59
	DJ26R	aacgaaaccaaactcccaac		
27+28	DJ27-28F	ccttgtggtttgagtggttg	330	59
	DJ27-28R	ctgctctccactctgtccaa		
29	DJ29F	ggctaaataacttttccccaaga	331	60
	DJ29R	gcatgtgcccgagtaagttc		
30	DJ30F	gaggtccttttctggcatga	332	60
	DJ30R	aacacgaggaacacgaggag		
31	DJ31F	aacacatggttgcttctattgg	333	60
	DJ31R	aagggttaagccatccgtgt		
32	DJ32F	gcctagacttgagatgctg	334	54
	DJ32R	cacctatttgcatcacca		

IV.V.III. Microarray on Genome Wide Human SNP 6.0

To receive a large-scale data for the haplotyping, we used the Affymetrix Genome Wide Human SNP Array, 6.0 featuring about 1.8 milion genetic markers. The chip carries more than 906 600 of SNP probes with a minor allele frequency > 5 % and over 946 000 of copy number variations. The amount used for genotyping on Affymetrix GWH SNP 6.0, for measurement of concentration and for electrophoresis was 1 ug with a concentration > 50 ng/ul). In advance of hybridization, we checked the quality of our samples to meet the critical criteria for the analysis. 40 samples met the quality control check and continued for further analyses. The hybridization process

includes following steps: 1/ DNA digestion with restriction enzyme StyI, 2/ligation to an adaptor with T4 DNA ligase, 3/PCR of the template with TITANIUM Taq DNA polymerase, 4/ Digestion with NsP restriction enzyme, 5/ Ligation with Nsp, 6/ PCR reaction, 7/ purification of PCR product, 8/ quantification, 9/fragmentation with DNAse, 10/ end-labeling with terminal deoxynucleotidyl transferase and 11/ hybridization of the target.

Genotype calling and quality check control

After the hybridization of DNA to the probes, the data were evaluated with use of Affymetrix Genotyping Console version 4.1. The loss of heterozygosity (LOH) was determined with the same software (Supplementary Figure 1). LOH is a method using a data from the hybridized probes (SNPs, CNVs) to indicate a genetic changes resulting from the loss of variability within the chromosome. It is mostly used in the cancer genomics where it shows the absence of heterozygosity in cancer cells, but it is also a tool for a detection of recessive disorders.

Haplotyping

The genotyping data were entered into PLINK 1.07 and SNPs on chromosome 10 were selected for a further analyses. Unphased genotype data of the whole cohort were entered into the Genotype Visualization and Algorithmic Tool (GEVALT) software, version 2. Phasing, linkage disequilibrium (LD) analysis and estimation of the haplotype block structure was performed utilizing the Genotype Resolution and Block Identification with Likehood (GERBILT) algorithm. A haplotype block of 21 SNPs encompassing the site of c.1013_1014delTG variant in *ABCC2* gene was selected to include following SNPs rs2756115, rs17112219, rs3184991, rs2093354, rs2804412, rs2804409, rs2756095, rs717620, rs4919395, rs2756103, rs4148389, rs4148389, rs17222744, rs2804398, rs2756109, rs11816875, rs2804397, rs2273697, rs2002042, rs4148396, rs4148399, rs7476245. The age of the ancestral variant c.1013_1014delTG in Slovakian Roma population was estimated using a standard Luria - Delbrück algorithm modified by Austerlitz in Mathematica 8 using the Mathematica notebook provided by F. Austerlitz. Variant`s age was estimated for a current population size of 400 000 – 450 000 Roma (Sproch et al, 2009).

IV. V. IV. Statistical analyses

Statistical analyses of the first study used calculation of ODDs ratio and linkage disequlibrium analyses. Regular statistical calculations we used in our studies were average, standard deviation or elementary quantification calculations.

IV.V.V. Linkage disequilibrium (D)

The calculation of linkage disequilibrium used in the first study describes a non-random association of two alleles in a chromosome locus/loci and reflect the DNA recombination in a population. For the calculation is necessary to know the frequency of each allele in the sample, which is determined by

p1 = x11 + x12 for allele A1

p2 = x21 + x22 for allele A2

q1 = x11 + x12 for allele B1

q2 = x12 + x22 for allele B2

Linkage disequlibrium D = x11 = p1q1

Linkage disequlibrium is presented when both loci and alleles are independent from each other and its denoted by D≠ 0. The maximum of D is reached when allelic frequencies are 0,5. genetic, the D is mostly calculated together with its normalized level D`

$$D' = \frac{D}{D_{\max}}$$

$$D_{\max} = \begin{cases} \min(p_1 q_1,\, p_2 q_2) & \text{when } D < 0 \\ \min(p_1 q_2,\, p_2 q_1) & \text{when } D > 0 \end{cases}$$

Linkage disequilibrium is mostly expressed by the correlation coefficient between the loci pairs as

$$r = \frac{D}{\sqrt{p_1 p_2 q_1 q_2}}$$

V. RESULTS

The PhD thesis is based on two papers published in journals with impact factor

1. Slachtova L, Kemlink D, Martasek P, Kabicek P. *Does Bilirubin Level Correspond to Interaction of c.-3279T>G and A(TA)7TAA Variants in UGT1A1 Gene?* Cell Mol Biol 2009, 55: 95-98 (IF 2009 = 1, 154)

2. Slachtova L, Seda O, Behunova J, Mistrik M, Martasek P. *Genetic and biochemical study of Dual hereditary jaundice: Dubin-Johnson & Gilbert syndrome. Haplotyping and founder effect of deletion in ABCC2* Eur J Hum Gen 2015, paper accepted, (IF 2015 = 4,349)

Cited in:

Fozza C, Pardini S, Coiana A, Rosatelli MC, Longinotti M. A novel pattern of uridine diphosphate glucuronosyltransferase polymorphisms associated with hyperbilirubinemia during nilotinib treatment. *Blood Cells Mol Dis.* 2013 Oct;51(3):162

Cellular and Molecular Biology™ **55**, N°1, 98-101
DOI 10.1170/T843

ISSN 1165-158X
2009 Cell. Mol. Biol.™

DOES BILIRUBIN LEVEL CORRESPOND TO INTERACTION OF c.-3279T>G AND A(TA)7TAA VARIANTS IN UGT1A1 GENE?

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Received December 31th, 2008; Accepted January 17th, 2009; Published February 16th, 2009

Abstract – Promoter variants c.-3279T>G and A(TA)7TAA show decreased level of expression of UDP-glucuronosyl transferase 1A1 (UGT1A1) and consequently reduced activity of the enzyme catalyzing glucuronidation of bilirubin in hepatocytes. Thus, coincidental occurence of both variants should lead to increase of hyperbilirubinemia or contribute to its manifestation. In this study, investigation of both variants in 101 patients and 84 controls in a Caucasian population was performed and the results were compared with serum bilirubin levels. Despite high linkage disequilibrium between the loci ($D' = 0.91$, $r^2 = 0.69$), we have proven an interaction between the variants increasing the odds ratio for [(TA)7]+c.[-3279T>G] homozygotes to 54.2.

Key words: c.-3279T>G, A(TA)7TAA, UGT1A1, Gilbert´s syndrome, bilirubin

INTRODUCTION

UGT1A1 catalyzes glucuronidation of bilirubin and other xenobiotics in hepatocytes and in this way it participates in heme degradation. The most frequent variant in the Caucasian population, two base insertion of TA nucleotides in TATAA box of UGT1A1 gene and its association with Gilbert´s syndrome, was firstly described in 1995 by Bosma *et al.* TATAA box region is the binding site of transcription factor IID, which iniciates the transcription. In vitro experiments revealed decreased transcription and reduced enzymatic activity in altered [(TA)7] allele of UGT1A1 up to 10-30% (1, 3, 4, 7, 18).

Impaired glucuronidation results in Gilbert´s syndrome - mild unconjugated hyper-bilirubinemia with fluctuating bilirubin levels from 20 to 50 μmol/l (rarely up to 100 μmol/l).

Abbreviations: **PBREM**, phenobarbital enhancer module; **UGT1A1**, UDP-glucuronosyl transferase 1A1; **A(TA)7TAA**, (TA)7; **OR**, odds ratio.

The frequency of altered [(TA7)] allele among the Caucasian population is 30-40%, resulting in the homozygous Gilbert´s genotype (TA)7/(TA)7 in 9-16% of them. Since only about 5% develops into Gilbert´s syndrome, other factors contributing to the hyperbilirubinemia development are necessary (3, 14, 17, 18).

Other promoter variant c.-3279T>G is located in the phenobarbital responsive enhancer module (PBREM) of the gene. This 51bp enhancer is regulated by the transcription factor constitutive active receptor in response to phenobarbital induction (8). As well as in the previous variant, the reduced activity of UGT1A1 in phenobarbital enhancer variant c.-3279T>G was shown experimentally (20). The coincidental occurence of more genetic defects and/or the effects of epidemiologic factors such as stress, infection, starvation, is supposed to be the cause of the manifestation of hyperbilirubinemia. The relation of more genetic defects and hyperbilirubinemia was well documented in the Japanese population in the most common change p.Gly71Arg and (TA)7 variant.

Also, a haplotype analysis of UGT1A1 gene in the Korean population was published, but distribution of UGT1A1 variants in the Caucasian population differs (12, 13, 20).

Maruo *et al* postulated that coincidental occurence of homozygous variants [(TA)7]+c.[-3279T>G] is the principal cause in development of Gilbert´s syndrome. However, Jirsa *et al* did not confirm this theory. We believe that either genetic or environmental factors can contribute to the development of hyperbilirubinemia. Then in vitro studies can not simulate in vivo bilirubin metabolism conditions properly and we should also be aware of individual differences between bilirubin metabolism in men and women. As we know, no correlation of bilirubin, PBREM and TATAA alterations in UGT1A1 was carried out in probands and controls (10, 11).

MATERIALS AND METHODS

(TA)7 and c.-3279T>G variants were investigated in 101 patients and 84 controls from a Caucasian population. Blood samples were taken with the signed informed consent of the participants. Genomic DNA was extracted from the peripheral blood leukocytes according to the standard procedures. Genotyping of TA repeats of UGT1A1 gene was performed by PCR and the sequencing with 5'-AACTCCCTGCTACCTTTGTGG-3'forward and 5'-TCAACAGTATCTTCCCAGCATGGG-3'reverse primers (product size 239 bp). Analysis of c.-3279T>G promoter variant was carried out by PCR and RFLP with the use of *Hpy81* restrictase (10). In all subjects, liver tests were checked (total serum bilirubin and its conjugated fraction, ALT, AST). Statistic analysis was performed by CubeX and odds ratio calculator (2, 6).

RESULTS

Criteria for probands group were determined according to the guidelines for Gilbert´s syndrome diagnostic – mild unconjugated hyperbilirubinemia with a lower limit of bilirubin 17 μmol/l without hemolysis or other liver injury. Control and proband groups were consistent in sex and age; the average age of controls was 16 years (SD±4.19) and in probands 17 years (SD±5.3). Average total serum bilirubin level in controls was 10.69 μmol/l (SD±4.0) compared with 46.7 μmol/l (SD±26) in probands.

Frequency of wild type [(TA)6] allele in controls was 67.86% versus [(TA)7] allele 32.14 %. Further frequency of allele T in c.-3279T>G variant was 61.31 % in contrast to allele G 38.69 % in controls. Linkage between alleles [(TA)7] and c.[-3279T>G] was D´= 0.79, r^2 = 0.47 in controls. No homozygote for [(TA)7]+c.[-3279G] was found in controls. However, two homozygotes (TA)7/(TA)7 was heterozygous for c.-3279T>G (see Table 1). Correlation of c.-3279 T>G and (TA)7 genotype and bilirubin levels was evident in (TA)6/(TA)7 heterozygotes, however the difference was not significant (see Table 2).

In the probands group (n = 101), allele [(TA)7] frequency was 97%, in the remaining 3% wild type was presented. Also, the wild type allele c.[-3279T] was in a minority (0.99%) in comparison with 99.01% frequency of allele c.[-3279T>G.] All wild type alleles in probands were presented in a heterozygous state. Linkage disequilibrium in probands expressed with D´was high (D´ = 1), but r^2 was very low (r^2 = 0.32). Correlation of genotype and bilirubin levels was not possible to carry out because of the uniformity of genotypes in this group.

Table 1. Distribution of genotypes in probands and controls

	Probands (n = 101)				Controls (n = 84)		
	T/T	T/G	G/G		T/T	T/G	G/G
6/6	-	-	-	6/6	25	6	1
6/7	-	2	4	6/7	4	37	9
7/7	-	-	95	7/7	-	2	0

Table 2. Average of bilirubin levels according to genotype in controls (in μmol/l)

	T/T	T/G	G/G
6/6	8,37 (±2,31)	8,47 (±0,81)	13
6/7	8,48 (±3,66)	10,58 (±4,39)	13,62 (±3,15)
7/7	0	12,3 (±4,53)	0

The overall survey of proband and control group was as follows. There is no doubt about the higher frequency of [(TA)7] allele in probands (97%) than in controls (32.14%) as well as the 99.01% frequency of c.[-3279T>G.] allele in probands compared with 38,69 % frequency of the same allele in controls. Linkage disequilibrium of two variants in both groups was significant D´= 0.91, r^2 = 0.69 (P<0.0001). The distribution of genotypes for the haplotypes formed by these 2 loci is significantly different between patients and controls – P = 0.00005, chi = 24.99, df = 4. The odds ratio (OR) for the risk allele c.[-3279T>G.] in the PBREM is 34.42 (95% confidence interval from 19.14 to 61.89), and 38.71 for the risk allele [(TA)7] in the TATAA box (95% confidence interval from 21.71 to 68.99). The odds ratio increases for the

homozygotes for both risk alleles to 54.20 (95% confidence interval from 30.15 to 97.45).

DISCUSSION

The aim of our study was to establish the role of c.-3279T>G on hyperbilirubinemia in humans. We have demonstrated that this variant is strongly associated with affection by the Gilbert's syndrome, with OR 34.42. This association signal is probably not only due to strong linkage disequilibrum with the other variant (TA)7, because we have shown additive efect of these two variants by demonstrating an increase of the OR for the homozygotes for both variants. We correlated bilirubin levels with coincidental occurence of both variants. Regular data collection and limit estimation is important in epidemiological studies and should not be underestimated. In Gilbert's syndrome, different results can be obtained with diverse criteria. Since the occurence of (TA)7 allele in the Causcian population is 30-40%, we cannot reject controls with (TA)7/(TA)7 with no signs of hyperbilirubinemia. Also the variability of bilirubin in an individual can influence the results, which is why we calculated with average bilirubin level obtained from at least three measurements.

In bilirubin-genotype comparison the most interesting was the effect of c.-3279T>G variant in conjuction with the (TA)6/(TA)7 in controls. In these individuals, the bilirubin levels were the following: (in µmol/l): 8.48 in PBREM wild type T/T; 10.58 in heterozygotes T/G and 13.62 in homozygotes G/G. This indicates the increasing tendency of bilirubin according to the PBREM genotype and which is also apparent the in (TA)6/(TA)6 controls (see Table 2). However, these results has not been found significant, probably due to the low number of examined controls. Bilirubin levelsin (TA)7 variant has been documented many times. In this study, the average serum bilirubin in probands with both homozygous variants (TA)7 and c.-3279T>G was 46.7 µmol/l (SD±26). The high SD of bilirubin level in probands is caused by the presence of about five individuals with bilirubin over 100 µmol/l in probands group. In these five probands, no defect in the structural region of UGT1A1 was found.

Results from both groups contained all genotypes with exception of homozygote (TA)7 together with homozygote wild type PBREM allele c.-3279T. However, according to high linkage disequilibrium D´=0.95 allele (TA)7 is linked with c.-3279T>G allele, this and other studies document genotype (TA)6/(TA)7 together with c-3279T>G/-3279T>G and even (TA)6/(TA)6 coincidentally with c-3279T>G/-3279T>G (data calculated from Jirsa *et al*) (10). The most interesting fact is that we found no homozygote (TA)7 together with homozygote wild type c.-3279T. In no other studies of Caucasians, African-Americans or Japanese with total number of investigated persons more than 800 found this genotype (5, 9, 10, 13). This is remarkable because homozygous wild type (TA)6 together with c.-3279T>G allelic variant does exist. However, controversially the homozygous wild type -3279T together with mutated homozygote (TA)7 does not occur.

ACKNOWLEDGEMENT

For the financial and material support we thank to the grant MSM 0021620806. We thank Prof. Milan Jirsa for critical reading of manuscript.

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Genetic and biochemical study of Dual hereditary jaundice: Dubin-Johnson & Gilbert syndrome. Haplotyping and founder effect of deletion in *ABCC2*

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Running title: Dual inherited jaundice: genetics and biochemistry

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ABSTRACT

Dual hereditary jaundice – a combination of Dubin-Johnson and Gilbert´s syndromes, is a rare clinical entity resulting from the compound defects of bilirubin conjugation and transport. We aimed to study the hereditary jaundice in 56 members from seven seemingly unrelated Roma families, to find the causal genetic defect and to estimate its origin in Roma population. Based on biochemical results of total and conjugated serum bilirubin and clinical observations, *ABCC2* gene, TATA box and phenobarbital enhancer (PBREM) of *UGT1A1* gene were analysed by sequencing, RFLP and fragment analysis. We found a novel variant c.1013_1014delTG in the eighth exon of *ABCC2* gene in 17 individuals in homozygous state. Dual defect NG_011798.1:c.[1013_1014delTG]; NG_002601.2:g.[175492_175493insTA] in homozygous state was found in four subjects. Biochemical analyses of porphyrins and coproporphyrin isomers in urine performed by HPLC showed inverted ratio of excreted coproporphyrin, with the predominance of coproporphyrin I (up to 100 %), typical for patients with Dubin-Johnson syndrome. Pursuant cultural and social specifics of the population led us to suspect a founder effect; therefore we performed a haplotype study using genotyping data from Affymetrix Genome-Wide Human SNP Array 6.0. As a result, we detected a common 86 kbp haplotype encompassing promoter and part of the *ABCC2* coding region among all families, and estimated the age of the ancestral variant to 178-185 years. In this study, we found a novel deletion in *ABCC2* gene, described genetic and biochemical features of dual hereditary jaundice and confirmed the existence of founder effect and common haplotype among seven Roma families.

Keywords: bilirubin, Dubin-Johnson syndrome, Gilbert´s syndrome, *ABCC2, UGT1A1,* coproporphyrin, Roma population, rare diseases

INTRODUCTION

Bilirubin excretion involves bilirubin conjugation with glucuronic acid (mediated via uridine diphosphate glucuronosyltransferase 1A1 - UGT1A1) followed by the transport of bilirubin conjugates from hepatocytes into the bile by MRP2 (multidrug resistance-associated protein 2, coded for by *ABCC2* gene). Genetic defects in bilirubin elimination pathway clinically manifested by jaundice are known as hereditary hyperbilirubinemias. Dubin-Johnson syndrome (DJS; OMIM #237500) is a rare autosomal recessive hyperbilirubinemia characterized by the absence of functional MRP2 protein at the canalicular membrane of hepatocytes.[1-4] MRP2 protects organisms from the toxic compounds of their catabolism and its intact function is a pitfall in anticancer therapy.[5-6] To date, more than 40 variants in *ABCC2* causing DJS have been described (Human Genome Mutation Database) and several SNPs with pharmacogenomics importance. [7,8]

The clinical profile of DJS comprises of: (1) Conjugated hyperbilirubinemia in blood (> 7 µmol/L), (2) inverted ratio of excreted coproporphyrin isomers I to III in urine (> 80 % of isomer I), (3) prolonged visualization of liver by technetium (Tc-99)-labeled hepatobiliary iminodiacetic acid ^{99m}Tc-HIDA and, (4) abnormal deposits of melanin-like pigment in lysosomes of hepatocytes. Liver function remains otherwise normal.[9] The rare DJS is highly prevalent among small populations in Iranian (1:20), Moroccan (frequency of carriers 1:100), or Ashkenazi Jews (www.health.gov.il). [3,10] Such a founder effect reflects the deviation from the natural selection and the population

isolation, potentiated by consanguineous marriages, resulting in a loss of genetic variability over generations.

Gilbert` s syndrome (GS; OMIM #143500) is relatively common among the general population. The frequency of TA insertion in TATA box (rs8175347) of *UGT1A1* gene (Ref. Seq. NG_002601.2) in Caucasians is around 30 %, with the 5 % manifestation by jaundice.[11] Reduced transcription of *UGT1A1* gene with 7 TA repeats (NG_002601.2:g.[175492_175493insTA]) compared to wild type 6 TA repeats. The reduction is often potentiated by simultaneous presence of variant c.-3279T>G (rs4124874) in PBREM, located in a promoter region of *UGT1A1*. Finally, it results in lower glucuronidation causing unconjugated hyperbilirubinemia with the total serum bilirubin > 20 µmol/L and usually < 80 µmol/L.[12,13]

Dual hereditary jaundice, a compound d`efect of bilirubin conjugation (GS) and transport (DJS), was firstly described by Cebecauerova *et al*.[14] Biochemical characteristics of dual hereditary jaundice correspond to predominant defect of bilirubin glucuronides transport, including conjugated and total bilirubin elevation and urinary coproporphyrin isomer I increase as mentioned above.

The population of Roma (Gypsies) is the largest minority in Europe of about 11 millions of individuals. A great majority of European Roma shares the same haplotypes - haplogroup H-M82 of chromosome Y and mitochondrial haplogroup M. Their common ancestors originate 1500 years ago from Northern India.[15-17]

In this work, we investigated the prevalence of dual hereditary jaundice among 56 members from seven seemingly unrelated Roma families. Here, we report the identification of a novel variant

in *ABCC2* gene together with a compound defect in *ABCC2* and *UGT1A1* genes and a shared haplotype among all seven families. These findings demonstrate the highest prevalence of DJS in Europe thus far. Moreover, this is the first study to describe biochemical and molecular genetic defects of bilirubin transport and coproporphyrin excretion in several individuals with dual hereditary jaundice.

PATIENTS AND METHODS

Patients. A total of 56 individuals from 7 unrelated Slovak Roma families were investigated for suspected hereditary hyperbilirubinemia. All participants signed the informed consent approved by the Ethics Committee of General University Hospital in Prague. The first proband was a girl from a large family with a history of noninfection jaundice, born from a consanguineous marriage of two cousins (Figure 1). Her biochemical tests showed total bilirubin (TBi) of 35-70 µmol/L, conjugated bilirubin (cBi) of 31-54 µmol/L (F 1.IV/17) and normal levels of alanine aminotransferase - ALT, and aspartate aminotransferase - AST. She was hospitalized at the age of nine months due to the severe bilirubinemia, acute respiratory infection and suspected anemia and treated with Unasyn, Dithiaden, Halixol, folic acid, Maltofer and glucose. The performed ^{99m}Tc-HIDA cholescintigraphy of liver and biliary tract revealed delayed visualization of liver and gallbladder, typical for Dubin-Johnson syndrome. Similarly, other individuals were tested for TBi and cBi (standard diazo- reaction), ALT and AST.

Genetic analyses. Genomic DNA was extracted from 7 mL whole blood in EDTA using standard procedures. All 32 exons and adjacent intron regions of *ABCC2* gene (NM_000392.4, exons were

numbered according Ref. Seq. NG_011798.1, Supplementary Table 1) were amplified using PCR, PP Master Mix polymerase with buffer (Top-Bio, Prague, Czech Republic) and 2.5 pM of primer; purified (Promega A9282 kit); and sequenced by ABI PRISM 3100 AVANT (Applied Biosystems, Big Dye Terminator 3.1 Sequencing Kit). Fragment analysis of TA insertion of TATA box of *UGT1A1* gene was performed (rs8175347, chr2:hg19:g.175492_175493insTA) (see Supplementary Table 1). PBREM enhancer variant c.-3279T>G (rs4124874; chr2:hg19:g.172270T>G) was amplified and assessed with RFLP as described previously. [12] All sequence variants were annotated according to reference sequences (NG_011798.1 for *ABCC2*, NG_002601.2 for *UGT1A1*), and the findings were submitted to ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar).

Coproporphyrin isomers analysis. Urine samples from 8 patients with jaundice were collected (100 mL per patient), and stored in dark at -80°C. Porphyrins and coproporphyrin isomers I and III were analyzed by HPLC with a fluorescence detector (excitation 405 nm, emission 620 nm), Chromsystems column (# 44100) and kit Porphyrins in Urine (# 44000, Chromsystems).

Microarray, haplotyping and variant´s age estimation. To assess a common haplotype, genomic DNA from 40 individuals was hybridized to Affymetrix Genome Wide Human SNP Array 6.0 according to manufacturer´s recommendation and further analysed. Genotype calling and loss of heterozygosity in 10q24 region was determined with Affymetrix Genotyping Console version 4.1. The genotyping data were entered in PLINK 1.07 [18] and SNPs present on chromosome 10 were selected for further analyses. After performing standard quality checks, unphased genotype data of the whole cohort were entered into the Genotype Visualization and Algorithmic Tool (GEVALT) software, version 2.[19] Phasing, linkage disequilibrium (LD) analysis and estimation of the

haplotype block structure was done utilizing the Genotype Resolution and Block Identification using Likehood (GERBILT) algorithm.[20] A haplotype block encompassing the site of c.1013_1014delTG variant in *ABCC2* gene included 21 SNPs. The age of the ancestral variant c.1013_1014delTG in Slovakian Roma population was estimated using a standard Luria - Delbrück algorithm modified by Austerlitz [21] in Mathematica 8 using the Mathematica notebook kindly provided by F. Austerlitz. Variant`s age was estimated for a current population size of 400 000 – 450 000 Roma.[22]

RESULTS

Biochemical blood tests included total and conjugated serum bilirubin, ALT and AST. ALT and AST ranged within normal levels, indicating the absence of liver damage. TBi in DJS patients was 18.8 - 72.2 µmol/L with cBi 9.5 - 55.2 µmol/L (Table 1). The percentage of cBi out of TBi in patients with dual defect DJS + GS were 28 %, 38 %, 43 % and 89 % (see Table 2). The last value 89 % was found in proband F1.IV/17 on medication with following TBi/cBi: 38/33; 70/55; and 35/31 µmol/L. Values of TBi and cBi of patients with the same *UGT1A1* and *ABCC2* haplotype varied (Table 1).

Molecular genetic analysis of the proband revealed the deletion in *ABCC2* gene c.1013_1014delTG (http://www.ncbi.nlm.nih.gov/clinvar/?term=SCV000195771), responsible for Dubin-Johnson syndrome (Figure 1). The causal variant is located in the eighth exon and, *via* a frameshift, it leads to a premature stop codon p.(Val338Glufs14*) resulting in a shortened protein product. Investigation of TATA box of *UGT1A1* of the same proband showed also

NG_002601.2:g.[175492_175493insTA] causing Gilbert´s syndrome. Next, family members and probands were investigated for the defect in *ABCC2* gene with the total result of 17 homozygous patients with the deletion c.1013_1014delTG and 30 heterozygous carriers as evident from Figure 1. The presence of the same genetic defect in all seven seemingly unrelated families suggested a founder effect of c.1013_1014delTG among the investigated Roma families. Moreover, four patients with homozygous variant in *ABCC2* gene were also homozygous for the TA insertion in TATA box of *UGT1A1* gene with a genotype NG_011798.1:c.[1013_1014delTG]; NG_002601.2:g.[175492_175493insTA] (Table 1). All patients with homozygous TA insertion were also homozygotes for c.-3279T>G variant in PBREM (Table 1 and Supplementary Table 2).

Pedigrees from seven investigated families are depicted onFigure 1. Consanguineous marriages between cousins were found in three of them (F 1, 4, 5). In five families, both parents were heterozygous carriers of variant c.1013_1014delTG. In next two families, fathers´ samples were not available, however the genotypes of offspring suggest heterozygous or homozygous occurrence of the mutated allele in both fathers.

Coproporphyrin isomers excretion. We analyzed coproporphyrin isomers in urine samples of 11 individuals: in eight patients with DJS caused by c.1013_1014delTG in *ABCC2* gene and in three heterozygous carriers. Significantly increased proportion of excreted coproporphyrin isomer I was observed in all DJS patients with values ranging from 89-100 % (Table 2). In heterozygous carriers, the ratio of excreted isomer I was 43-57 %, compared to about 25-30 % in healthy controls (control data from literature).[23,24] No differences were found in excrection of coproporphyrin isomers in patients with DJS compared to thouse with dual defect of DJS + GS.

Microarrays and haplotyping. The analysis of chromosome 10 genotypes in *ABCC2* region revealed a shared 86 kbp haplotype upstream and within *ABCC2* gene, transmitted together with the deletion c.1013_1014delTG (Figure 2, Table 3). Furthermore, the loss of heterozygosity was found in all homozygous patients with DJS with use of CNV analysis from Affymetrix Genotyping Console. Loss of heterozygosity in DJS patients was in all cases in concordance with both genetic and biochemical data.

Estimation of variant´s age. Age of ancestral variant c.1013_1014delTG was estimated using the Luria-Delbrück algorithm modified by Austerlitz.[20,21] Population size of Slovakian Roma inhabitants was set at 400 000 – 450 000 [22] and the frequency of disease allele was estimated between 0.01 – 0.001. Using these data, our results show that the variant originated 7.1-7.4 generations ago, which corresponds to 177.5 – 185 years ago (Supplementary Table 3).

DISCUSSION

Our study describes compound defect of *ABCC2* and *UGT1A* genes, causing Dubin-Johnson and Gilbert`s syndrome. The frequency of TA insertion in *UGT1A1* in Caucasians is about 15 %.[25] Similar distribution may be assumed in the Roma population, although the accurate frequency has not been established yet. The new variant c.1013_1014delTG described herein is located in the eighth exon of *ABCC2* gene. A two base pair deletion leads to frameshift and truncation of the encoded MRP2 protein, from original size of 1545 amino acids to the reduced size of 352 residues. Deletion truncates protein´s two of total three membrane spanning domains (MSD2 and MSD3) and one ATP binding site, and newly formed mRNA probably subjects to a rapid degradation.[4] The excretion of conjugated bilirubin into the bile is performed mainly *via* ABCC2

efflux pump with minor elimination *via* ABCG2.[26,27] However, ABCG2 does not provide sufficient substitution of impaired ABCC2, and bilirubin glucuronides are transported by upregulated basolateral MRP3 into the blood.

Biochemistry. Results of total bilirubin and its conjugated fraction correspond to presence/absence of genetic defect in bilirubin pathway (Table 1). All DJS patients with homozygous presence of c.1013_1014delTG had elevated cBi (9-55 µmol/L) as well as TBi (19-72 µmol/L). Both TBi and cBi showed high variability within the same genotype. Fluctuating bilirubin levels are present in both - DJS and GS. Many factors affecting hyperbilirubinemia have been described, with the most important TA insertion, found in four our patients with DJS and in two healthy DJS carriers.

Effect of a double defect. To date, dual hereditary jaundice has been described only in a single case by Czech authors Cebecauerova *et al.* with no additional studies up today. [15] Our study describes the coincidental defects in two genes of bilirubin metabolism in another four patients. The percentages of cBi out of TBi in our DJS + GS patients were 28 %, 38 %, 43 % and 89 %, respectively (Table 1). We suppose, that coincidental defects of bilirubin conjugation and transport result in decreased amount of cBi available for its transport, therefore the percentage of cBi from TBi is < 50 % in DJS + GS compared to > 50 % in DJS only (Table 1, Supplementary Table 2). Except the last value of 89 % (baby girl proband, data collected under medication), our findings are in agreement with the previous study. Both proteins UGT1A1 and MRP2 are responsible for the drug`s biotransformation, which may explain a higher cBi and TBi in patient with dual defect under medication. Certain drug precautions should be taken in advance of

pharmacotherapy in patients with dual hereditary jaundice including irinotecan, atazanavir, cisplatin, vincristine or doxorubicin to prevent its toxicity or sensitivity. [28,29,]

Coproporphyrin. Urinary excretion of coproporphyrin reflects the heme degradation and excreted isomers are presented as isomers I – IV with the most significant isomers I and III. The proportion of coproporphyrin isomer III in urine samples of healthy adults is 70-80 %, compared to ~ 20-30 % in DJS patients, who excrete about 80-97 % of coproporphyrin I. Under normal conditions, excretion of coproporphyrin isomers depends on maturation of hepatobiliary system and changes within the first days of newborn's life.[30,31]

We have investigated coproporphyrin in 8 patients with DJS caused by c.1013_1014delTG in *ABCC2* and all of them showed shifted ratio excreted coproporphyrin isomers in urine (89-100 %, Table 2). Several studies show > 90 % predominance of coproporphyrin I in urine of patients with DJS, but to our knowledge, no data show results of 100 %. [32,33] Our patient with 100% excretion of isomer I was one year old boy with total serum bilirubin 50 µmol/L and its conjugated fraction 21 µmol/L. Coproporphyrin isomers were analysed from two independent samples with the same result. Before the era of molecular genetics, heterozygous carriers of DJS were only detectable by changed isomers of coproporphyrin in urine. Compared to 20-30 % in healthy controls, heterozygotes secrete about 40 % of isomer I. Average percentage of isomer I in our DJS carriers was 43-57 %, which is similar to data from literature.[23,24,35] Excretion pattern of coproporphyrin isomers in four patients with dual hereditary jaundice was the same as in other DJS patients with no defect in *UGT1A1* (see Table 2). Our findings for coproporphyrin I was 92, 97, 99 and 100 % in patients with dual defect compared to 89, 93, 96 and 98 % in DJS patients with heterozygous NG_002601.2:g.175492_175493insTA and 89 % in DJS patient with the wild type *UGT1A* gene.

Haplotyping and dating an ancestral variant. After the finding an identical genetic defect, we have suspected the founder effect of c.1013_1014delTG variant among Roma families. The consanguinity was observed in 3 of 7 families and all families lived within tens of kilometres of one another (Supplementary Figure 1). We have identified a common haplotype spanning 86 kbp segregating with the deletion in *ABCC2* and a loss of heterozygosity in *ABCC2* region in patients with DJS (Figure 2). Several haplotype analyses of *ABCC2* had been performed due to its pharmacogenomic impact, even one concerning the impact of *ABCC2* haplotype on coproporphyrin excretion, but only using SNPs with proven pharmacogenomics effect.[36,37] There are more than 130 SNPs within *ABCC2* and their importance *in vitro* has mostly not been described yet. Our study clearly shows, that in addition to the *ABCC2* haplotype, which is shared in our DJS patients, other factors may also influence coproporphyrin excretion in urine, such as SNPs in other genes or undiscovered environmental factors.

Dating the ancestral variant c.1013_1014delTG in Slovakian Roma population revealed the probable origin of variant 178 – 185 years ago. Allelic association estimates the age of the ancestral variant from haplotypic data, without specification of the population growth rate.[21]

Conclusion

Our study describes defects in *UGT1A1* and *ABCC2* genes corroborating genetic findings with biochemical results in more individuals with dual hereditary jaundice. In 17 Roma patients with DJS we have detected a new deletion NG_011798.1:c.[1013_1014delTG], which represents the highest prevalence of DJS in Europe. Four of our patients were homozygous for a dual defect

NG_011798.1:c.[1013_1014delTG]; NG_002601.2:g.[175492_175493insTA]. We also uncovered the existence of the founder effect of *ABCC2* variant in all patients from seven families and a shared haplotype 86 kbp and estimated the age of the deletion to 178 – 185 years.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Authors would like to thank Hynek Strnad for his assistance with the raw data, Frederic Austerlitz for his Mathematica notebook and Sudha Veeraraghavan for the critical reading of the manuscript. This work was financially supported by grants UNCE 204011, PRVOUK P24/LF1/3, RVO-VFN 641652012 and further by BIOCEV (CZ.1.05/1.1.00/02.0109). O.S. is supported by MSMT project LK11217.

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Tables (attached)

Table 1 Total and conjugated bilirubin (in μmol/L) and genotype of patients with/out Dubin-Johnson[1] and Gilbert´s[2] syndrome. Legend [1] +/+ homozygote for c.1013_1014delTG in *ABCC2*; [2] 6/6 healthy, 6/7 heterozygote, 7/7 homozygote for g.175492_175493insTA in *UGT1A1*; **bold - double homozygotes**

Table 2 Excretion of coproporphyrin isomers I and III in urine and serum bilirubin in patients with Dubin-Johnson (+/+) and Gilbert´s syndrome (7/7) and in healthy carriers (+/-) or (6/7)

Table 3 A shared haplotype in family 2

SUPPLEMENTARY (attached)

S Table 1 Primers and PCR conditions

S Table 2 Total and conjugated bilirubin (in µmol/L) and genotypes of heterozygotes and healthy individuals. Legend DJS -/- healthy; +/- heterozygote; +/+ homozygote for c.1013_1014delTG in *ABCC2*; GS 6/6 healthy, 6/7 heterozygote, 7/7 homozygote for g.175492_175493insTA in *UGT1A1*

S Table 3 Estimation of age of deletion c.1013_1014delTG in *ABCC2* in Slovakian Roma

S Figure 1 Map of the Slovakia with indicated occurrence of founder effect of c.1013_1014delTG in *ABCC2*

Titles and legends to figures

Figure 1a Sequences of deletion in *ABCC2* c.1013_1014delTG (Ref Seq NG_011798.1; NM_000392.4) 1. homozygous variant c.1013_1014delTG in patient with DJS 2. heterozygous variant in carrier 3. wild type

Figure 1b Pedigree of the families with Dubin-Johnson syndrome ■homozygote, ◨Heterozygote,□healthy,□ male,○ female,◇ unknown – abortion; N – not investigated; arrow – proband; double bond indicates consanguinity;

Figure 2 The span of a common haplotype in families with DJS encompassing c.1013_1014delTG in *ABCC2* gene with indication of linkage disequilibrium (r^2). Black diamonds without numerical annotation correspond to complete linkage disequilibrium between the SNPs. Only polymorphic SNPs are shown.

Figure 1a

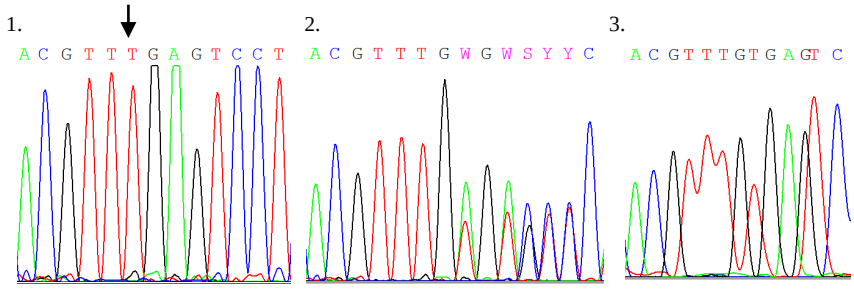


Figure 1b

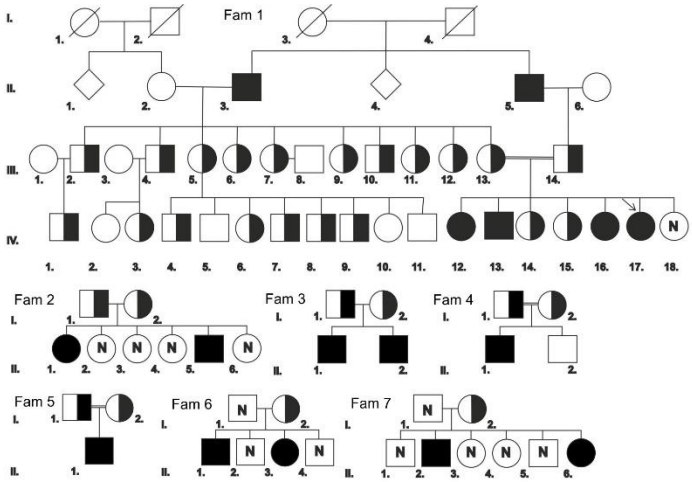


Figure 2

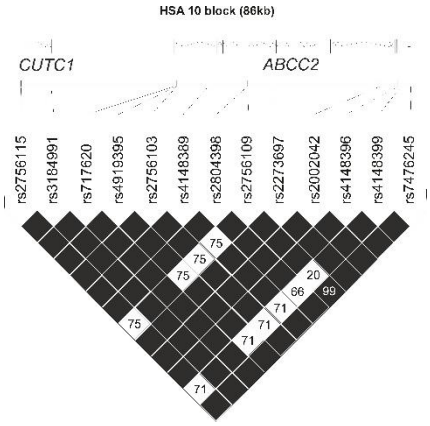


Table 1

Family	Genealogy	DJS[1]	GS[2]	PBREM	cBi	Tbi	% cBi of Tbi
F1	II/2	-/-	6/6	T/T	1.9	5.7	33.3
	II/3	+/+	6/7	T/G	28.7	46.5	61.7
	II/5	+/+	6/7	T/G	55.2	72.2	76.5
	II/6	-/-	6/7	T/G	2.3	7.6	30.3
	III/1	-/-	6/7	T/G	1.8	3.6	50.0
	III/2	+/-	6/7	T/G	2.0	12.3	16.3
	III/3	-/-	6/6	T/T	0.1	4.6	2.2
	III/4	+/-	6/7	T/G	2.8	7.2	38.9
	III/5	+/-	6/6	T/T	2.3	6.5	35.4
	III/6	+/-	6/7	T/T	1.8	5.1	35.3
	III/7	+/-	6/6	T/T	1.0	2.8	35.7
	III/8	-/-	6/6	T/T	2.4	9.5	25.3
	III/9	+/-	6/7	T/G	0.3	9.0	3.3
	III/10	+/-	6/7	T/G	1.2	1.5	80.0
	III/11	+/-	6/6	T/T	0.7	5.7	12.3
	III/12	+/-	6/6	T/T	0.2	3.7	5.4
	III/13	+/-	6/7	T/G	0.3	5.0	6.0
	III/14	+/-	6/7	T/G	0.2	5.5	3.6
	IV/1	+/-	7/7	G/G	1.5	6.8	22.1
	IV/2	-/-	6/7	T/G	3.0	3.8	78.9
	IV/3	+/-	6/6	T/T	0.2	4.7	4.3
	IV/4	+/-	6/7	T/G	-	-	-
	IV/5	+/-	6/7	T/G	-	-	-
	IV/6	+/-	6/7	G/T	-	-	-
	IV/7	+/-	6/7	T/G	-	-	-
	IV/8	+/-	6/7	T/G	1.8	5.8	31.0
	IV/9	+/-	6/7	T/G	-	-	-
	IV/10	-/-	6/7	T/G	3.0	5.5	54.5
	IV/11	-/-	6/7	T/G	-	-	-
	IV/12	+/+	6/6	T/T	16.7	28.1	59.4
	IV/13	**+/+**	**7/7**	**G/G**	**17.7**	**46.7**	**37.9**
	IV/14	+/-	6/6	T/T	1.3	4.5	28.9
	IV/15	+/-	6/7	T/G	3.2	12.7	25.2
	IV/16	+/+	6/7	T/G	9.5	18.8	50.5

	IV/17	**+/+**	**7/7**	**G/G**	**31.0**	**35.0**	**89.0**
Family	Genealogy	DJS[1]	GS[2]	PBREM	cBi	Tbi	% cBi of Tbi
F2	I/1	+/-	7/7	G/G	-	-	-
	I/2	+/-	6/7	T/G	-	-	-
	II/1	+/+	6/7	T/G	11.4	32.4	35.2
	II/5	**+/+**	**7/7**	**G/G**	**16.9**	**60.4**	**28.0**
F3	I/1	+/-	6/7	T/G	-	-	-
	I/2	+/-	6/7	T/G	-	-	-
	II/1	**+/+**	**7/7**	**G/G**	**21.4**	**49.5**	**43.2**
	II/2	+/+	6/6	T/T	-	-	-
F4	I/1	+/-	6/7	G/G	-	-	-
	I/2	+/-	6/7	G/G	-	-	-
	II/1	+/+	6/6	T/G	28.0	42.0	66.7
	II/2	-/-	6/7	T/G	-	-	-
F5	I/1	+/+	6/6	T/G	32.7	39.9	82.0
	I/2	+/-	6/7	T/G	-	-	-
	II/1	+/+	6/7	T/G	-	-	-
F6	I/1	+/-	6/6	T/T	-	-	-
	II/1	+/+	6/7	T/G	-	-	-
	II/3	+/+	6/7	T/G	17.6	43.5	40.5
F7	I/2	+/-	6/6	T/G	-	-	-
	II/2	+/+	6/6	T/T	32.4	57.4	56.4
	II/6	+/+	6/6	T/T	-	-	-

Table 2

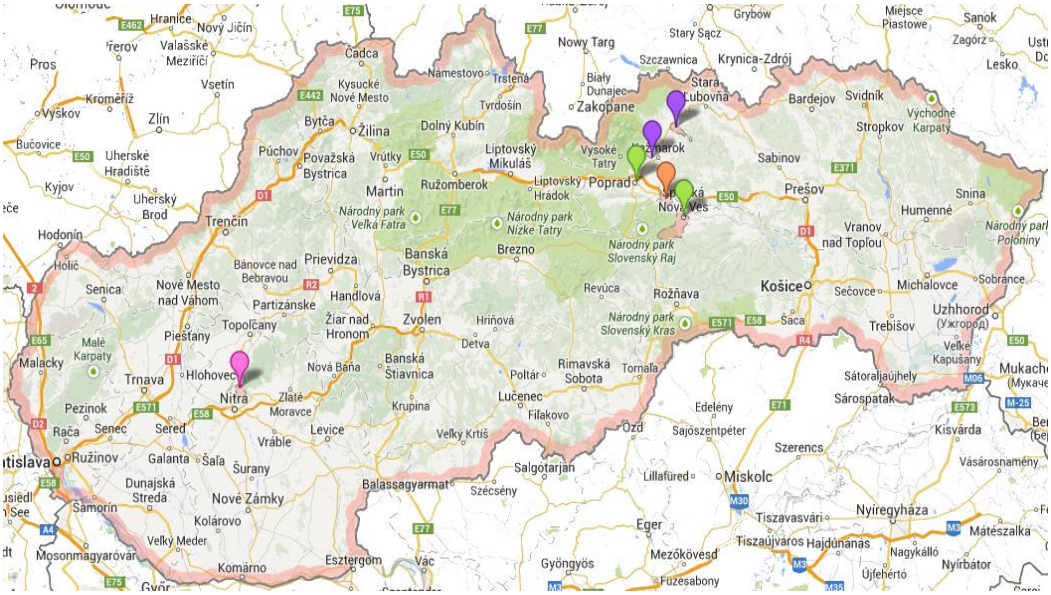
	F1.IV/13	F1.IV/17	F2.II/5	F3.II/1	F1.II/5	F1.IV/16	F2.II/1	F3.I/1	F4.I/1	F4.I/2	F4.II/1
Total porphyrins [nmol/L]	212	348	258	78	587	230	205	91	93	58	133
Uroporphyrin [nmol/L]	88	80	78	13	118	74	84	10	6	6	19
Coproporphyrin [nmol/L]	124	268	180	63	469	156	121	78	75	52	110
Isomer I (%)	**99**	**97**	**92**	**100**	**98**	**96**	**93**	**43**	**53**	**57**	**89**
Isomer III (%)	**1**	**3**	**8**	**-**	**2**	**4**	**7**	**57**	**47**	**43**	**11**
Conjug. bilirubin [µmol/L]	17.7	31.0	16.9	21.4	55.2	9.5	11.4	-	-	-	28.0
Total bilirubin [µmol/L]	46.7	35.0	60.4	49.5	72.2	18.8	32.4	-	-	-	42.0
DJS	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/-	+/-	+/+
GS	7/7	7/7	7/7	7/7	6/7	6/7	6/7	6/7	6/7	6/7	6/6

Ref. ranges: Total porphyrins <300 nmol/L; uroporphyrin < 40 nmol/L; coproporphyrin <150 nmol/L; Conjug. Bilirubin < 7 µmol/L, total bilirubin < 20 µmol/L

Table 3

	I/1 father		I/2 mother		II/1 daughter		II/5 son	
rs2756115	T	C	T	C	C	C	C	C
rs17112219	G	G	G	G	G	G	G	G
rs3184991	T	T	T	T	T	T	T	T
rs2093354	A	A	A	A	A	A	A	A
rs2804412	G	G	G	G	G	G	G	G
rs2804409	T	T	T	T	T	T	T	T
rs2756095	A	A	A	A	A	A	A	A
rs7176201	C	C	C	C	C	C	C	C
rs4919395	G	A	G	A	A	A	A	A
rs2756103	A	C	A	C	C	C	C	C
rs4148389	A	G	A	G	G	G	G	G
rs17222744	A	A	A	A	A	A	A	A
rs2804398	A	T	A	T	T	T	T	T
rs2756109	T	T	T	T	T	T	T	T
rs11816875	G	G	G	G	G	G	G	G
rs2804397	G	G	G	G	G	G	G	G
c.1013_1014delTG	W	*	W	*	*	*	*	*
rs2273697	A	G	A	G	G	G	G	G
rs2002042	C	C	C	C	C	C	C	C
rs4148396	C	T	C	T	T	T	T	T
rs4148399	T	G	T	G	G	G	G	G
rs7476245	G	G	G	G	G	G	G	G

Supplementary:



VI. DISCUSSION

VI.I. Aim 1, Paper 1 - Does Bilirubin Level Correspond to Interaction of c.-3279T>G and A(TA)7TAA Variants in UGT1A1 Gene?

Functional studies show, that promoter variant c.-3279T>G, as well as A(TA)7TAA decrease the transcription of *UGT1A1* up to 60 %, resulting in consequently reduced glucuronidation of bilirubin and affecting its elimination as a toxic product (Bosma et al, 1995, Sugatani et al, 2002). Therefore, coincidence of both variants should lead to significant increase of hyperbilirubinemia. Combined genetic defect affecting glucuronidation of bilirubin and its elimination was described on the compound defects of A(TA)7TAA and other variants, located in a coding region of *UGT1A1* (Kamisako, 2004).

In our cohort of 101 patients and 84 controls from general Caucasian population we have studied the impact of coincidental occurrence of both genetic defect located in promoter region of *UGT1A1* on serum bilirubin and calculated the linkage disequlibrium of both variants. It is important to highlight, that presented data are describing genetic defects in Caucasian population. In Japanese population, the prevalent variant causing Gilbert`s syndrome is G211A (Koiwai et al, 1995).

The frequency of [(TA)7] allele in healthy controls was 32%, which responds to the data from literature (Bosma et al, 1995, Jirsa et al, 2006, Kabicek 2007). Regarding c.-3279T>G variant, frequency of this variant was 39% among healthy population. As expected, significantly higher incidence of both variants was found in patients with GS – 99 % carried variant c.-3279T>G variant and 97 % had [(TA)7] allele. The linkage between both variants in controls was D´= 0.79, r^2 = 0.47 compared to linkage in patients with GS D´ = 1, but $r^2$was very low (0.32). The ODDs ratio of c.-3279T>G variant and Gilbert`s syndrome was OR 34.42, confirming the strong association of PBREM promoter variant and GS.

The linkage of c.-3279T>G and A(TA)7TAA in promoter region of UGT1A1 was previously suggested by Maruo et al (Maruo et al, 2004), who postulated the hypothesis, that the presence of both variants is necessary for the manifestation of Gilbert`s syndrome. However, next study conducted by Czech authors Jirsa et al on a larger cohort showed, that even when the linkage of both variant is strong, it is not necessary for clinical manifestation of GS (Jirsa et al, 2006).

The effect of both promoter variants c.-3279T>G and A(TA)7TAA in *UGT1A1* on serum bilirubin is summarized in Table 2 (Slachtova et al, 2009).

	T/T	T/G	G/G
6/6	8,37 (±2,31)	8,47 (±0,81)	13
6/7	8,48 (±3,66)	10,58 (±4,39)	13,62 (±3,15)
7/7	0	12,3 (±4,53)	0

With the development of molecular genetic techniques, current data confirm the importance of various genetic defects on glucuronidation or bilirubin elimination. Several studies based on haplotyping techniques show the importance of genotyping variants involved in glucuronidation and elimination of toxic compounds. Bilirubin is transformed into its soluble form solely by UGT1A1. However, importance of haplotyping doesn`t involve the elimination of only bilirubin, but especially the clearance of other toxic compounds as the UGTs are the general drug metabolizing enzymes (Lankisch 2009).

VI.II. Aim 2 – Paper 2 - To characterize the Dubin-Johnson syndrome on both molecular genetic and biochemical levels; To perform a mutation analysis of *ABCC2* in patients with DJS and its effect on bilirubin elimination.

In literature, DJS has been characterized in a cohort of 101 Israeli patients, collecting biochemical, clinical and family history data (Shani et al, 1970). However, there is no study describing the defects on molecular genetic data, the relationship of mutations in *ABCC2,* impact of the mutated protein on direct excretion of bilirubin or coproporphyrin isomers.

Our study describes the biochemical and molecular genetics relationship on 56 investigated members from 7 Slovakian Roma families, which is the largest cohort described in Europe up today. Our data suggest the common ancestral mutation allele with the origin of 178-185 years ago. In the beginning of our study, we hoped to obtain more variable cohort with various mutations, analyse the impact of the protein damage on cellular level and compared the data within the cohort. However, when analysing our data, we found, that the high incidence of rare

Dubin-Johnson syndrome is caused by the effect of one founder mutation, and that all probands share the common haplotype of 86 kbp encompassing *ABCC2* gene. According the Table…, and in concordance with literature, conjugated bilirubin is presented in all DJS patients with homozygous presence of c.1013_1014delTG had elevated cBi (9-55 µmol/L) as well as TBi (19-72 µmol/L). (Slachtova et al, 2015, Dubin and Johnson, 1952); however it is clear that the bilirubin levels are fluctuating even within the same haplotype, suggesting that bilirubin elimination does not reflect only the genotype, but also the influence of environmental factor, age of the probands or nutrition.

VI. III – Aim 3 and 4 – Paper 2 To study the excretion of coproporphyrin isomers in patients with DJS and to compare it with the genetic defect in *ABCC2;* to describe genetic and biochemical characteristics of Dual hereditary jaundice

Coproporphyrin isomers in urine are excreted as isomers I – IV with the most significant isomers I and III. In healthy adults, the proportion of coproporphyrin isomer III in urine is ~ 70-80 %, compared to ~ 20-30 % in DJS patients, who excrete about 80-97 % of coproporphyrin I. Under normal conditions, excretion of coproporphyrin isomers depends on maturation of hepatobiliary system and changes within the first days of newborn's life (Koskelo et al, Kondo et al 1976).

In our study, we have investigated coproporphyrin isomers in 8 patients with DJS caused by c.1013_1014delTG in *ABCC2* and all of them showed shifted ratio excreted coproporphyrin isomers in urine (89-100 %, Table 2). Several studies show up to > 90 % predominance of coproporphyrin I in urine of patients with DJS, but no data shows results of 100 % (Ben Ezzer et al, 1973, Shani et al, 1970). Our patient with 100% excretion of isomer I was one year old boy with total serum bilirubin 50 µmol/L and its conjugated fraction 21 µmol/L. Coproporphyrin isomers were analysed from two independent samples with the same result. Unafected heterozygotes secrete about 40% of isomer I compared to 20-30 % of isomer I in healthy controls. Average percentage of isomer I in our DJS carriers was 43-57 %, which is in concordance with a data from literature. Surprisingly, excretion pattern of coproporphyrin isomers in four patients with dual hereditary jaundice was the same as in other DJS patients with no defect in *UGT1A1* Patients with dual defect of NG_011798.1:c.[1013_1014delTG]; NM_000463.2:c.[-54_-53insTA]

had the ratio of coproporphyrin I 92, 97, 99 and 100 % , compared to 89, 93, 96 and 98 % in DJS patients with only heterozygous NG_002601.2:g.175492_175493insTA and 89 % in DJS patient with the wild type *UGT1A* gene. This data suggest, that the excretion of coproporphyrin isomers depends not only on genotype of the patients but also on the other factors.

Dual hereditary jaundice has been described in a single case by other Czech authors. Our study aimed to collect more data on this rare condition, affecting both conjugation and transport of the bilirubin. In our study we are describing four DJS + GS patients. Supposed effect on the ratio of conjugated and excreted bilirubin is decreased amount of cBi available for its transport; therefore the percentage of cBi from TBi is < 50 % in DJS + GS compared to > 50 % in DJS only. We found a ratio of conjugated bilirubin fraction of 28 %, 38 %, 43 % and 89 %. The last value of 89 % is from a probands whose data were collected under medication, probably affecting the bilirubin clearance (Slachtova et al, 2015).

VII. CONCLUSIONS AND FUTURE PERSPECTIVE

Inherited hyperbilirubinemias are the metabolic disorders, mostly manifested in children; therefore their early diagnosis and adequate therapy is of great interest. Currently, there are known all genes responsible for the protein defects, resulting in an increased levels of bilirubin circulated in a blood stream. However, thanks to the rarity of some of hyperbilirubinemias, the estimation of the diagnosis still may take years. The importance of the knowledge of such a disorder is especially in avoiding certain pharmacotherapy, whose effect may change a mild condition to a life threating consequences. During other studies, we would like to highlight several recommendation:

- early molecular diagnosis testing should be taken into account already in non-typical jaundice of newborns
- the molecular genetics testing should consider the population specifics and medical history should already include this information
- as all of the proteins associated with inherited hyperbilirubinemias are also very important entities in biotransformation of toxic compounds and drug metabolizing enzymes, molecular genetic testing should be regularly performed before indication specific pharmacotherapy. Namely in a treatment of oncologic patients or in young adolescents taking hormonal contraceptives.
- genetic counselling in enclosed population with the risk of founder effect mutations about the increased risk of transmission of recessive disorders in consanguineous marriages.

Genetic and biochemical part of our study of inherited hyperbilirubinemias raised following questions to be study:

- what factors, except the genetic ones are affecting the excretion of coproporphyrin isomers?
- what is the cause of inverted ratio of excreted coproporphyrin isomers in patients with DJS and RS?
- in case of Dubin-Johnson syndrome, what is the importance of localization of the mutation in *ABCC2* on excretion of coproporphyrin isomers?

VIII. PUBLICATION ACTIVITY

VIII. I. Papers not related to the PhD thesis, published in journals with IF

Boraska, Franklin, Floyd et al*; A genome-wide association study of anorexia nervosa*, Mol Psychiatry. 2014 Feb 11

Huckins LM, Boraska V, Franklin CS, Floyd JA, Southam L; GCAN; WTCCC3, Sullivan PF, Bulik CM, Collier DA, Tyler-Smith C, Zeggini E, Tachmazidou I; GCAN; WTCCC3. *Using ancestry-informative markers to identify fine structure across 15 populations of European origin.* Eur J Hum Genet. 2014 Oct;22(10):1190-200

Šlachtová L, Kaminská D, Chval M, Králík L, Martásek P, Papežová H: Stress perception and (GT)n repeat polymorphism in HO-1 gene are both risk factors in eating disorder development. Folia Biologica, Folia Biol 2013;59(6):233-9.

Martásková D, Šlachtová L, Kemlink D, Záhoráková D, Papežová H. *Polymorphisms in Serotonin-Related Genes in Anorexia Nervosa. The First Study in Czech Population and Meta-analyses with Previously Performed Studies.* Folia Biologica 2009; 55:192-7

Cited 17x in:

Family-Based Clinical Associations and Functional Characterization of the Serotonin 2A Receptor Gene (HTR2A) in Autism Spectrum Disorder

By: Smith, Ryan M.; Banks, Wesley; Hansen, Emily; et al.

AUTISM RESEARCH Volume: 7 Issue: 4 Pages: 459-467 Published: AUG 2014

Preliminary evidence for the role of HTR2A variants in binge eating in young women

By: Koren, Rachel; Duncan, Alexis E.; Munn-Chernoff, Melissa A.; et al.

PSYCHIATRIC GENETICS Volume: 24 Issue: 1 Pages: 28-33 Published: FEB 2014

Multiple Regulatory Variants Modulate Expression of 5-Hydroxytryptamine 2A Receptors in Human Cortex

By: Smith, Ryan M.; Papp, Audrey C.; Webb, Amy; et al.

BIOLOGICAL PSYCHIATRY Volume: 73 Issue: 6 Pages: 546-554 Published: MAR 15 2013

The Genetics of Eating Disorders

By: Trace, Sara E.; Baker, Jessica H.; Penas-Lledo, Eva; et al.

Edited by: NolenHoeksema, S

ANNUAL REVIEW OF CLINICAL PSYCHOLOGY, VOL 9 Book Series: Annual Review of Clinical Psychology Volume: 9 Pages: 589-620 Published: 2013

Serotonin neurotransmission in anorexia nervosa

By: Haleem, Darakhshan Jabeen

BEHAVIOURAL PHARMACOLOGY Volume: 23 Issue: 5-6 Pages: 478-495 Published: SEP 2012

Antipsychotic Agents in the Treatment of Anorexia Nervosa: Neuropsychopharmacologic Rationale and Evidence from Controlled Trials

By: Brewerton, Timothy D.

CURRENT PSYCHIATRY REPORTS Volume: 14 Issue: 4 Pages: 398-405 Published: AUG 2012

Examining associations between disordered eating and serotonin transporter gene polymorphisms

By: Munn-Chernoff, Melissa A.; McQueen, Matthew B.; Stetler, Gary L.; et al.

INTERNATIONAL JOURNAL OF EATING DISORDERS Volume: 45 Issue: 4 Pages: 556-561 Published: MAY 2012

Food Addiction and Obesity: Evidence from Bench to Bedside

By: Liu, Yijun; von Deneen, Karen M.; Kobeissy, Firas H.; et al.

JOURNAL OF PSYCHOACTIVE DRUGS Volume: 42 Issue: 2 Pages: 133-145 Published: JUN 2010

Genetic Findings in Anorexia and Bulimia Nervosa

By: Hinney, Anke; Scherag, Susann; Hebebrand, Johannes

Edited by: Bouchard, C

GENES AND OBESITY Book Series: Progress in Molecular Biology and Translational Science Volume: 94 Pages:241-270 Published: 2010

A reward-centred model of anorexia nervosa: A focussed narrative review of the neurological and psychophysiological literature

By: O'Hara, Caitlin B.; Campbell, Iain C.; Schmidt, Ulrike

NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS Volume: 52 Pages: 131-152 Published: MAY 2015

Identification of determinants of referral and follow-up body mass index of adolescent patients with anorexia nervosa: evidence for the role of premorbid body weight

By: Hebebrand, Johannes

EUROPEAN CHILD & ADOLESCENT PSYCHIATRY Volume: 24 Issue: 5 Pages: 471-475 Published: MAY 2015

JEPEG: a summary statistics based tool for gene-level joint testing of functional variants

By: Lee, Donghyung; Williamson, Vernell S.; Bigdeli, T. Bernard; et al.

BIOINFORMATICS Volume: 31 Issue: 8 Pages: 1176-1182 Published: APR 15 2015

Anorexia runs in families: does this make the families responsible? A commentary on 'Anorexia runs in families: is this due to genes or the family environment?' (Dring, 2014)

By: Dodge, Elizabeth; Simic, Mima

JOURNAL OF FAMILY THERAPY Volume: 37 Issue: 1 Special Issue: SI Pages: 93-102 Published: FEB 2015

Perspectives of Genetic Research in Eating Disorders Using the Example of Anorexia nervosa

By: Hinney, Anke; Volckmar, Anna-Lena

PSYCHOTHERAPIE PSYCHOSOMATIK MEDIZINISCHE PSYCHOLOGIE Volume: 65 Issue: 1 Pages: 8-10 Published: JAN 2015

Candidate Gene-Environment Interaction Research: Reflections and Recommendations

By: Dick, Danielle M.; Agrawal, Arpana; Keller, Matthew C.; et al.

PERSPECTIVES ON PSYCHOLOGICAL SCIENCE Volume: 10 Issue: 1 Pages: 37-59 Published: JAN 2015

Neuro- and Social-Cognitive Clustering Highlights Distinct Profiles in Adults with Anorexia Nervosa

By: Renwick, Beth; Musiat, Peter; Lose, Anna; et al.

INTERNATIONAL JOURNAL OF EATING DISORDERS Volume: 48 Issue: 1 Pages: 26-34 Published: JAN 2015

A novel pattern of uridine diphosphate glucuronosyltransferase polymorphisms associated with hyperbilirubinemia during nilotinib treatment

By: Fozza, Claudio; Pardini, Simonetta; Coiana, Alessandra; et al.

BLOOD CELLS MOLECULES AND DISEASES Volume: 51 Issue: 3 Pages: 162-162 Published: OCT 2013

VIII. II. Abstracts

Slachtova L, Lohse M, Johnson S, Veeraraghavan S. *Biophysical biochemical and functional studies of a novel fungal TEC1 paralog.* In Biophysical Journal, vol. 108, issue 2, p. 510a, February 7-11th, Baltimore, MD, USA

Slachtova L, Bulant J, Chval M, Martasek P, Papezova H. *What is the role of BDNF in anorexia nervosa?* X. Mezinárodní interdisciplinární konference o poruchách příjmu potravy a obezitě, 26. – 28. 3. 2015, Praha

Huckins L, Hatzikotoulas K, Thornton L, Southam L, GCAN, Collier D, Sullivan P, Bulik CM, Zeggini E. *Core-exome Chip study of low-frequency variants identifies genome-wide significant hits associated with anorexia nervosa.* In ASHG 2014, October 18-22th, San Diego, USA

Huckins L, Mitchell K, Thornton L, WTCCC3, GCAN, Collier D, Sullivan P, Bulik CM. *Probing the shared polygenic underpinnings of anorexia nervosa and five other major psychiatric disorders.* In XXIst World congress of psychiatric genetics, October 17 – 21th, 2014, Boston, USA

Papezova H, Slachtova L, Yamamotova A, Kaminska D, Chval M, Martasek P. *Geneticke modulatory v poruchach prijmu potravy*, X. Sjezd Psychiatricke spolecnosti, 12. – 15. 6. 2014 Spindleruv mlyn

Šlachtová L, Šeda O, Mistrík M, Behúňová J, Martásek P. *Haplotype study of double jaundice – Dubin Johnson and Gilbert syndrome in Roma families. Founder effect, dating an ancestral mutation in ABCC2 and biochemical impact on bilirubin metabolism,* IV. Student scientific conference, 28. 4. 2014 Ostravská univerzita v Ostravě, Lékařská fakulta

Slachtova L, Baxova A, Martasek P *De novo mutation c.545T>C within WNT5A gene in mother with autosomal dominant form of Robinow syndrome*, Mutation Detection 22.nd – 26th April 2013, Lake Louise, Canada

H. Papezova, L. Kralik, L. Slachtova, D. Martaskova, M. Chval, P. Martasek *Haem oxygenase 1 promoter polymorphism (GT) in Anorexia and Bulimia nervosa,* World Congress of Biological Psychiatry, 23. – 27. 6. 2013, Kyoto, Japan

Slachtova L, Papezova H, Chval M, Martasek P. *Eating, body and weight concerns associated with BDNF Val66Met*, 9th International Eating Disorders and Obesity Conference, 21. – 23. 3. 2013, Prague

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Šlachtová L, Yamamotová A, Uhlíková P, Martásková D, Papezova H. ***Klinické a genetické koreláty vnímání vlastního těla u pacientek s poruchami příjmu potravy,*** 53. Psychofarmakologická konference, 5. – 9. ledna 2011, Lázně Jeseník (2. místo – Nejlepší poster)

Skopová J, Šlachtová L, ***Akutní jaterní porfyrie s psychiatrickou symptomatikou a rizika podávání některých farmak na provokaci akutního záchvatu,*** 53. Psychofarmakologická konference, 5. – 9. ledna 2011, Lázně Jeseník

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Papezova H, Slachtova L, Martaskova D, Yamamotova A, Martasek P. *Pathologic self-perception and G196A polymorphism in BDNF gene in patients with eating disorders*, 10th World congress of Biological Psychiatry, 29 May – 2 June 2011, Prague

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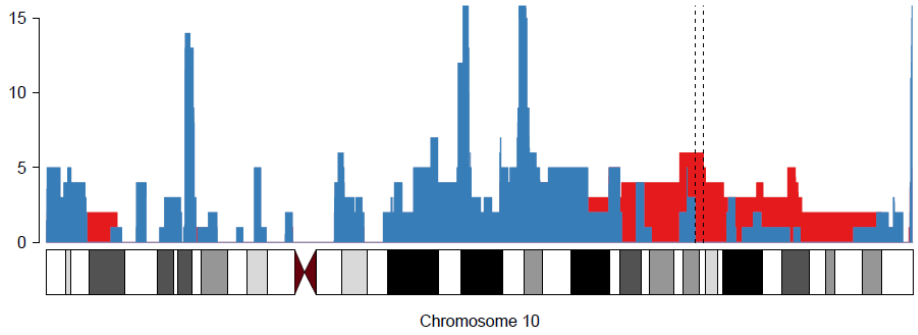
IX. ATTACHMENTS – SUPPLEMENT

Supplementary Figure 1

Loss of heterozygosity in 10q24 region encoding *ABCC2* in patient F1.IV/17 with DJS



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