5. Biochemistry, molecular biology and molecular genetics of hyperbilirubinemia

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Abstract. Hyperbilirubinemia is the most common problem encountered in terms newborns which is mainly caused by abnormal liver function, hemolysis or genetic defect. Unconjugated bilirubin is produced mainly by the turnover of erythrocytes, after that it is transported by organic anion transporter polypeptide (OATP) to the liver, where unconjugated bilirubin is conjugated by uridine diphosphoglucuronate glucuronosyl transferase 1A1 (UGT1A1) before being excreted into the bile. The presence of mutations and polymorphism in UGT1A1 gene are associated with Crigler-Najjar and Gilbert syndrome. The Gilbert syndrome is an asymptomatic unconjugated hyperbilirubinemia that is most commonly caused by polymorphism in UGT1A1 gene worldwide. In Asian patients, the other mutations like G71R contribute significantly to hyperbilirubinemia, however this mutation was absent in Indian population in our study. Ala 72 Pro of exon 1 was found to be the most common mutation of UGT1A1 gene in our population. There is a spectrum of mutation in UGT1A1 gene that has been characterized worldwide which is associated with neonatal hyperbilirubinemia. In this context we characterized 21 mutations out of which 16 were novel mutations reported only in Indian population. The another gene which has been implicated in our study is OATP gene 6 mutations

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which was identified in 57 hyperbilirubinemic neonates, out of which 4 were novel. Another genetic defect, G6PD deficiency an X linked abnormality is the most commonly seen genetic defect affecting 400 million individuals worldwide. G6PD deficiency is also a known causative factor for hyperbilirubinemia. Most polymorphic mutations predominate in specific regions of the world: G6PD A- (376G/202A) is prevalent in Africa and Southern Europe, G6PD Mediterranean (C563T) in Mediterranean countries, and G6PD Viangchan (G871A) in Asian countries. In our study we found Mediterranean and Orissa mutations to be most common.

1. Introduction

The incidence and severity of neonatal hyperbilirubinemia is significantly higher in Asians, more so in North Indians, than in Caucasians [1,2] and frequently encountered clinical abnormality in the newborn, ranging between physiologic range of neonatal jaundice and pathologic hyperbilirubinemia, requiring further etiologic investigation and treatment to prevent long-term sequelae such as kernicterus [3]. Despite all standard investigations, no cause is identified in 48–58% of these cases [2, 3]. Genetic factors have been implicated in such situations; however, their contribution in Indian population has not been reported. Genetic variants involving red blood cell enzyme glucose-6-phosphate dehydrogenase G6PD (EC 1.1.1.49) and bilirubin conjugating enzyme uridinediphosphoglucuronate-glucuronosyltransferase 1A1 (UGT1A1, EC 2.4.1.17) have been commonly associated with neonatal hyperbilirubinemia [4,5]. The usual polymorphism described in Caucasians is additional thymine-adenine (TA) insertions in the normal sequence A(TA)6TAA of the TATAA box promoter of the UGT1A1 gene [6]. However, in East Asians, missence mutations in the coding area of the UGT1A1 gene, especially transition at nucleotide position 211 of exon 1 (Gly71Arg) is the most common mutation [7]. The types, prevalence, and importance of various mutations of UGT1A1 gene vary across different regions of world. UGT1A1 is the rate-limiting enzyme for the conjugation of bilirubin with glucuronic acid in its excretion process into the bile [1]. Bilirubin is mainly produced in its unconjugated form by the turnover of erythrocytes. It may be transported by the organic anion transporter 2 (OATP 2). Organic anion transporters (OATs) or organic anion transporting polypeptides (OATPs) play an essential role in the elimination of numerous endogenous and exogenous organic anions from the body. The OAT family is expressed in the kidney, liver, brain and placenta [8]. Organic anion transporter 2 (OATP2) is involved in the hepatic uptake of a broad array of endogenous compounds, such as taurocholate, leukotriene C4, prostaglandin E2, conjugated steroid, thyroid hormone and peptide. Recently, OATP2 has also been shown to mediate the cellular uptake of bilirubin and its
glucuronide conjugates [9]. In red blood cells, glucose-6-phosphate dehydrogenase (G6PD) catalyzes NADP to its reduced form, NADPH, in the pentose phosphate pathway [10]. Limited production of NADPH increases the vulnerability of red blood cells to oxidative stress, which may shorten RBC’s life span [11]. The main symptom of G6PD deficiency in adults is hemolytic anemia, which usually occurs after exposure to certain medications (antimalarias, primaquine, sulfonamides, nitrofurantoin and other drugs), foods (especially fava beans) or even infection (hepatitis viruses A and B, cytomegaloviruses, pneumonia and others). Whatever the cause of the acute hemolysis in G6PD deficiency, it is clinically characterized by fatigue, back pain, anemia and jaundice [10,12]. There are approximately 400 million people suffering from G6PD deficiency throughout the world [13]. The deficiency occurs with high frequency in Africa, the Mediterranean (including in Italians, Greeks, Arabs and Sephardic Jews), the Middle East and Southeast Asia. A study conducted in the United States estimated that 30% of jaundiced infants who have permanent brain damage are G6PD deficient [3]. However, Kaplan et al., suggested that impaired bilirubin conjugation and delayed clearance by the liver have a considerable contribution to neonatal jaundice [14]. G6PD deficiency is an X-linked recessive disease, thus, the disease usually affects males but there are some female patients [15]. More than 160 different mutations have been demonstrated so far, most of which are missense mutations http://bminfor.tongji.edu.cn/mutdb/.

2. Biochemistry of bilirubin metabolism

The term bilirubin is derived from the Latin words for bile (bilis), and red (ruber). Städeler first used it in 1864 to describe the orange-red colored bile pigment. When bilirubin accumulates in the body it causes a yellow discoloration of the skin, sclerae and other tissues, referred to as jaundice (from the French jaunisse) or icterus (from the Greek ikteros), and high levels of bilirubin in the blood, termed as hyperbilirubinemia. The systemic name of Unconjugated bilirubin (UCB) (bilirubin IXa) is 1’8’-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid [16]. UCB is a nearly symmetrical at carbon atom 10 (Fig. 1).

UCB preferably has a “ridge-tile” conformation, i.e. is shaped like a partially open book which was identified by analysis of X-ray diffraction [18]. Six internal hydrogen bonds make the molecule insoluble in water because the hydrophilic polar COOH and NH groups are not available for attachment of H2O and the hydrophobic hydrocarbon groups are on the outside of the molecule [19].
2.1. Bilirubin production and transport

Bilirubin is the end product of heme catabolism. The major source of heme (75-80%) is hemoglobin from breakdown of erythrocytes. Senescent erythrocytes are removed from the circulation and destroyed in the reticuloendothelial system (RES), mainly localized in the spleen, liver and bone marrow. Once the hydrophobic UCB leaves the reticuloendothelial system, over 99.9% of bilirubin is bound to albumin in plasma in a non-covalent fashion and transported to the liver. Albumin has a high affinity binding site for UCB. In the absence of albumin, the aqueous solubility of UCB at pH 7.4 is less than 0.1 μmol/l, emphasizing the importance of albumin for preventing unconjugated hyperbilirubinemia, which is considered toxic.

2.2. Hepatic uptake of bilirubin

Albumin delivers UCB to the liver where fenestrae in the sinusoidal endothelial cells allow albumin-bound substances to reach the subendothelial space of Disse [20]. Hepatocytes have a highly efficient capacity for removing UCB from plasma. The uptake of UCB into the hepatocyte results from dissociation from albumin and transfer across the plasma membrane [19]. Several proteins have been suggested as putative UCB transporter, including the organic anion transport protein (Oatp2 / Slc21a6) [9] and the bilirubin/BSP binding protein (BBBP) which also transports other organic anions such as bromosulfophthalein (BSP) [21].
2.3. Bilirubin conjugation

In order to excrete bilirubin efficiently into bile, conjugation is required to convert the non-polar, water-insoluble UCB (at pH 7.4) to water-soluble conjugate. Glucuronic acid is the major conjugating group. Bilirubin glucuronides are present as mono- and diglucuronides. The enzyme bilirubin-uridinediphosphoglucuronosyltransferase (UDPGT, UGT1A1), primarily located in the endoplasmic reticulum, catalyzes the transfer of one or two glucuronic acid(s) from UDPglucuronate (UDPGA) to UCB, forming, respectively, bilirubin monoglucuronides (BMG, ~20%) or bilirubin diglucuronides (BDG, ~80%) that are excreted into bile [22,23].

The uridinediphosphate (UDP)-glycosyltransferases are a group of enzymes that catalyze the transfer of sugars (glucuronic acid, glucose, and xylose) to a variety of acceptor molecules (aglycones). In vivo, the most common reaction occurs by transfer of glucuronic acid moiety from UDP glucuronic acid (UDPGA) to an acceptor molecule. This process is termed either glucuronidation or glucuronosylation. When the enzymes catalyse this reaction, they are also referred to as UDP-glucuronosyltransferases (UGTs). Glucuronidation is an important step in the elimination of many important endogenous substances from the body, including bilirubin, bile acids, steroid hormones, thyroid hormones, retinoic acids, and biogenic amines such as serotonin. Many of these compounds are also substrates for sulfonylexferases (SULTs). The Uridine-diphosphoglucuronate-glucuronosyltransferases (UGTs) belong to the enzyme group of glycosyltransferases. UGTs in mammals have been classified into three families, based on the sequence similarity and structure of the gene: the UGT1, UGT2 and UGT8. Within these families there are several isoforms. Only two isoforms in the UGT1A family (UGT1A1 and UGT1A4) have bilirubin as a substrate but in humans only UGT1A1 plays a significant role in bilirubin glucuronidation [24].

2.4. Pathophysiology of neonatal jaundice

Jaundice or icterus can be defined as a yellowish discoloration of skin and sclera. This phenomenon is an indication of excess bilirubin in the blood. Clinical jaundice appears when the bilirubin level reaches about 85 µmol/L. Initially jaundice tends to appear in the face alone but as the hyperbilirubinemia gets worse, other areas of the body become yellow as well. In newborns, it typically follows a cephalocaudal progression. Hyperbilirubinemia can be divided into two types depending on the conjugation of bilirubin to glucuronide molecules: i.e. unconjugated hyperbilirubinemia and conjugated hyperbilirubinemia.
2.5. Unconjugated hyperbilirubinemia (Indirect hyperbilirubinemia)

Unconjugated hyperbilirubinemia becomes clinically apparent with visible jaundice at plasma bilirubin levels of about 85 μmol/L [19]. Normal plasma total bilirubin levels in human adults range from 5 to 17 μmol/L. For the majority of neonates, unconjugated hyperbilirubinemia is a benign transitional phenomenon of no overt clinical significance [25]. However, in some cases and in the presence of risk factors such as prematurity, hemolytic disease or inherited deficiency of UGT1A1, plasma UCB concentration may rise to hazardous levels leading to kernicterus or bilirubin-induced neurologic damage (BIND).

2.6. Conjugated hyperbilirubinaemia (Direct hyperbilirubinaemia)

This is an elevation of the serum bilirubin which is conjugated with glucuronide molecules. This water-soluble bilirubin diglucuronide is ready for excretion in bile and may increase when cholestasis occurs.

2.7. Common causes of neonatal jaundice

2.7.1. Physiological jaundice

Physiological jaundice is the most common cause of neonatal jaundice. Physiological jaundice most commonly occurs on day 3 to day 5 of life and decreases by the end of the first week after birth. It is usually associated with mild to moderate jaundice believed to be caused by a variety of factors: cessation of bilirubin clearance of placenta, immaturity of hepatic enzymatic systems at birth which decreases the ability to conjugate bilirubin, decreased hepatic uptake of bilirubin, larger red cell mass, shortened erythrocyte life span of cells carrying foetal haemoglobin and increased enterohepatic circulation [26]. In vitro studies have shown unequivocally that bilirubin is an antioxidant. In our study, we hypothesized that bilirubin serves a physiological role as an antioxidant in vivo, [27]. Lipid peroxidation and antioxidant enzymes were significantly lower in babies with STB <200mg/L compared to controls. Total antioxidant capacity (TAC) had a positive and MDA had a negative correlation with STB till 200 mg/L. However, TAC had a negative and MDA had a positive correlation with bilirubin>200 mg/L and in babies with acute bilirubin encephalopathy. Elevated level of MDA, SOD and catalase and significantly decreased level of reduced glutathione and TAC were observed in STB>200mg/L group. Antioxidant enzymes were also
significantly inhibited in babies with bilirubin encephalopathy. Post phototherapy, MDA production and antioxidant level were significantly increased whilst total antioxidant capacity and reduced glutathione were significantly decreased compared to pre-phototherapy values. Taken together, this study propounds that bilirubin acts as a physiological antioxidant till 200 mg/L concentration in full-term neonates. It is conjectured that beyond 200 mg/L, it can no longer be considered physiologic. However the cause of pathological jaundice needs to be identified and treated [27].

3. Biochemistry and physiology of G6PD

The G6PD enzyme catalyzes an oxidation/reduction reaction. Oxidation/reduction reactions function in transferring electrons from one molecule to another; oxidation is the loss of electrons and reduction is the gain of electrons. As illustrated in fig. 2, the G6PD enzyme functions in catalyzing the oxidation of glucose-6-phosphate to 6-phosphogluconate, while concomitantly reducing nicotinamide adenine dinucleotide phosphate (NADP+ to NADPH); or, in terms of electron transfer, glucose-6-phosphate looses two electrons to become 6-phosphogluconate and NADP+ gains two electrons to become NADPH. This is the first step in the pentose phosphate pathway. This pathway, or shunt, as it is sometimes called, produces the 5-carbon sugar, ribose, which is an essential component of both DNA and RNA. There are other metabolic pathways, however, that can produce ribose if there is a deficiency in G6PD [28].

In addition to producing the 5-carbon sugar ribose, G6PD is also responsible for maintaining adequate levels of NADPH inside the cell. NADPH is a required cofactor in many biosynthetic reactions. NADPH is also used to keep glutathione, a tri-peptide, in its reduced form (Fig. 2). Reduced glutathione acts as a scavenger for dangerous oxidative metabolites in the cell; it converts harmful hydrogen peroxide to water with the help of the enzyme, glutathione peroxidase [28]. There are other metabolic pathways that can generate NADPH in all cells, except in red blood cells where other NADPH-producing enzymes are lacking [29]. This has a profound effect on the stability of red blood cells since they are especially sensitive to oxidative stresses in addition to having only one NADPH-producing enzyme to remove these harmful oxidants. Total serum bilirubin levels in G-6-PD deficient neonates can rise gradually and eventually lead to jaundice.
Figure 2. Diagram for the regeneration of NADPH (reduced form) from NADP in the presence of G-6-PD enzyme. This is a part of the antioxidant defence mechanism. GSH: Reduced Glutathione; GSSG: Oxidized Glutathione [30].

4. Molecular genetics of UGT1A1

4.1. UGT1A1 locus and structure

UGT1A1 gene has been mapped on chromosome 2q37 [31] as represented in Fig. 3, UGT1A1 consists of 5 exons. Exons 2 to 5 are common exons of other isoforms within the UGT1A group (UGT1A1-13) and exon 1 is a unique exon for UGT1A1 (Fig. 3). The four common exons at 3’ end encode the carboxy terminal domain for all isoforms of UGT which bind to UDP-glucuronic acid. The unique exon for each isoform at 5’ end encodes the N-terminal domain for the enzyme which specifies the substrate for the isoform. At least 13 exons (for UGT1A1 until UGT1A13) are located upstream of exon 2 [32] cDNA encoding human UGT1A1 gene was cloned and functionally characterized. It is 1.602 kb long and encodes peptide of 533 amino acids [33,34]. A decrease in enzyme activity caused by defects or lesions in the UGT1A1 gene results in unconjugated hyperbilirubinaemia.
This large complex on chromosome 2 contains at least 13 substrate-specific first exons (A1, A2, etc.). Since four of these are pseudogenes, nine UGT1 isoforms with differing substrate specificities are expressed. Each exon 1 has its own promoter and encodes the amino-terminal substrate-specific ~286 amino acids of the various UGT1-encoded isoforms, and common exons 2–5 that encode the 245 carboxyl-terminal amino acids common to all of the isoforms. mRNAs for specific isoforms are assembled by splicing a particular first exon such as the bilirubin-specific exon A1 to exons 2 to 5. The resulting message encodes a complete enzyme, in this particular case bilirubin-UDP-glucuronosyltransferase (UGT1A1). Mutations in a first exon affect only a single isoform. Those in exons 2–5 affect all enzymes encoded by the UGT1 complex. In humans, three forms of inheritable unconjugated hyperbilirubinemic diseases exist: Crigler-Najjar syndrome type I, Crigler-Najjar syndrome type II, and Gilbert’s syndrome.

4.2. Crigler-Najjar syndrome

Defects of the UGT1A1 gene may occur in any of its five exons and contribute to Crigler-Najjar syndrome, either type I or II [35]. Crigler-Najjar syndrome type I is the severe form and was described by Crigler and Najjar.
in 1952 as a potentially lethal hyperbilirubinaemia (serum bilirubin 20–50 mg/dL) without liver disease or overt haemolysis. Crigler-Najjar syndrome type II is an intermediate form with moderate elevations of the level of bilirubin (7–20 mg/dL). It is also known as Arias syndrome as it was described by Arias in 1962. It is commonly caused by a severe, but an incomplete lack of UGT1A1 activity in the liver [36]. The prevalence of Crigler-Najjar disease is estimated at 0.6-1 per million live births. Mutations in any of 5 exons (or rarely in introns or promoter region) can cause Crigler-Najjar syndrome type I or II. Approximately 60 mutations (point mutations, deletions, insertions) in the UGT1A1 gene have been identified. Indicating that Crigler-Najjar syndrome is genetically heterogeneous, while there is a homogeneity of its clinical presentation. In all Crigler-Najjar patients studied homozygosity for UGT1A1*7 is associated with the Crigler-Najjar syndrome type II.

4.2.1. Treatment

Phototherapy is the preferred long-term treatment for Crigler-Najjar disease type I, but has logistic difficulties. If plasma unconjugated levels cannot be kept below 450-500 μmol/L, liver transplantation may be necessary to prevent irreversible brain damage due to kernicterus. During exacerbations of jaundice, several measures in addition to continuous high-intensity phototherapy are taken to manage the disease safely, including albumin infusion if the bilirubin-albumin molar ratio is above 0.7, and avoidance of drugs that displace bilirubin from albumin [37]. Before the introduction of phototherapy, all patients with Crigler-Najjar syndrome type I died from kernicterus [38]. In recent years, neurological outcome of Crigler-Najjar syndrome has improved if treatment is started early and adequately. Combined data from recent surveys suggest that 23-47% of patients with Crigler-Najjar syndrome have neurologic damage ranging from mild to severe, 28-50% of patients will need one or multiple exchange transfusions, and 9-38% die of complications related to the disease [37].

4.3. Gilbert syndrome

Gilbert syndrome was first described by Augustin Gilbert and Lereboullet in 1901. Patients with Gilbert syndrome commonly have mild and chronic unconjugated hyperbilirubinaemia with normal liver function and without overt haemolysis [39]. It is an inherited disorder of bilirubin metabolism. This benign condition of young adults does not require therapy and is characterized by fluctuating unconjugated hyperbilirubinemia in
response to psychological stress, infection, fasting, or physical activity. The levels of unconjugated serum bilirubin are lower than in Crigler-Najjar’s syndrome, and the hepatic bilirubin UGT activity is reduced to 60–70% of an unaffected individual. Gilbert syndrome is considered to be a harmless disease, but it may be a risk factor for neonatal jaundice, especially in combination with haemolytic disorders, such as G-6-PD deficiency and ABO incompatibility [40]. There is still controversy whether Gilbert syndrome is inherited as a recessive or dominant trait. It was assumed that it was inherited as an autosomal dominant trait while a study by Bosma et. al., 1995 [41] suggested it an autosomal recessive trait. There may be different degrees of severity of the enzyme defect depending on the particular mutation. Some mutations are known to cause Crigler-Najjar syndrome type II in the homozygous state and Gilbert syndrome in the heterozygous state.

The incidence of Gilbert syndrome in the general population is about 3% to 10% [42,43]. Hsieh and colleagues found that the TATA box mutation and G71R underlies the molecular background of Gilbert syndrome in the Taiwanese population. Simultaneous occurrences of more than one mutation were also found in this population which resulted in higher level of bilirubin and caused a more severe form of Gilbert syndrome.

4.4. A(TA)7TAA promoter polymorphism and neonatal hyperbilirubinemia

The most common genetic polymorphism encountered with Gilbert’s syndrome in Caucasians is an additional TA insertion in the TATAAA box of the UGT1A1 gene promoter. The usual sequence is (TA)6; insertion of additional (TA) sequence, resulting in (TA)7 or occasionally (TA)8 causes progressively diminished expression of the UGT1A1 enzyme. The importance of this polymorphism varies with different ethnic populations. Bancroft et. al., were the first to confirm an association between Gilbert’s syndrome, as indexed by the variant promoter, and neonatal jaundice [44]. Using a transcutaneous jaundice meter to evaluate jaundice, they found that homozygosity for the variant A (TA)7TAA gene promoter caused a significantly greater increase in the transcutaneous jaundice index during the first two days of life than in controls, but did not result in higher peak jaundice index levels or in higher incidence of hyperbilirubinemia. Roy-Chowdhury et al., in a study of predominantly breast-feeding Greek neonates in whom direct Coombs’ positivity, ABO incompatibility and G6PD deficiency had been excluded, found significantly higher STB values at 96 hours of life in those homozygous for the variant A(TA)7TAA promoter (10.2 ± 1.4 mg/dL) and intermediate values in heterozygotes (8.9 ± 3.1
mg/dL) compared with homozygous normal A(TA)6TAA controls (7.0 ± 3.2 mg/dL, p=0.005) [45]. Laforgia et al., found a significantly higher frequency of homozygosity for the variant A(TA)7TAA promoter in neonates (hemolytic conditions excluded) with STB concentrations > 13.0 mg/dL compared with controls whose STB values did not exceed that concentration (26.8% vs. 12.2%, p<0.05) [46]. However, in this study peak STB values did not differ according to promoter genotype. However studies of the relationship of UGT promoter polymorphism to prolonged jaundice have not yielded consistent results. In Scotland population, it was found that 31% of neonates (almost all of whom were breast feeding) with prolonged jaundice (STB levels >5.8mg/dL>14 days of life) were homozygous for the 7/7 Gilbert’s syndrome promoter genotype compared with only 6% of a control group with acute jaundice (p<0.05) [47]. On the other hand, in the two studies on Turkish population [48, 49], homozygosity for the 7/7 promoter genotype was encountered in only 8% and 4% of those with prolonged jaundice, compared with 6% and 9.3% of controls respectively [48].

In our study 127 neonates were enrolled (77 hyperbilirubinic, 50 controls). The incidence of (TA)n polymorphism was higher in babies with hyperbilirubinemia [89.6% vs. 50 % or (95% CI, 3.2 -24.1)]. Presence of variant (TA)n promoter (adjusted OR, 10.6; 95% CI, 3.3 -34.2), G6PD deficiency (adjusted OR, 20.6; 95% CI, 3.6-117.3) and history of jaundice in sibling requiring phototherapy (adjusted OR, 12.6; 95% CI, 1.1-141.6) where independent risk factors for bilirubin levels > 18 mg/dL [50].

We found Ala72Pro (214G>C) polymorphism at the codon position 72 of the exon 1 of bilirubin UGT1A1 gene [50]. 214G>C of exon 1 was found to be the most common polymorphism of UGT1A1 gene in our population. The neonates who carry variation 214G>C in the UGT1A1 gene are at high risk for experiencing severe hyperbilirubinemia. It has been seen that polymorphisms found in exon 1 might reduce the affinity of the ligand, bilirubin, for the catalytic site in the amino-terminal half of the molecule, which is encoded by exon 1 of the UGT1A1 gene. UGT1A1 enzyme activities of the G71R variations in the heterozygous or homozygous state were decreased to 60.2 and 32% of normal respectively [43]. These decreased enzyme activities are thought to cause delayed elimination of bilirubin and ultimately occurrence of hyperbilirubinemia [51]. The conversion of G→A at nucleotide 211 (G71R) is the predominant variation and is highly associated with neonatal hyperbilirubinemia and adult hyperbilirubinemia in Taiwanese population [52]. Pro364Leu (1091C>T) was mostly reported in Chinese and Twainese population. Homozygous variant of nucleotide 1091C>T polymorphisms of UGT1A1 gene was a significant risk factor associated with severe hyperbilirubinemia among Indian, Malaysian and Chinese newborns.
In our population we found 17 mutations in which 1 was already reported (D359N) and 16 of them were novel mutations. These 16 novel mutations included nine missense mutations (A64S, D70H, G71E, S143N, D146Y, S191P, R257K, W335R and L489V), five silent mutations (I57I, K114K, S334S, T349T and T482T), one frame shift mutation (1363 ins C) and one nonsense mutation (E534X). Table 1 shows the identified and characterized the UGT1A1 mutations in the Indian population. The UGT1A1 polymorphisms have recently acquired significance because they predispose individuals to altered metabolism. Exons 2-5 are shared by other UGT1A1 transcripts and isozymes that mediate metabolism of xenobiotics involved in bilirubin glucuronidation. The identification of these novel mutations in the UGT1A1 gene, increasing the mutational spectrum of UGT1A1 allelic variants, contributes to a better understanding of the molecular pathology of disorders characterized by unconjugated hyperbilirubinemia.

5. Organic anion transporting polypeptide (OATP)

The organic anion transporting polypeptides (rodents: oatps; human; OATPs) represents a family of protein responsible for the membrane transport of a large number of endogenous and xenobiotic compounds with xenobiotic compounds with diverse chemical characteristics. The exact transport mechanism(s) of the OATPs, has not yet been worked out. However, studies with rat OATP suggest that they act as organic anion exchanger.

5.1. Molecular biology OATP2

OATP2 gene, located at chromosome 12p12 [53]. It consists of 14 exons (Fig. 4). The GenEMBL EST database revealed a sequence (GenBankTM accession number T73863) obtained from a human liver library with significant homology to hOATP. The insert of this clone did not contain full-length coding sequence. Thus, an oligonucleotide based on EST T73863 was used to screen for full-length clones using the Gene Trap method. A 2.8-kilobase cDNA was identified containing 2076 base pairs and encodes a polypeptide of 691 amino acids. Oatp1 and oatp2 are glycoproteins with 12 putative transmembrane domains.

According to the hydropathy analysis, all Oatps/OATPs contain 12 transmembrane domains with both the amino and the carboxy terminal parts located intracellularly. However, the predicted 12-transmembrane domain model for any Oatp/OATP has not been proven experimentally.
Table 1. UGT1A1 mutations identified and characterized in the Indian population.

<table>
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<th>Mutation</th>
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<th>GenBank accession Number</th>
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<td>6</td>
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Figure 4. Human chromosome 12 (http://www.genome.ucsc.edu/cgi-bin/hgTracks?chr12) showing the location of OATP2 gene.

5.2. OATP substrates

Substrates of OATP transporters include a variety of endogenous compounds of differing chemical structures and a wide variety of drug classes. Oatp1 and oatp2 primarily transport bile acids and derivatives, steroids, peptidomimetics, glucuronides and anionic estrogen conjugates. Oatp1 is capable of transporting endogenous organic anions such as the bile
acid taurocholate, estradiol 17-glucuronide, the steroid hormone estrone-3-sulfate and enalapril. Oatp2 is similar to oatp1 in that it transports many of the same substrates as oatp1, but demonstrates different specificities for some drugs such as digoxin.

5.3. Severe hyperbilirubinemia with mutations in the OATP2 gene

Recently it was demonstrated that OATP 2 is responsible for the transportation of organic anions into hepatocytes. This mechanism may also be involved in the transportation of unconjugated bilirubin. Genetic polymorphism for the organic anion transporter protein OATP-2 correlates with a 3-fold increased risk for developing marked neonatal jaundice. Combination of the OATP-2 gene polymorphism with a variant UDPGT1A1 gene further increases this risk to 22-fold. High prevalence of c.388A>G (73.4%) and c.521T>C (14.0%) variants of OATP 2 gene has been reported among Chinese in mainland China. A study from Taiwan showed that variation at c.388A>G was a risk factor associated with unconjugated hyperbilirubinemia in newborns [54]. Variations at c.571T>C and c.597C>T were detected in 26% and 50%, respectively, of a Chinese population and 50.0% and 42.9%, respectively, of a Japanese Population [55]. A variant of Organic anion transporter polypeptide 2 gene has been incriminated in unconjugated hyperbilirubinemia in Taiwanese population [56]. Buyukkale G et. al., found that in males with the 388 A>G mutation of OATP-2, unconjugated bilirubin in plasma was significantly increased compared with females who had the same mutation [57]. Other studies also suggested that c.388G>A of OATP2 was the most common and highly polymorphic in Asian Population with special reference to Malaysian, Chinese and Taiwanese population [54,56,58]. The frequency of c.521T>C variant was found to be 13% in Chinese, Taiwanese and Japanese population. A388G and T521C polymorphisms have been previously reported in association with unconjugated hyperbilirubinemia [59] and at allele frequencies intermediate of European American and African American descent [60]. Taiwanese carry the variations only at nucleotide 388 and 521. Variation at nucleotide 521T>C reduces the transportation function for estrone-3-sulphate to <50% of normal in an experiment using HeLa cells. Several variants were found at a relatively high incidence (A388G> C463A>T521C >A2000G> G1463C), and their genotypic frequencies appear to be dependent on race. Interestingly, Tirona et. al., reported that Gly488Ala (OATP-C protein) amino acid substitution takes place in an area of the protein that could be considered a signature motif for all members of OATP transporter family. However, these SNPs were found to have modest changes in OATP transporter activity [61].
In our Laboratory 57 hyperbilirubinemic neonates presented with bilirubin level of > 20mg/dL and 50 control subjects were enrolled for investigation frequency of OATP2 gene polymorphism. The c. 388G>A variant was most common, and c.463C>A was the least common. Surprisingly 523T>C variant instead of 521T>C was observed with 31.5% frequency in exon 5 of OATP 2 gene in our population. Additionally 2 novel polymorphism in exon4 of OATP2 gene viz C.370T>A and c.411G-T were also characterized on DNA sequencing of OATP2 gene from hyperbilirubinemic subjects. Compound variants variations in OATP2 gene were associated with significant hyperbilirubinemia in hyperbilirubinemic infants. Notably one nonsense novel mutation c.369T >A in exon 4 of OATP2 gene was found in 4 in hyperbilirubinemic patients.

6. Glucose 6 phosphate dehydrogenase (G6PD) gene

G6PD, in its active enzyme form, is made up of either two or four identical subunits, each having a molecular mass of about 59 KDa. This is more than three times as large as the hemoglobin molecule, which is the principal oxygen carrying molecule in humans.

6.1. Molecular biology of G6PD

It is important to learn about the genetics of G6PD deficiency since this determines whether someone will be affected by this condition. In humans, there are 23 pairs of chromosomes which direct various physical and metabolic traits. One of the 23 pairs of chromosomes is the X and Y- chromosome pair (also known as the sex chromosomes) which determine sex of an individual. The X-chromosome is especially important because it carries genes that are critical to human survival. An important gene located on the X-chromosome is the gene for the G6PD enzyme [29].

The G6PD gene is located at the telomeric region of the long arm of the X chromosome (band Xq28), close to the genes for haemophilia A, congenital dyskeratosis, and colour blindness (Fig. 5). The gene was cloned in 1986 [62], and consists of 13 exons and 12 introns, spanning nearly 20 kb in total (Fig. 5). It encodes 515 amino acids, and a GC-rich (more than 70%) promoter region. The 5’ untranslated portion of the mRNA corresponds to exon I and part of exon II; the initiation codon is in exon II.30 In the promoter region, there are several binding sites for the transcription factor SP1—GGCGGG and CCGCCC sequences- similar to those in other housekeeping gene promoters.
Figure 5. Representation of X-chromosome and the position of the G6PD gene at the Xq28 locus (http://wiki.medpedia.com/Glucose-6-phosphatedehydrogenase (G6PD) Diagram of the G6PD locus, spanning ~18 kb. Exons are shown as blackened boxes, and introns and noncoding regions are shown as unblackened boxes [63].

6.2. Molecular genetics of G6PD

All X-linked genetic conditions, such as G6PD deficiency, are more likely to affect males than females. G6PD deficiency will only manifest itself in females when there are two defective copies of the gene in the genome. As long as there is one good copy of the G6PD gene in a female, a normal enzyme will be produced and this normal enzyme can then take over the function that the defective enzyme lacks. When a certain heritable trait is expressed in such a manner, it is called a recessive trait. In males, however, where there is only one X-chromosome, one defective G6PD gene is sufficient to cause G6PD deficiency.

G6PD deficiency is one of the most common enzyme deficiency known; affecting hundreds of millions of people. Although originally it was distributed in Africa, south Europe, the Middle East, migration of population groups and ease of travel in modern times have resulted in virtually worldwide distribution. G6PD deficiency is known to have over 400 variant alleles, or different forms of the same gene [64]. A mutant G6PD enzyme may be different from person to person; mutations can be in the form of point mutations or can range from one to several base pair deletions as well as replacements in the DNA. Different populations have different types of mutations, but within a specific population, common mutations are usually
shared. For example, in Egypt there exists only one type of allele, called the "Mediterranean" variant, among the population, whereas in Japan there is a different variant with a different type of mutation prevalent within that population, this one called the "Japan" variant [29].

Potentially most devastating conditions associated with G-6-PD deficiency in the neonate is bilirubin encephalopathy, or kernicterus. In the informal Kernicterus Registry at least 22% of neonates with kernicterus had documented G-6-PD deficiency, emphasizing the important role of this enzyme deficiency in the pathogenesis of this condition. Serum conjugated bilirubin fractions were found to be low in G-6-PD deficient neonates who developed hyperbilirubinemia and is likely secondary to impairment of bilirubin conjugation and clearance by the liver leading to indirect hyperbilirubinemia. Polymorphic mutations affect amino acid residues throughout the enzyme and decrease the stability of the enzyme in the red blood cells, possibly by perturbing protein folding. However, severe mutations mostly affect amino acid residues at the dimer interface or the residues interacting with a structural NADP molecule that stabilizes the enzyme. Most of the G6PD-deficient neonates who had suffered from hyperbilirubinemia carried the mutation at nucleotide 1376.

6.3. G-6-PD deficiency in India

G-6-PD deficiency was first reported in India from the Parsi population of Mumbai in the year 1963 by Baxi et al., [65]. The prevalence rate varies between 0-28% in different castes, tribes and ethnic groups [66]. The prevalence reported in neonates born at a tertiary hospital of Ludhiana in North India was 3.9% [67]. A higher incidence of G-6-PD deficiency is seen in the North and West as compared to South India. About 13 different variants have been characterized biochemically, but at DNA level 6 G-6-PD variants have been reported. G-6-PD Mediterranean (536 C→T) is the most common variant in India followed by G-6-PD Orissa (131 C→G) and G-6-PD Kerala Kalyan (949 G→A). G-6-PD Chatham (1003 G→A), G-6-PD Jammu (871 G→A) and G-6-PD Insuli (989 G→A) (with normal G-6-PD activity) were found to be additional very rare variants in the Indian population.

G6PD deficiency has been associated with 5.1-18.2% of cases with severe indirect neonatal hyperbilirubinemia in northern India [68, 69, 70]. Only about 20% of babies with G-6-PD deficiency develop jaundice requiring phototherapy. So then, what is the determining factor of which baby will and will not develop jaundice? These mutations may be one of the
determining factors whether or not a baby with G-6-PD deficiency will develop severe jaundice.

7. Future perspectives

In all likelihood, additional genes are involved in neonatal hyperbilirubinemia, their identification and characterization are needed. Knowledge of each susceptibility gene polymorphism is essential for understanding more fully the molecular pathogenesis of neonatal hyperbilirubinemia, providing genetic markers for clinical risk assessment, and characterizing potential novel therapeutic targets, all meritorious lines of future investigations. The degree of genetic heterogeneity and variant coexpression across UGT1A1, OATP2 and G6PD gene observed in this cohort underscore the likely complex polygenic nature of neonatal hyperbilirubinemia. A more comprehensive study is warranted. However, due to strong evidence in the literature, genetic polymorphisms still should be considered in the clinical management, especially in those neonates who have existing risk factors, in order for early intervention to take place and to prevent neurotoxicity.

References