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2.1 Neonatal Hyperbilirubinemia

Neonatal jaundice is a common phenomenon among newborn infants. Most of the neonatal jaundice presents during the first week of life. Many babies develop only mild jaundice which is believed to be physiological. This physiological jaundice is thought to be due to an immaturity of the liver function in the newborn infant and due to the shorter life span of red blood cells carrying foetal haemoglobin. On the other hand, many babies develop more severe jaundice, putting them at risk of developing bilirubin encephalopathy and kernicterus (Boo et al., 2009; American Academy of Pediatrics Subcommittee on Hyperbilirubinemia, 2004). Many factors have been implicated in the pathogenesis of severe jaundice. The best known causes include haemolysis (such as rhesus incompatibility, ABO incompatibility or glucose-6-phosphate dehydrogenase (G-6-PD) deficiency), liver dysfunction (such as sepsis, Crigler-Najjar syndrome or metabolic disorders) and conditions which increase the enterohepatic circulation of bilirubin (such as breast feeding jaundice).

It is well known that in many cases of moderate or severe neonatal jaundice, the routine screening for common causes (including G-6-PD deficiency, blood grouping, direct Coombs test and full blood picture) does not identify any cause. If in these cases there is neither a history of poor breast feeding, these babies are often labelled as having excessive physiological jaundice or more correctly idiopathic pathological jaundice (Van Rostenberghe et al., 2006). The term ‘idiopathic’ implies that the cause is unknown. Recent evidence in literature suggests that causal factors of this type of neonatal jaundice may be due to point mutations in exons or mutations in the promoter region of the uridine-diphosphate glucuronosyltransferase 1 (UGT1A1) gene. UGT1A1 gene encodes the enzyme responsible for the rate limiting step for the bilirubin excretion.

Furthermore it is becoming increasingly clear that haemolysis in G-6-PD deficiency (and also ABO incompatibility) tends to be very mild and there is not much difference between babies developing neonatal jaundice and those not developing neonatal jaundice (Jalloh et al., 2005; Kaplan and Hammerman, 2005a). 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? It has been shown
in some populations that again point mutations or mutations in the promoter region of the
UGT1A1 gene are very important associated factors (Kaplan et al., 1997).

In different populations, different mutations in the UGT1A1 gene have been identified
and allelic frequencies of these mutations varied among the different population
(Carvalho et al., 2010; Sun et al., 2007; Akaba et al., 1998; Odell, 1980). No population
study had been conducted so far regarding these mutations in any South-East Asian
population. Since these mutations may be one of the determining factors whether or not a
baby with G-6-PD deficiency will develop severe jaundice, it was felt that it is important
to know in the Indian population whether mutations in this gene are common and
whether they are related to the occurrence of jaundice.

UGT1A1 is not only responsible candidate which involved in the development of
unconjugated hyperbilirubinaemia. Glucose-6-phosphate dehydrogenase (G6PD)
deficiency also shortens the mean life span of erythrocytes and/or low-grade haemolysis
may occur, which both may lead to an increased production of bilirubin. Importantly, the
organic anion transporter polypeptide 2 (OATP2) is involved in the transportation of
unconjugated bilirubin which is associated with hyperbilirubinemia in infants (Cui et al,
2001) Genetic variations in the G6PD and OATP2 genes were both additive risk factors
for unconjugated hyperbilirubinaemia, however genetic variation in the UGT1A1 gene
was acknowledged as the major risk (Prachukthum et al., 2009; Huang et al., 2005).

The observation of jaundice in some infants with hypertrophic pyloric stenosis may also
be related to a Gilbert-type variant. 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. Studies also suggest that
polymorphisms in the gene for glutathione-S-transferase (ligandin) may contribute to
higher levels of total serum bilirubin. That is the reason why this population study was
undertaken in a North Indian population to determine the frequency of mutations in the
UGT1A1 gene, OATP 2 gene and G6PD gene in a group of jaundiced babies and in a
group of babies with nonjaundiced as controls (Kaplan et al., 2007; Huang et al., 2004).
Certain factors present in the breast milk of some mothers may also contribute to increased enterohepatic circulation of bilirubin (breast milk jaundice). β-glucuronidase may play a role by uncoupling bilirubin from its binding to glucuronic acid, thus making it available for reabsorption. Data suggest that the risk of breast milk jaundice is significantly increased in infants who have genetic polymorphisms in the coding sequences of the UDPGT1A1 or OATP2 genes. Although the mechanism that causes this phenomenon is not yet agreed on, evidence suggests that supplementation with certain breast milk substitutes may reduce the degree of breast milk jaundice (Huang et al., 2004).

2.2 Bilirubin

The term bilirubin is derived from the Latin words for bile (bilis), and red (ruber). Städelier (Städelier et al., 1864) 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, hyperbilirubinemia.

2.2.1 Bilirubin Metabolism

2.2.1.1 Bilirubin chemistry

The systemic name of UCB (bilirubin IXa) is 1’8’-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-α,δ-dipropionic acid (Gourley et al., 1993; Ludwig, 1957). (Roy Chowdhury et al., 2010; Rudiger, 1971). Its molecular weight is 584.7 gram. UCB is a nearly symmetrical eat carbon atom 10 (Figure 2.1) (Ostrow et al, 1994).

UCB preferably has a “ridge-tile” conformation, i.e. is shaped like a partially open book. UCB structure was identified by analysis of X-ray diffraction (Bonnett et al., 1976). Six internal hydrogen bonds make the molecule insoluble in water because the hydrophilic polar COOH and NH groups are not available for attachment of H20 and the hydrophobic hydrocarbon groups are on the outside of the molecule (Maisels et al., 1994). When the hydrogen bonds are opened at, for example, an alkaline pH or by addition of (m) ethanol, diphylolin or caffeine, UCB becomes more labile, more polar and
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water-soluble. This allows UCB to react rapidly with the diazo reagent, the basis for measurement of unconjugated or indirect bilirubin by the Van den Bergh reaction (van den Bergh et al., 1916). UCB exists as three species with different degrees of ionization (H2B or diacid, HB- or monoanion, and B2- or dianion (Ostrow et al., 1994). The integrity of the hydrogen-bonded structure requires the interpyrrolic bridges at positions C4 and C15 to be in the trans or Z configuration (Z for zusammen).

Figure 2.1 Structure of unconjugated bilirubin. From McDonagh and Lightner (McDonagh et al., 1985)

2.2.1.2 Bilirubin production

Bilirubin is the end product of heme catabolism. The major source of heme (75-80%) is hemoglobin, from breakdown of erythrocytes. Other heme sources include cytochromes, peroxidase, catalase, myoglobin, and ineffective erythropoiesis (Bissell, 1986; Berk et al., 1976). The life span of erythrocytes is approximately 120 days in adults and 90 days in neonates (Gourley, 1993). Senescent erythrocytes are removed from the circulation and destroyed in the reticuloendothelial system (RES), mainly localized in the spleen, liver and bone marrow. In the RES, heme is phagocytized by macrophages. Macrophages contain microsomal heme oxygenase and cytosolic biliverdin reductase, two essential enzymes for degradation of heme to bilirubin. Heme oxygenase catalyzes the first step in
heme degradation: the opening of the porphyrin ring structure at the a-methene bridge (Figure 2.2).

Figure 2.2 Bilirubin production from heme catabolism (Tenhunen et al., 1970).

The intermediate blue-green pigment formed, biliverdin IXa, is water-soluble and nontoxic. The iron (Fe) is recycled and carbon monoxide (CO) is excreted by the lungs (Ludwig GD 1957). In mammals, biliverdin IXa is reduced by NADPH-dependent biliverdin reductase to produce bilirubin IXa, also known as unconjugated bilirubin (UCB). Why the nontoxic, water-soluble biliverdin is converted to the non-water-soluble and potentially toxic UCB is unclear. One hypothesis involves the need for products of fetal heme degradation to cross the placenta (Gartner, 1999; Ives, 1999). Biliverdin cannot, whereas the more lipophilic UCB can cross the placenta UCB production can be assessed by measurement of CO formation. Conversion by heme oxygenase of one heme molecule to biliverdin produces one molecule of CO. Production rate of UCB is
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approximately 6-8 mg/kg per 24 hours in healthy full-term infants, and 3-4 mg/kg per 24 hours in healthy adults (Maisels et al., 1971; Bloomer et al., 1970). Infants produce more UCB per kg body weight because of their higher red blood cell (RBC) count, the relatively larger fraction of hepatic heme proteins, and the shorter life span of fetal RBC’s. Fetal hemoglobin (HbF), which has a higher affinity for oxygen than “adult” HbA, is broken down postnatal in the relatively oxygen-rich environment. Apart from CO measurements, UCB production rate can be derived from turnover of radioisotopically labelled bilirubin, under steady-state conditions.

2.2.1.3 Bilirubin Transport

Once the hydrophobic UCB leaves the reticuloendothelial system, over 99.9% is bound to bilirubin in plasma in a non-covalent fashion and transported to the liver. Albumin has a high affinity binding site for UCB. Beyond a molar ratio of 1:1, which is equivalent to a plasma UCB concentration of approximately 600 μmol/l, UCB can bind to albumin at additional lower affinity binding sites (Brodersen, 1979; Brodersen, 1980). 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 unbound (i.e. free) UCB, which is considered toxic. Recently, high-density lipoprotein (HDL) has been reported to be the principal nonalbumin carrier of UCB in human plasma. The affinity of HDL for UCB is primarily the result of binding to apolipoprotein D (Goessling and Zucker, 2000).

2.2.1.4 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 (Sorrentino and Berk, 1988). 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 (Maisels, 1994). This transfer of UCB is carrier mediated, although controversy exists regarding the exact mechanism (Sorrentino and Berk, 1988; Berk et al., 1987). Several proteins have been suggested as putative UCB transporter, including the organic anion transport protein (Oatp2 / Slc21a6) (Cui et al., 2001; Wang et al., 2003) and the bilirubin/BSP binding protein (BBP) which also transports other organic anions such as bromosulfophthalein (BSP) (Stremmel et al., 1983).
Once within the hepatocyte, UCB is bound by the major cytosolic binding protein for UCB, glutathione S-transferase, traditionally referred to as ligandin or Y-protein (Boyer, 1989; Habig et al., 1974). UCB flux across the hepatocyte membrane is bidirectional. Binding to glutathione S-transferase decreases the unbound fraction and thereby the reflux of UCB and conjugated bilirubin back into plasma (Wolkoff et al., 1979; Crawford et al., 1988).

### 2.2.1.5 Bilirubin conjugation

Figure 2.3 shows metabolism of Bilirubin in the hepatocyte. In order to excrete bilirubin efficiently into bile, conjugation is required to convert the non-polar, water-insoluble

![Figure 2.3 Hepatocellular bilirubin transport.](image)

UCB (at pH 7.4) to water-soluble conjugate. Glucuronic acid is the major conjugating group (Gordon et al., 1976). Traces of other conjugates (e.g. glucose and xylose...
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Conjugates have been identified in human bile, (Spivak and Carey, 1985) and higher proportions of glucose and xylose conjugates are present in rat and dog bile. Bilirubin glucuronides are present as mono- and diglucuronides. The enzyme bilirubin-uridine diphosphoglucuronosyltransferase (UDPGT, UGT1A1, EC 2.4.1.17), 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 (Burchell and Coughtrie, 1989; Roy Chowdhury et al., 2010).

2.2.1.6 Bilirubin excretion

Conjugation is an important step in UCB catabolism. Efficient biliary secretion of bilirubin requires conversion to polar conjugates. A very small amount of UCB is excreted into bile without conjugation, where it rapidly associates with mixed micelles (Roy Chowdhury et al., 2002). UCB in bile is seldom more than 2% of total bilirubin and is believed to derive in large part from hydrolysis of secreted conjugates in the biliary tree. Conjugated bilirubin leaves the hepatocyte via Mrp2 (multidrug resistant protein 2, Abcc2). Mrp2 is an ATP-dependent transporter that carries conjugated bilirubin across the canalicular membrane into the biliary tree. Absence of Mrp2 in patients with Dubin-Johnson syndrome, and in analogous rat models (the TR- rat and the Eisai hyperbilirubinuria rat), causes conjugated hyperbilirubinemia (Paulusma et al., 1997; Paulusma et al., 1996; Kuroda et al., 2004). However, Mrp2 cannot be the only canalicular transporter that is able to excrete conjugated bilirubin, because in the TR- rat organic anion transport was found to be preserved (Jansen et al., 1993). Mrp3 (multidrug resistant protein 3, Abcc3) is considered an important candidate for basolateral excretion of conjugated bilirubin (Donner and Keppler, 2001). Conjugated bilirubin is retained in hepatocellular and cholestatic disorders. Increased plasma levels of conjugated bilirubin result in formation of a bilirubin-albumin complex called d-bilirubin, (Brett et al., 1984) which reacts directly with diazo reagents, as does conjugated (i.e. direct) bilirubin (van den Bergh et al., 1916). Multidrug resistant protein 1 (Mrp1, Abcc1) is a proven exporter of UCB that requires glutathione as a co-factor. Mrp1 protects cells against UCB-induced cytotoxicity (Gennuso et al., 2004; Cekic et al., 2003; Rigato et al., 2004; Calligaris et al., 2006).
2.2.1.7 Intestinal metabolism and enterohepatic circulation of bilirubin

Conjugated bilirubin is hydrolyzed in the intestine to UCB, which can be reabsorbed into the enterohepatic circulation (EHC), (Lester and Schume, 1963\textsuperscript{a}; Lester and Schume, 1963\textsuperscript{b}) (Figure 2.4). Hydrolysis of conjugated bilirubin to UCB can occur nonenzymatically under the influence of mild alkaline conditions as in the duodenum or jejunum, (Halamek and Stevenson, 2002) and enzymatically by β-glucuronidase. Endogenous tissue β-glucuronidase exists in the enteric mucosa and liver, (Musa \textit{et al.}, 1984) but the major part of enzyme activity is of bacterial origin (Saxerholt and Midtvedt, 1984; Kent \textit{et al.}, 1972). In neonates, a relative lack of bacterial flora and a high mucosal β-glucuronidase activity increase the enterohepatic circulation of UCB. β-glucuronidase is present in human breast milk and was thought to exaggerate jaundice in breastfed infants (Gourley \textit{et al.}, 1986). However, the small amounts of enzyme in milk relative to the large amounts of mucosal β-glucuronidase would not be expected to add much to the overall activity (Maisels, 1994). UCB in the intestine not only results from deconjugation of conjugated bilirubin. UCB can also diffuse from the blood into the intestinal lumen across the mucosa, (Kotal \textit{et al.}, 1997; Schmid and Hammaker, 1963) particularly when plasma UCB levels are high. Preventing enterohepatic circulation of UCB is one of the strategies for treatment of unconjugated hyperbilirubinemia.

Conjugated bilirubin must be hydrolyzed to UCB before the tetrapyrrole ring can be reduced to the colorless urobilinogens by intestinal anaerobic bacteria (3 Clostridia species and Bacteroides fragilis) (Vitek \textit{et al.}, 2006\textsuperscript{a}; Vitek \textit{et al.}, 2005).

Urobilinogen can be oxidized to the yellow-orange urobilin. The brown color of feces is due to dipyrrolic oxidative derivatives of UCB, the mesobilifuscins. Absence of urobilinogen in feces and urine indicates complete obstruction of the bile duct. Oxidation-reduction of the various unsaturated bonds in bilirubin results in a large family of related colorless reduction-oxidation products known as urobilinoids (Billing, 1986). The formation of urobilinoids is important for the removal of bilirubin from the body because the majority of urobilinoids is excreted via the feces. A small portion is reabsorbed across the intestinal mucosa into the enterohepatic circulation and subsequently excreted by liver and kidney. Urobilinogen can also undergo enterohepatic circulation (Lester and Schume, 1965\textsuperscript{a}; Lester \textit{et al.}, 1965\textsuperscript{b}). Conjugated bilirubin cannot be reabsorbed into the portal circulation.
2.3 Bilirubin Toxicity and Antioxidant Properties

2.3.1 Bilirubin neurotoxicity

It is generally accepted that UCB bound to albumin or other plasma (lipo) proteins is not toxic. Unbound, i.e. free UCB is toxic when the concentration is higher than its aqueous solubility (70 nM) (Ostrow et al., 2004) also showed toxicity to cultured astrocytes (Gennuso et al., 2004). The diacid (H2B) is considered the toxic agent because the dianion (B2-) and monoanion (HB-) do not diffuse readily into cells. B2- and HB- are relatively more water-soluble because of, respectively, 2 and 1 open internal hydrogen bond(s). At pH 7.4 in plasma, there is <2% dianion and >80% diacid (Ostrow et al., 1994). Free H2B can diffuse bidirectionally, passively and rapidly across membranes,
The mechanism of UCB neurotoxicity is not fully understood. Brain damage probably results from a combination of risk factors. Free UCB not only enters brain tissue when the UCB binding capacity in plasma is exceeded, or when displacing substances (e.g., sulfonamides or free fatty acids) compete for bilirubin binding sites on albumin (Maisels, 1994). At bilirubin/albumin ratio’s below 1.0 toxicity can also occur if free UCB levels increase steeply (Ostrow et al., 2003). Acidosis is considered a risk factor for the development of bilirubin encephalopathy, although the effect of acidosis on UCB-albumin interaction is controversial (Roth and Polin, 1988). The adverse effects of acidosis appear secondary to rapid deposition of insoluble H2B precipitates in tissues. An increased permeability of the blood-brain barrier via disruption of tight junctions by hyperosmolality, hypercapnia, asphyxia or hypertension can increase entry of free UCB (and even albumin-bound UCB) in the brain (Bratlid et al., 1984). Possibly hyperthermia and septicemia have similar effects (Levine et al., 1983). Recently, it has been suggested that transporter molecules in the blood-brain barrier actively pump free UCB out of the central nervous system and maintain a concentration gradient of UCB from cerebrospinal fluid to plasma (Ostrow et al., 2003). Indirect support for this hypothesis can be derived from the observation that UCB induces expression and translocation of multidrug resistance-associated protein 1 (Mrp1, Abccl) in astrocytes (Gennuso et al., 2004). Intracellular UCB levels may also be diminished by oxidation, conjugation or binding to cytosolic proteins (glutathione-S-transferases) (Ostrow et al., 2003). Regional UCB deposits in the brain are probably mainly explained by regional differences in exporters of UCB (Ostrow et al., 2004; Ostrow et al., 2003) but may also relate to differences in lipid composition, blood flow (Bratlid, 1990) or bilirubin oxidation (Hansen, 2000).

The exact mechanism of UCB toxicity at the cellular level is still under debate. In the past, UCB was shown to impair mitochondrial function and to interfere with RNA/DNA synthesis and carbohydrate metabolism in the brain. However, these studies were performed at extremely elevated, i.e. not physiologically relevant, free UCB concentrations. More recent papers showed that UCB decreases cell membrane potential and disrupts transport of neurotransmitters (Ostrow et al., 2004; Cashore, 1990). UCB also inhibits protein (Schenker et al., 1986) and interferes with intracellular calcium homeostasis (Shapiro, 2005) and glutamate efflux (Falcao et al., 2005). Microglia cells and astrocytes damaged by UCB produce cytokines that may contribute to brain toxicity.
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(Fernandes et al., 2004; Fernandes et al., 2006; Gordo et al., 2006). Free UCB induces apoptosis at levels as low as 71-85 nmol/l. Since damage to neurons and astrocytes can occur at free UCB concentrations near or modestly above aqueous saturation, (Ostrow et al., 2003) treatment of jaundiced neonates should be intensified if at physical examination early signs of bilirubin encephalopathy are detected, even if plasma UCB levels are only moderately elevated.

2.3.2 Bilirubin toxicity to other organs

Apart from the brain, UCB may also have deleterious effects on other organs. Bilirubin crystals and necrosis result in impaired urinary concentration with polyuria (Call and Tisher, 1975; Odell et al., 1967). The liver is relatively resistant to UCB toxicity. This is being re-examined, but may be due to the high conjugating activity or high degree of protein binding in this organ (Schenker et al., 1986). Dental enamel dysplasia or green discoloration of the teeth may occur (Perlstein, 1960). Patterns of bilirubin deposition have also been found in heart, lung, adrenal, pancreas, testes and skin (Bernstein and Landing, 1962; Harper et al., 1986). UCB can inhibit cartilage metabolism and growth in vitro, (Vassilopoulou-Sellin et al., 1989) and may inhibit cellular immune responses (Rola-Pleszczynski et al., 1975).

2.3.3 Bilirubin as antioxidant

Bilirubin may not only be a potentially toxic metabolite from heme degradation, it may also be good for you (Vassilopoulou-Sellin et al., 1989). The first suggestion that UCB might have a physiologic function was made in 1937 when UCB appeared to be part of a protective mechanism designed to overcome (pneumococcal) infection (Farah, 1937). Subsequently many have demonstrated antioxidant properties of UCB. UCB inhibits auto-oxidation of unsaturated fatty acids, (Bernhard et al., 1954) scavenges peroxyl radicals, (Stocker et al., 1987) may prevent oxidative membrane damage, and detoxifies singlet oxygen (McDonagh, 1990). Infants with illnesses believed to enhance free-radical production (e.g. sepsis, asphyxia) had a significantly lower daily rise in mean plasma bilirubin levels than control infants consistent with the hypothesis that bilirubin is consumed as an antioxidant (Maisels, 1994; Benaro and Bowen, 1991). Conjugated bilirubin and biliverdin also have antioxidant properties (McDonagh, 1990; Stocker and Peterhans, 1989). Heme oxygenase induction protected human hepatocytes against warm
and cold hypoxia. The proposed mechanisms by which heme oxygenase exerts its cytoprotective effects include its abilities to degrade the prooxidative heme, to produce biliverdin and subsequently bilirubin, and to generate carbon monoxide, which has antiproliferative and anti-inflammatory as well as vasodilatory properties (Morita, 2005). Antioxidant enzymes were also significantly inhibited in bilirubin encephalopathy babies. Post phototherapy, MDA (Malondialdehyde) production and antioxidant levels were significantly increased whiles total antioxidant capacity and reduced glutathione were significantly decreased compared to pre-phototherapy values (Shekeeb shahab et al., 2008).

In vitro exposure of neurons and astrocytes to free UCB showed neuroprotection at free UCB levels below aqueous saturation (70 nM) (Dore and Snyder, 1999). UCB was shown to inhibit oxidation of low density lipoprotein more effectively than a vitamin E analogue, (Wu et al., 1994) hence it was postulated that UCB may reduce atherogenesis. Elevated bilirubin levels, (Hopkins et al., 1996; Sedlak and Snyder, 2004; Vitek et al., 2002; Mayer 2000; Novotny and Vitek, 2003; Vitek et al., 2006) and (inducers of) heme oxygenases (Morita, 2005) are associated with a diminished risk of atherosclerosis and appear also negatively related to the risk of cancers and demyelinating neuropathies (Rigato et al., 2005).

### 2.4 Clinical aspects 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 90 μmol/L. Initially jaundice tends to appear in the face alone but as the hyperbilirubinaemia gets worse, other areas of the body become yellow as well. Generally it is assumed that jaundice reaching the abdomen indicates that the bilirubin level is about 250μmol/L. If the legs are jaundiced it means there is severe hyperbilirubinaemia.

Hyperbilirubinemia can be divided into two types depending on the conjugation of bilirubin to glucuronide molecules: i.e. unconjugated hyperbilirubinemia and conjugated hyperbilirubinemia.
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2.4.1 Unconjugated hyperbilirubinemia (Indirect hyperbilirubinemia)

Hyperbilirubinemia can either be unconjugated or conjugated, or involves elevation of both UCB and conjugated bilirubins, as the vast majority of conjugated hyperbilirubinemia. Conjugated hyperbilirubinemia always involves a pathophysiological mechanism located after the level of hepatic conjugation, including secretory defects and bile duct obstructions. Examples of conjugated hyperbilirubinemia include inherited syndromes with reduced biliary secretion of conjugated bilirubin (Dubin-Johnson syndrome, Rotor syndrome), obstructive jaundice (tumor/stones), and Benign Recurrent Intrahepatic Cholestasis (Roy Chowdhury et al., 2010). This introduction will be limited to unconjugated hyperbilirubinemia, which can result from increased UCB production, decreased hepatic uptake, decreased conjugation or increased enterohepatic circulation of UCB.

Unconjugated hyperbilirubinemia becomes clinically apparent with visible jaundice at plasma bilirubin levels of about 85 μmol/l. (Maisels, 1994) Normal plasma total bilirubin levels in human adults range from 5 to 17 μmol/l (Gollan and Knapp, 1985). Neonatal jaundice starts at the head and progresses in a cephalocaudal manner to the trunk, arms, legs, palms and soles (Maisels, 1994). Increased heme catabolism contributes to jaundice in the first days after birth (Maisels and Kring, 2006). For the majority of neonates, unconjugated hyperbilirubinemia is a benign transitional phenomenon of no overt clinical significance (Watchko, 2006; Watchko, 2004). 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). Although several guidelines for the management of unconjugated hyperbilirubinemia have been published, definitive data on “safe” plasma UCB concentrations have not been established.

2.4.2 Increased bilirubin production

Neonates have an increased UCB production compared with adults, mainly because of a higher erythrocyte count and a shorter erythrocyte life span. Other causes of increased bilirubin production include hemolysis due to blood group incompatibility, due to structural or biochemical erythrocyte defects, or due to sepsis. Extravasation of blood
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(cephalhematoma, intracranial hemorrhage) and polycythemia contribute to a high bilirubin load.

2.4.3 Decreased hepatic uptake

A reduced capacity of net hepatic uptake may contribute to the pathogenesis of physiologic jaundice. In newborn monkeys, deficiency of ligandin and reduced clearance of BSP were demonstrated in the first days of life (Levi et al., 1970). In humans, this deficiency is of less importance than an absolute deficiency of bilirubin conjugation, (Halamek and Stevenson, 1997) or a relative deficiency of conjugation due to a mismatch between increased supply of UCB in the neonatal period and conjugation capacity. In Gilbert syndrome some patients have a reduced hepatic uptake of bilirubin (Roy Chowdhury et al., 2010; Berk et al., 1972; Martin et al., 1976).

2.4.4 Decreased conjugation

In the first ten days of life, UGT1A1 activity is usually less than 0.1% of adult values (Kawade and Onishi, 1981). Then UGT1A1 activity increases exponentially to adult values at 6 to 14 weeks of life. The postnatal increase in plasma UCB levels appears to play an important role in the initiation of bilirubin conjugation (Rosenthal et al., 1986). Gilbert syndrome, described in 1901 by Gilbert, (Gilbert and Lereboullet, 1901) is a mild recurrent unconjugated hyperbilirubinemia that usually does not become manifest until after the second decade of life. In Gilbert syndrome UGT1A1 activity is approximately 20-30% of normal and in some patients an additional reduced hepatic uptake of bilirubin has been demonstrated (Roy Chowdhury et al., 2010; Maisels, 1994; Berk et al., 1972; Martin et al., 1976). The prevalence of patients with Gilbert syndrome ranges between 2 and 12% (Okolicsanyi et al., 1993; Sieg et al., 1987). The mode of inheritance is most likely autosomal recessive (Powell et al., 1967; Bosma et al., 1995). A polymorphism (an extra TA in the TATAA box) in the promoter region of the UGT1A1 gene appears to be necessary for Gilbert syndrome but not sufficient for the complete manifestation of the syndrome (Bosma et al., 1995; Rajmakers et al., 2000). To increase plasma UCB concentration, a concomitant decrease in hepatic uptake and/or increase in UCB production is needed. Some patients have an increased bilirubin turnover rate due to subclinical hemolysis (Okolicsanyi et al., 1993). The majority of patients are anicteric because plasma UCB concentrations are usually less than 50-85 μmol/l. Intercurrent
illnesses and fasting may exaggerate the unconjugated hyperbilirubinemia and cause manifest jaundice. Administration of phenobarbital reduces the unconjugated hyperbilirubinemia, but does not enhance UGT1A1 activity in Gilbert patients (Roy Chowdhury et al., 2010).

2.4.4 Increased enterohepatic circulation

Delayed intestinal transit due to starvation, delayed passage of meconium, pyloric stenosis or Hirschsprung’s disease increases the enterohepatic circulation of UCB (Kotal et al., 1996). Increased intestinal motility allows less time for UCB absorption. Frequent feedings (De Carvalho et al., 1982) and rectal stimulation (Cottrell and Anderson, 1984) are associated with lower plasma UCB levels. The absence of anaerobic bacterial flora in the neonatal intestine, with limited conversion of UCB to urobilinogen, greatly enhances the enterohepatic circulation of UCB. In older children and adults a comparable situation occurs during treatment with broad-spectrum antibiotics that suppress the anaerobic flora (Vitek et al., 2005). Breast feeding enhances the enterohepatic circulation of UCB via several mechanisms. The first few days, intake is limited, leading to delayed passage of meconium and decreased stool weight (De Carvalho et al., 1992). Breast milk contains β-glucuronidase which converts conjugated bilirubin to UCB (Gourley and Anend, 1986). Breast milk is thought to alter the bacterial colonization of the intestine leading to decreased formation of urobilinogen (Yoshioka et al., 1965; Yoshioka et al., 1983). UGT1A1 polymorphisms or Gilbert syndrome may be an underlying cause of breast milk jaundice (Kotal et al., 1996; Maruo et al., 2000). Free fatty acids in breast milk have been suggested to contribute to neonatal jaundice through inhibition of UGT1A1 (Gourley, 1992; Arias and Gartner, 1964). However, it is not easy to envision how intestinal free fatty acids would affect the liver, given the physiological post-absorptive transport of intestinal fatty acids in the form of chylomicron triglycerides. Rather, the association between jaundice and free fatty acids in milk may be based on the presence of lipase activity in breast milk. Lipases in breast milk, in particular bile salt stimulated lipase, may increase the amount of free fatty acids and enhance fat absorption. According to our hypothesis that unabsorbed fat captures UCB in the intestine, a lower fraction of unabsorbed fat in the intestinal lumen will result in less UCB capture, more enterohepatic circulation and subsequently less fecal excretion of UCB (Verkade, 2002).
2.4.5 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 (Thilo, 1999). Conjugated hyperbilirubinaemia more commonly occurs after the first week of life, especially when the unconjugated hyperbilirubinaemia has decreased.

2.5 Common causes of neonatal jaundice

2.5.1 Physiological jaundice

Physiological jaundice most commonly occurs on day 3 to day 5 of life and decreases 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 volume, shortened erythrocyte life span of cells carrying foetal haemoglobin and increased enterohepatic circulation (Hintz et al., 2001).

Physiological jaundice is the most common cause of neonatal jaundice. It tends to be more severe in Asian populations than in Caucasian and Black populations (Zaghloul and Schulze, 2001). Quite often however, the term is used incorrectly to denote severe cases of neonatal jaundice. If screening for common causes does not reveal any cause, neonatal jaundice tends to be labelled as excessive physiological jaundice. A better term for this severe jaundice might be idiopathic pathological jaundice (Van Rostenberghe et al., 2006).

2.5.2 Pathological causes

2.5.2.1 Haemolytic causes

2.5.2.1(a) ABO incompatibility

ABO incompatibility most commonly occurs when a baby with blood group A or B is born to a mother with type O blood group. In these cases antibodies made by the mother against the blood group antigens of the baby may cross the placenta and cause
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haemolysis. The presence of these maternal antibodies in the baby’s blood is tested through the direct Coombs test, using heterologous anti-immunoglobulin (Kaplan, 2004). The Coombs test however has a high false negativity rate that may be related to a relatively low expression of the blood group antigens on the red blood cells of the neonate or to relatively low titres of antibodies. If jaundice occurs in ABO incompatibility, it typically starts on day 1 after birth (Thilo, 1999).

2.5.2.1(b) Rhesus incompatibility

Rhesus incompatibility occurs when a Rhesus negative mother carries a Rhesus positive baby. This condition results in severe anaemia antenatally, causing hydrops fetalis. It is also called erythroblastosis fetalis (Thilo, 1999).

2.5.2.1(c) Glucose-6-phosphate dehydrogenase deficiency

The enzyme glucose-6-phosphate dehydrogenase (G-6-PD) deficiency can be found in every cell in our body. It is used to protect cells (especially red blood cells) against oxidative damage. The glutathione which is oxidised is used for neutralisation of toxic agents such as hydrogen peroxide and organic peroxidases (Mehta et al., 2000). To maintain the process, oxidized glutathione is directly changed back to the reduced form using hydrogen ions from NADPH. NADPH is formed from nicotinamide adenine dinucleotide phosphate (NADP) and this process is catalyzed by G-6-PD. Therefore, NADPH formation is disturbed in G-6-PD deficiency and this leads to fragility of the red blood cells in the presence of oxidative stresses. This process is illustrated in Figure 2.5. Residual G-6-PD activity and additional antioxidant enzymes such as catalase prevent significant haemolysis under physiological conditions but under oxidant stress conditions, severe haemolysis may occur (Mehta et al., 2000, Kaplan and Hammerman, 2002b).

G-6-PD deficiency is a major cause of neonatal jaundice (Mehta et al., 2000) and is common in many developing countries with limited access to modern medical care. Half of the morbidity and mortality which is associated with neonatal jaundice may be caused by G-6-PD deficiency (Kaplan et al., 2001c).

Classically it was believed that the neonatal jaundice in babies with G-6-PD deficiency is due to haemolysis. However in the majority of cases there is no identifiable oxidative
stress and haemolysis has been found to be very mild and similar in G-6-PD deficient babies developing jaundice and those not developing jaundice (Jalloh et al., 2005).

![Diagram for the regeneration of NADPH](image)

**Figure 2.5** 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 (Kaplan and Hammerman, 2002c).

The presence of other risk factors for jaundice in combination with G-6-PD deficiency may become the crucial factor determining whether or not a baby with G-6-PD deficiency gets jaundiced. Such factors may include a decrease in bilirubin conjugation that may be related to promoter polymorphism in the gene which controls the bilirubin conjugating enzyme UDP-glucuronosyltransferase (Kaplan et al., 2001b). These findings were also supported by other studies (Bosma et al., 1995; Beutler et al., 2002).

Total serum bilirubin levels in G-6-PD deficient neonates rise gradually and eventually leading to jaundice. This gradual onset jaundice may cause the jaundice to be missed in babies discharged early from the postnatal ward. This is the reason that in Malaysia, all babies are screened and the G-6-PD deficient babies are monitored for 5 days in the ward, so phototherapy can be started early so as to prevent the need for exchange transfusion.

**2.5.2.1(d) Other causes of haemolysis**

Another common cause of haemolysis is the extravasation of blood due to birth trauma. Extravasated blood is rapidly broken down and may cause severe hyperbilirubinaemia.
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2.5.2.1 Non-haemolytic causes of neonatal jaundice

The common causes of jaundice without haemolytic disorder can be classified as follows:

a) Increase of bilirubin production which is related to polycythaemia and swallowed blood.

b) Decrease of bilirubin clearance, which is caused by UDPGT deficiency, resulting in Crigler-Najjar and Gilbert syndrome. Decreased bilirubin clearance is also seen in hypothyroidism and breast feeding which is associated with jaundice.

c) Unknown or multiple pathogeneses include altitude, racial differences, prematurity and bowel obstruction.

2.6. Kernicterus and Bilirubin-induced neurologic dysfunction (BIND)

In 1847, Hervieux was the first to report yellow staining of brain nuclei in a severely jaundiced baby (Hansen et al., 1987). In 1985, Orth observed bilirubin pigment at autopsy in the brains of severely jaundiced infants (Orth, 1875). The term kernikterus (from the German kern, nucleus, and the Greek ikterus, jaundice), was first used in 1903 by Schmorl, who described similar yellow staining of brain nuclei in infants who died with severe neonatal jaundice (Schmorl, 1903). The regions commonly affected are the basal ganglia (globus pallidus, nucleus subthalamicus), the hippocampus, various nuclei in the brain stem (a.o. oculomotor, cochlear, vestibular and olivary nuclei) and cerebellum (nucleus dentatus) (Turkel, 1990; Haymaker et al., 1961; Volpe, 1995).

Originally kernicterus was a pathologic diagnosis, later the term was also used for the acute and chronic neurological syndrome. Classic acute kernicterus in neonates is characterized by three phases (Maisels, 1994; Shapiro, 2005; Connolly and Volpe, 1990; Gerrard, 1952). In the first few days the infant becomes lethargic, hypotonic and sucks poorly. In the second phase, the infant becomes hypertonic with retrocollis and opisthotonus, frequently develops a fever and high-pitched cry, and may develop seizures. In the third phase, usually after one week, hypertonia gradually becomes less pronounced and is replaced by hypotonia. Chronic signs of kernicterus, so called longterm sequelae, include choreoathetosis, vertical gaze paralysis, sensorineural deafness and dental dysplasia (the ‘tetrad of Perlstein’), (Perlstein, 1960) asymmetric spasticity, motor delay and mental retardation. Subtle encephalopathy is referred to as
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Bilirubin-induced neurologic dysfunction (BIND) (Shapiro, 2005; Volpe, 2001). BIND can present with hearing loss, lowered IQ (Seidman et al., 1991; Naeye, 1978) and abnormal cognitive function (Odell et al., 1970; van de Bor et al., 1992; Boggs et al., 1967; Scheidt et al., 1977). Recently, plasma UCB levels up to ~510 μmol/l treated with phototherapy or exchange transfusion were not associated with adverse neurodevelopmental outcomes in infants born at or near term (Newman et al., 2006). Patients with Crigler-Najjar disease have a life-long risk of developing kernicterus. The risk increases especially during adolescence when phototherapy becomes less effective and compliance gets worse, and during intercurrent infectious illnesses. In some children with Crigler-Najjar disease type I there may be a late clinical presentation of bilirubin encephalopathy with cerebellar symptoms as presenting feature (Labrune et al., 1992).

2.7 Treatment of Unconjugated Hyperbilirubinemia / Crigler najjar Syndrome

2.7.1 Phototherapy

Phototherapy was discovered in 1956 when a nurse in England noticed that when jaundiced infants were exposed to sunlight they became less yellow (Cremer et al., 1958). Pediatric resident Cremer et al. subsequently demonstrated the efficacy of phototherapy by exposing preterm infants to blue fluorescent lights, which dropped plasma bilirubin levels (Cremer et al., 1958). In the mid 1960’s other therapeutic trials followed (Broughton PMG et al., 1965) and since then phototherapy has been used extensively for treatment of unconjugated hyperbilirubinemia. Phototherapy detoxifies bilirubin by converting UCB to photoisomers that are less hydrophobic than UCB. The photoisomers are a better substrate for Mrp2 and therefore can be excreted into bile without being conjugated first (McDonagh et al., 1990). Phototherapy increases the amount of UCB in bile (Ostrow, 1971; Zenone et al., 1982). When bilirubin molecules in the skin absorb (phototherapy) light, 3 photochemical reactions can occur: configurational and structural photoisomerization, (Figure 2.6) and photooxidation. In configurational photoisomerization, one (or both) of the double bonds at carbon atoms C4 and/or C15 in the bilirubin molecule is (are) opened, converting it from the ZZ configuration to a ZE, EZ or EE configuration (Z for zusammen, E for entgegen). When this occurs, the polar N and O groups are exposed, making the UCB-photoisomer less...
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hydrophobic than UCB and therefore a better substrate for transport into the bile via Mrp2. The predominantly formed 4Z, 15E isomer is an unstable molecule that readily reverts back. This reverse reaction is relatively slow when the isomer is bound to albumin, but occurs rapidly in bile and intestinal lumen (Maisels, 1994). In structural photoisomerization, intramolecular cyclization of bilirubin occurs to form the non-reversible photoisomer lumirubin (McDonagh et al., 1982; Costarino et al., 1985). Lumirubin is cleared much more rapidly from plasma than the 4Z, 15E isomer, and is therefore considered mainly responsible for the decline in plasma UCB levels during phototherapy (Ennever et al., 1985; Onishi et al., 1986). Photo-oxidation of UCB involves hydroxylation and cleavage of –CH= bridges yielding mono- and dipyroles that are small, polar and can be excreted in the urine (Lightner et al., 1984). Photo-oxidation is a slow process and appears to play a minor role in the photocatabolism of UCB in vivo (Maisels, 1994; Lightner et al., 1984).

Figure 2.6 Phototherapy-induced photoisomerization of bilirubin. A: configurational photoisomerization. B: structural photoisomerization to lumirubin. From: McDonagh and Lightner (McDonagh et al., 1985).

Several types of fluorescent lights have been used for phototherapy, including daylight, broad-spectrum, white, green, (special) blue and violet (Ennever, 1990; Tan, 1989). Efficacy results have been contradictory, but special (narrow spectrum) blue lights are generally considered superior because bilirubin absorbs light maximally in the blue range (from 420-500 nm) with a peak absorption at about 440-460 nm. White light is preferred by some clinicians because blue light distorts skin color, which makes it difficult to assess cyanosis and jaundice in the neonate. Phototherapy is most efficient in the first 24-48 hours of treatment. The declining efficacy after 48 hours is probably related to configurational photoisomers that have been reverted to UCB, undergo enterohepatic circulation and increase the UCB load to be cleared by the liver (Maisels, 1994; Brown
et al., 1985). Furthermore, phototherapy is less effective at lower plasma UCB concentrations due to depletion of the bilirubin pool in the skin, which is the main target for phototherapy (Rubaltelli and Carli, 1971; Vogl, 1974). The efficacy of phototherapy also depends on the body surface area exposed to the light. Therefore, double-sided phototherapy with a conventional overhead lamp plus a “biliblanket” reduces plasma UCB levels more rapidly (Maisels, 1994). Whether phototherapy should be given continuously or intermittently is not quite clear. Some studies reported continuous phototherapy to be more effective, but this was not confirmed by others. Since migration of bilirubin to the skin takes one to three hours (Vogl, 1974) and is probably the rate-limiting step, intermittent phototherapy should be effective (Maisels, 1994). Intermittent phototherapy to in vitro human cells in tissue culture, however, caused more damage to DNA than continuous phototherapy (Santella et al., 1978).

Since phototherapy was introduced almost 50 years ago, serious long-term side effects such as skin cancers have not been observed. However, phototherapy-induced DNA damage to human cell lines in vitro does occur and bilirubin was found to enhance this damage (Rosenstein and Ducore, 1984; Sideris et al., 1981). Short-term phototherapy has relatively minor side effects and is considered safe (Tan, 1991).

Phenomena that have been attributed to or associated with phototherapy include retinal damage if the eyes are not shielded from light by eye patches, (Messner et al., 1978) diarrhoea and decreased gut transit time, (Brown et al., 1970; Rubaltelli et al., 1973) increased insensible water loss, (Oh and Karecki, 1972) temperature instability, patent ductus arteriosus (Barefield et al., 1993; Rosenfeld et al., 1986) and the “bronze baby syndrome”, which appears to be due to accumulation of photodegradation products (bilifuscins) when their biliary excretion is impaired by concomitant cholestasis (Halamek and Stevenson, 1997; Onishi et al., 1982).

Patients with Crigler-Najjar disease type I have to undergo daily phototherapy up to 12 hours per day. Type II patients usually only need a few hours of phototherapy per day, if any. Long-term phototherapy has considerable disadvantages. Phototherapy becomes less effective with age, due to a decrease in surface area to body mass ratio, (Strauss et al., 2006; Yohannan et al., 1983) due to a large tissue reservoir of UCB, (Strauss et al., 2006) due to skin alterations, (Van Der Veere et al., 1997; Van Der Veere et al., 1996)
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and due to a diminishing compliance to the intensive phototherapy regimen which has a profound impact on the quality of (social) life (Van Der Veere et al., 1996).

2.7.2 Exchange transfusion

Phototherapy has greatly reduced the need for exchange transfusion. With this technique, approximately 85% of circulating red blood cells will be replaced (when replacing 160 ml/kg BW), and plasma UCB levels will generally be reduced by 50% (Halamek and Stevenson, 1997). The exchange transfusion physically removes defective red blood cell and UCB, which diffuses from the extravascular space (i.e. tissue pool) into plasma. Indications for exchange transfusion include symptoms and signs characteristic of acute bilirubin encephalopathy, dangerously high or rapidly rising plasma UCB concentrations despite phototherapy, and progressive anemia due to hemolysis (Gourley, 1997 and Ives, 2010). The mortality rate from the procedure is around 0.3%. Significant morbidity is associated with ~5% of exchange transfusions (Keenan et al., 1985; Hovi and Siimes, 1985). Complications include cardiac and vascular complications such as cardiac arrest and thrombosis of the portal vein in case the exchange transfusion was done via a catheter in the umbilical vein, metabolic and coagulation disturbances, transmission of infectious diseases, graft versus host disease and necrotizing enterocolitis.

In the management of Crigler-Najjar disease, generally exchange transfusions are not required. Sometimes exchange transfusions are used in the neonatal period when the diagnosis is not yet clear. Incidentally, exchange transfusions are performed when plasma UCB concentration is dangerously increased and/or albumin concentration decreased, for example during intercurrent (febrile) illnesses or around surgery (Strauss et al., 2006; Van Der Veere et al., 1996).

2.7.3 Phenobarbital

Phenobarbital is an anti-epileptic drug that enhances the three steps in hepatic bilirubin metabolism independently: uptake and storage of UCB by the hepatocyte, conjugation, and biliary secretion (Crawford et al., 1988; Ostrow, 1972; Catz and Yaffe, 1968; Yaffe SJ et al., 1966; Wolkoff et al., 1978). Net uptake and storage is enhanced via an increased concentration of ligandin. Conjugation is enhanced via induction of UGT1A1. Biliary secretion is most likely enhanced due to induction of Mrp2. Phenobarbital is a
CAR (constitutive androstane receptor) agonist. Wagner et al. (Wagner et al., 2005) showed that phenobarbital, and other CAR agonists, induce Mrp2.

Phenobarbital is used to distinguish between type I and II Crigler-Najjar disease. In type I patients, phenobarbital is not effective because there is no residual enzyme activity that can be enhanced. In the animal model of type I Crigler-Najjar disease, phenobarbital decreases plasma UCB levels, despite the absence of residual enzyme activity, but this has been demonstrated to be due to a shift of the bilirubin pool to the liver. Phenobarbital is effective in type II Crigler-Najjar disease. It usually decreases plasma UCB concentration by 30% or more (Sinaasappel and Jansen, 1991). Side effects include sedation, and induction of cytochrome P450 enzymes which accelerate the metabolism of many drugs, vitamins, clotting factors and estrogenic and androgenic hormones (Ostrow, 1972).

Apart from its use for treatment of type II Crigler-Najjar disease, phenobarbital is not used anymore for treatment of unconjugated hyperbilirubinemia. Originally, it was given to pregnant mothers before delivery or to the infant within 24 hours after birth to limit the severity of unconjugated hyperbilirubinemia and the need for exchange transfusions (Valaes et al., 1990; Vest et al., 1970; Trolle et al., 1968). However, phototherapy is more effective than phenobarbital and combining phototherapy with phenobarbital did not reduce plasma UCB levels more rapidly than phototherapy alone (Valdes et al., 1971). Furthermore, the effect of phenobarbital does not start until a few days after administration (Valaes and Harvey-Wilkes, 1990).

2.7.4 Decreasing UCB production

UCB production can be decreased via inhibition of heme oxygenase (HO), the rate-limiting enzyme in the catabolism of heme to UCB. In theory, inhibition of biliverdin reductase could also be used to decrease UCB production. However, inhibitors of biliverdin reductase have not been explored, probably because their use would cause green babies. HO inhibitors such as tin (Sn)- and zinc (Zn)-protoporphyrin and -mesoporphyrin are synthetic heme analogues (Yao and Stevenson, 1995). A single dose inhibits HO for several days. The inhibition of heme degradation does not result in accumulation of heme because heme is excreted into bile (Berglund et al., 1988). Phototoxicity involves production of free radicals and other reactive oxygen species
which can cause cell damage, presumably via accelerated lipid peroxidation (Dennery et al., 1993).

In Crigler-Najjar patients, early administration of heme oxygenase inhibitors is expected to be more effective than initiation in adolescence, because in the latter case, total-body amount of UCB is many times greater than the amount of UCB in the intravascular space (Maisels, 1994).

2.7.5 Intestinal capture of UCB

UCB gets into the intestinal lumen via one of three routes: 1) biliary secretion of conjugated bilirubin, subsequently deconjugated to UCB; 2) biliary secretion of UCB: a very small amount of UCB can be excreted into bile (Roy Chowdhury et al., 2010; Clarenburg and Kao, 1973) 3) transepithelial diffusion: UCB can diffuse from the blood into the intestinal lumen across the intestinal mucosa along concentration gradients, particularly when plasma UCB concentrations are high as in Crigler-Najjar disease.

Intestinal capture of UCB followed by fecal excretion reduces the enterohepatic circulation of UCB and subsequently decreases plasma UCB concentration.

2.7.6 Bilirubin oxidase

Bilirubin oxidase was used in several experimental ways for treatment of unconjugated hyperbilirubinemia. Bilirubin oxidase administration or induction of bilirubin oxidation is currently not applied as therapeutic strategy for neonatal jaundice or Crigler-Najjar disease.

2.7.7 Hepatocyte transplantation

Since liver architecture and function, except for deficiency of UGT1A1 activity, are normal in Crigler-Najjar disease type I, hepatocyte transplantation might be safer and less invasive than liver transplantation. Correction of Crigler-Najjar disease requires only partial replacement of UGT1A1 activity (Gupta and Chowdhary, 1992). So far, hepatocyte transplantation has been performed in two patients with Crigler-Najjar disease type I. The first patient was a 10 year old girl in whom UGT1A1 activity was restored to 5.5% of normal after hepatocyte transplantation via percutaneous infusion through the portal vein. Afterwards, maximum plasma UCB levels dropped from 455 to
239 µmol/l, and she required 6-7 hours of phototherapy instead of 10-12 hours (Fox et al., 1998). Long-term results are awaited. More recently, the second patient, a 9 year old boy, received an allogenic hepatocyte transplantation (Ambrosino et al., 2005). Initially, plasma UCB levels decreased from 530 to 359 µmol/l. However, he was treated for cellular rejection and later he received a liver transplantation because of poor compliance to phototherapy. Although hepatocyte transplantation was safe and partially effective in these two patients, problems with long-term efficacy, rejection and immune suppression may prevent future use in Crigler-Najjar disease.

2.7.8 Liver transplantation

Several patients with Crigler-Najjar syndrome type I have undergone liver transplantation (Kaufman et al., 1986; Pett and Mowat, 1987; Shevell, 1987; Whitington et al., 1993; Wolff et al., 1986; Schauer et al., 2003). Successful liver transplantation effectively restores UGT1A1 activity which results in low or normal plasma UCB levels and eliminates the need for phototherapy. However, these benefits have to be weighed against the risks and complications of liver transplantation. The one year survival after liver transplantation is between 85 and 90%, (Kelly, 1994) although over the past years survival has improved (Rand and Olthoff, 2003; Kuang et al., 1996). Possible complications include rejection, infection, bleeding, thrombosis and biliary complications (Kelly et al., 1994). To reduce the risk of rejection, patients receive lifelong immunosuppressive medication, which increases the risk of lymphoproliferative disease and late infections, and has side effects as nephrotoxicity and hyperlipidemia (Kuang et al., 1996). Two types of liver transplantation are used. In orthotopic liver transplantation the patient’s own liver is removed and a donor liver is inserted in its place. In auxiliary liver transplantation, (part of) the patient’s own liver is left in situ, but supported by the transplantation of a non-affected donor graft (Rela et al., 1999; Terpstra, 1993). The theoretical advantage of the latter procedure is that, if gene therapy would become available in the future, this could still be applied to the native liver, allowing possible withdrawal of immunosuppression.

2.7.9 Gene therapy

Since Crigler-Najjar disease is caused by molecular lesions of a single gene and partial enzyme replacement would be enough to significantly lower plasma UCB
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concentrations, numerous strategies of gene transfer, using viral and non-viral vectors, have been developed in the Gunn rat model. Although several of these strategies resulted in a long-term correction in plasma UCB levels, none of them have been translated into a clinical trial. This has been mainly due to concerns regarding long-term safety and vector toxicity. The structure of the UGT1A1 gene has been elucidated and the gene was successfully cloned in 1991 (Ritter et al., 1991; Bosma et al., 1992; Van Es et al., 1993). Although these vectors efficiently integrate UGT1A1 into the host’s genome, the technique was not very effective in larger animals. Another approach involved the use of adenoviral vectors that localize to the liver and do have the ability to infect non-dividing cells.

The results of gene therapy thus seem promising in Gunn rats. Currently, however, the results of clinical trials must be awaited before any of these strategies can be applied in Crigler-Najjar patients.

2.7.10 Scope of this thesis

Although both genetic and environmental factors contribute to the development of neonatal hyperbilirubinemia, increasingly, the importance of genetically determined conditions is being recognized. Gene variants reported in association with an increased risk for neonatal hyperbilirubinemia include those of

(1) The hepatic bilirubin-conjugating enzyme uridine-diphosphateglucuronosyl transferase 1A1 (UGT1A1).

(2) The red blood cell enzyme glucose-6-phosphate dehydrogenase (G6PD).

(3) The hepatic organic anion transporter polypeptide 2 (OATP2).

In view of these facts, it can be hypothesize that genetic factors could be the possible candidate in the development of severe neonatal hyperbilirubinemia. Henceforth present study is planned to investigate the identification and molecular characterization of mutations and polymorphism in G6PD, UGT1A1, and OATP 2 genes in neonatal hyperbilirubinemia. In view of the above lacunae, the present study will be focused on identification of known mutations in prominent genes (UGT1A1, OATP 2 and G6PD) proposed in pathogenesis of hyperbilirubinemia in in-term and late-term neonates.
Additionally unknown mutations will also be identified and characterized in UGT1A1 gene.

Since the UGT1A1, OATP2 and G6PD gene is the focus of this study, a more in depth review on the UGT1A1 gene is presented below.

2.8 Molecular Genetics of UGT1A1 gene in Hyperbilirubinemia

2.8.1 Molecular Biology of UGT1A1 gene

The uridine diphosphate (UDP)-glycosyltransferases (EC2.4.21.17) are a group of enzymes that catalyze the transfer of sugars (glucuronic acid, glucose, and xylose) to a variety of acceptor molecules (aglycones). The sugars may be attached at aromatic and aliphatic alcohols, carboxylic acids, thiols, primary, secondary, tertiary, and aromatic amino groups, and acidic carbon atoms. 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). The structure and function of the enzymes have been the subject of several reviews (Burchell et al., 1995; Burchell et al., 1997; Mackenzie et al., 1997; Tukey and Strassberg, 2000).

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 sulfonyltransferases (SULTs) (Burchell et al., 1997).

The interplay between glucuronidation and sulfonylation (sulfation) of steroid and thyroid hormones and the corresponding hydrolytic enzymes, b-glucuronidase and sulfatase, may play an important role in development and regulation. The UGTs are expressed in many tissues, including liver, kidney, intestine, colon, adrenals, spleen, lung, skin, testes, ovaries, olfactory glands, and brain. Interactions between drugs at the enzymatic level are most likely to occur during the absorption phase in the intestine and liver or systemically in the liver, kidney, or intestine.
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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 (Meech and Mackenzie, 1997; Kadakol et al., 2000). 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 (Bosma et al., 1994).

2.8.2 UGT1A1 Locus and structure

UGT1A1 gene is originally mapped on chromosome 2q37 (Kiang et al., 2005) as represented in Figure 2.7 and is the focus of the current study. It 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 (Figure 2.8). The four common exons at 3’ end encode the carboxyterminal 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 (Ritter et al., 1992; Mackenzie et al., 1997; Watchko et al., 2002; Gong et al., 2001) cDNA encoding human UGT1A1 gene was cloned and functionally characterized in 1991. It is 1.602 kb long and encodes peptide of 533 amino acids (Strassburg et al., 1998; Mackenzie et al., 1997).

A decrease in enzyme activity caused by defects or lesions in the UGT1A1 gene results in unconjugated hyperbilirubinaemia (Sutomo et al., 2002). Several mutations in UGT1A1 gene have been reported to be associated with Gilbert syndrome (e.g. the TATA box mutation (A(TA)7TAA), G71R and P229G) (Hsieh et al., 2001). For the purpose of this study three mutations were studied.

Figure 2.9 shows the coding sequence of the human UGT1A1 gene and the deduced amino acid sequence of the human UGT1A1 protein. All splice junctions followed the GT/AG rule.
Figure 2.7  Human chromosome 2 (http://www.ensembl.org/Homo_sapiens/mapview?chr=2)
**Figure 2.8 Structural organization of human UGT1 gene complex.** 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. (Harrison's principles of internal medicine, 17th ed.)

### 2.8.3 Clinical syndromes associated with mutations in the UGT1A1 gene

In humans, three forms of inheritable unconjugated hyperbilirubinemic diseases exist: Crigler-Najjar syndrome type I, Crigler-Najjar syndrome type II, and Gilbert’s syndrome (Table 2.1). Decreased bilirubin clearance is attributed to Crigler-Najjar and Gilbert syndrome, due to lack of UDPGT enzyme in the liver.
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Figure 2.9 cDNA sequence of Human UGT1A1 gene with corresponding deduced amino acids (http://www.ncbi.nlm.nih.gov/nuccore/BC128415.1)
2.8.3.1 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 (Kadakol et al., 2000). 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 and Najjar, 1952^). 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 incomplete lack of UGT1A1 activity in the liver (Seppen et al., 1994). The therapeutic approach for crigler-Najjar syndrome type I and type II is different (Table 2.1). The prevalence of Crigler-Najjar disease is estimated at 1:1,000,000.145 In the Netherlands there are approximately 20 patients. The gene encoding for UGT1A1 lies on chromosome 2. Mutations in any of 5 exons (or rarely in introns or promoter region) can cause Crigler-Najjar disease type I or II. Approximately 60 mutations (point mutations, deletions, insertions) in the UGT1A1 gene have been identified; (Guillemette, 2003; Bosma et al., 1994; Kadakol et al., 2000; Labrune et al., 1994; Seppen et al., 1994) indicating that Crigler-Najjar disease is genetically heterogeneous, while there is a homogeneity of its clinical presentation.

Table 2.1 The clinical classification of unconjugated hyperbilirubinemia^ (Tukey and Strassberg, 2000)

<table>
<thead>
<tr>
<th>Determinants</th>
<th>Crigler-Najjar type 1</th>
<th>Crigler-Najjar type 2</th>
<th>Gilbert unconjugated disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>Rare</td>
<td>Very rare</td>
<td>% of population</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Unconjugated</td>
<td>Unconjugated/unconjugated</td>
<td>Unconjugated/unconjugated</td>
</tr>
<tr>
<td>Serum direct bilirubin levels</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Liver histology</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Inheritance</td>
<td>Autosomal recessive</td>
<td>Autosomal recessive</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Affected gene</td>
<td>UGT1A1 coding region and or</td>
<td>UGT1A1 coding region and or</td>
<td>UGT1A1 coding region and or</td>
</tr>
<tr>
<td></td>
<td>promoter</td>
<td>promoter</td>
<td>promoter</td>
</tr>
<tr>
<td>Effect of mutations</td>
<td>Absence of activity</td>
<td>10% of activity</td>
<td>60% of activity</td>
</tr>
<tr>
<td>Clinical response to induction</td>
<td>No induction</td>
<td>Induction with phenobarbital</td>
<td>Induction with phenobarbital self-limiting stress induced</td>
</tr>
<tr>
<td>Therapy</td>
<td>Blood exchange transfusion</td>
<td>Phenobarbital hormone</td>
<td>Not necessary</td>
</tr>
<tr>
<td></td>
<td>Stem cell transplantation</td>
<td>transplantation</td>
<td></td>
</tr>
<tr>
<td>Progress</td>
<td>Untreated death</td>
<td>Variable</td>
<td>Excellent</td>
</tr>
</tbody>
</table>

*Some of the percentages and genes are based on the differences between the three types of disease.*
Phototherapy is the preferred long-term treatment for Crigler-Najjar disease type I, but has considerable disadvantages. If plasma UCB 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 (Strauss et al., 2006). Before the introduction of phototherapy, all patients with Crigler-Najjar disease died from kernicterus (Crigler and Najjar, 1952a). In recent years, neurological outcome of Crigler-Najjar disease is good if treatment is started early and adequately. Combined data from recent surveys suggest that 23-47% of patients with Crigler-Najjar disease 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 (Strauss et al., 2006; Nazer et al., 1998; Suresh and Lucey, 1997; Van Der Veere et al., 1996).

2.8.3.2 Gilbert syndrome

Gilbert syndrome was first described by Augustin Gilbert and Pierre Lereboullet in 1901. (Runkel et al., 1978). Patients with Gilbert syndrome commonly have mild and chronic unconjugated hyperbilirubinaemia with normal liver function and without overt haemolysis (Sugatani et al., 2002). 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 (Sutomo et al., 2002, Kaplan et al., 2001; Beutler et al., 2002). There is still controversy whether Gilbert syndrome is inherited as a recessive or dominant trait. Schmid, (1995) assumed that it was inherited as an autosomal dominant trait while a study by Bosma et al., (1995) suggested an autosomal recessive trait.
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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 (Koiwai et al., 1995). Phenobarbital treatment has been found to increase the enzyme activity resulting in a decrease in the serum bilirubin level (Black and Sherlock, 1970).

The incidence of Gilbert syndrome in the general population is about 3% to 10% (Iyanagi et al., 1998; Yamamoto et al., 1998). Hsieh and colleagues found that the TATA box mutation and G71R underlies the molecular background of Gilbert syndrome in the Taiwanese population.

Simultaneous occurrence of more than one mutations were also found in this population which resulted in higher level of bilirubin and caused a more severe form of Gilbert syndrome (Hsieh et al., 2001).

2.8.4 Worldwide presence of mutation in UGT1A1 gene

UGT1A1 is an important enzyme that is primarily responsible for the glucuronidation of bilirubin in the liver. Cloned, expressed UGT1A1 is a glycosyltransferase that is also capable of catalyzing the formation of bilirubin xylosides and glycosides in the presence of UDP-xylose and UDP-glucose, respectively (Senafi et al., 1994). In vivo, glucuronidation predominates, but bilirubin xylosides and glucosides have been identified in human bile. However, the observation of physiological hyperbilirubinemia in neonates and the hereditary unconjugated hyperbilirubinemas in children and adults have led to the investigation of bilirubin metabolism and have ultimately proved to be the driving force of the discovery of the human UGT1A1 gene locus (Burchell et al., 1997; Chouinard et al., 2006; Court et al., 2004). Although the human UGT1A1 locus potentially encompasses nine functional transferase genes, only one isoform, UGT1A1, is involved in inherited diseases of bilirubin metabolism (Burchell et al., 1997; Chouinard et al., 2006; Lampe et al., 2000). With the exception of minor bilirubin UGT activity detected in vitro with expressed UGT1A4 (Chouinard et al., 2006), only UGT1A1 is capable of forming bilirubin glucuronides. Because a number of patients suffering from a complete loss of bilirubin glucuronidation exhibit homozygous mutations of the UGT1A1 first exon only, it is not likely that additional bilirubin UGTs exist in humans (Wilson et al., 2004). Mutations of the UGT1A1 first exon (Veenendaal
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Macdonald et al., 1995; Upton et al., 1980; Baber et al., 1978; Yu and Perel, 1980; Spahn et al., 1989; Bannier et al., 1985; Agrawal et al., 2009) lead to a selective effect on the UGT1A1 gene product. Because bilirubin glucuronidation can be completely abrogated by such a mutational event (Yu and Perel 1980), there appears to be no substitute isoform capable of bilirubin glucuronidation and the reported bilirubin activity of UGT1A4 by in vitro experiments does not appear to have any biological significance. Polymorphisms in the UGT1A1 gene have been extensively studied because of a rare inborn error of bilirubin metabolism resulting in Crigler-Najjar syndrome. Type I Crigler-Najjar patients typically require liver transplantation, whereas Type II patients can be treated with UGT1A1 inducers such as phenobarbital. Gilbert syndrome is an asymptomatic unconjugated hyperbilirubinemia that is most often caused by a genetic polymorphism in the promoter region of the UGT1A1 gene in Caucasians and Africans. Decreased expression of UGT1A1 in Gilbert’s patients is a result of the UDP-Glucuronosyltransferases presence of a (TA)7TAA allele (UGT1A1*28) in place of the more prevalent (TA)6TAA allele (Monaghan et al., 1996; Guillemette, 2003). Persons who are homozygous for the (TA)7TAA express approximately 70% less UGT1A1 enzyme in the liver. A second mutation at −3279 C>T in a phenobarbital response enhancer module (PBREM) also is linked with Gilbert syndrome and is often in linkage disequilibrium with UGT1A1*28 in Caucasians and Japanese (Kitagawa et al., 2005; Ferraris et al., 2006). Larger screening studies have demonstrated that this regulatory defect occurs in approximately 2–19% of various populations (Guillemette C, 2003). In Asian patients, other mutations in the UGT1A1 gene besides the (TA)7TAA genotype contribute significantly to hyperbilirubinemia, including UGT1A1*6 (211 G>A, G71R) (Urawa et al., 2006; Akaba et al., 1998). Drugs that are substrates for or inhibit UGT1A1 may cause a further increase of unconjugated bilirubin concentrations, especially in patients with Gilbert syndrome. For example, the HIV protease inhibitors atazanavir and indinavir are known to increase bilirubin levels (Rotger M et al., 2005). Lankisch et al. recently found that atazanavir treatment increased median bilirubin concentrations from 10 to 41 mM (p < 0.001) (Lankisch et al., 2006). Bilirubin levels exceeding 43 mM were observed in 37% of the 106 patients. Hyperbilirubinemia >43 mM was significantly associated with three non-1A1 mutations UGT1A3-66C, UGT1A7-57G, and UGT1A7*2 along with UGT1A1*28, although these variants are not typically in linkage disequilibrium in other populations. Six patients expressing all four
mutations had bilirubin levels >87 mM, a level that may require discontinuation or dosage adjustment. UGT1A3 is a weak catalyst of bilirubin glucuronidation, whereas UGT1A7 would not be expected to contribute given its extrahepatic tissue distribution.

Older studies in persons with mild hyperbilirubinemia (meeting the criteria for Gilbert syndrome, but not genetically determined) demonstrated a decreased clearance rate for drugs that are glucuronidated. Clearance of acetaminophen (APAP; also catalyzed by other UGT enzymes, especially UGT1A5) was decreased by 30% in six subjects with Gilbert syndrome (de Morais et al., 1992). In contrast, a small study by Ullrich et al., demonstrated no difference in the APAP-glucuronide/acetaminophen ratio in urine of 11 persons with Gilbert syndrome (Ullrich et al., 1987). A more recent study in genotyped patients also found no difference in the glucuronide/acetaminophen urinary ratio (Rauchschwalbe et al., 2004). Racemic (S/R) lorazepam clearance (catalysed by UGT2B7 and UGT2B15) was 30–40% lower in persons with Gilbert syndrome (Herman et al., 1994). A modest decrease (32%) in lamotrigine oral clearance was observed in persons with Gilbert’s syndrome (Posner et al., 1989). However, lamotrigine is glucuronidated by cloned, expressed UGT1A3 and UGT1A4, but not by UGT1A1 (Green and Tephly, 1996; Green et al., 1998). In general, these studies were conducted in a small number of Gilbert syndrome subjects. A distinct heterogeneity may be present in persons exhibiting mild hyperbilirubinemia that could include patients with Crigler-Najjar Type II syndrome who have mutations in the UGT1A1-coding region, persons who are homozygous for UGT1A1*28, or in patients with a higher than normal breakdown of heme. The role of UGT1A1*28 polymorphism and irinotecan toxicity has been extensively investigated in Japan by Ando et al. (Ando et al., 1998) and in the United States by Remmel et al. Innocenti, (Innocenti and Rarain, 2006). Irinotecan is a prodrug that is rapidly converted by esterases to active phenolic compound, SN-38. SN-38 glucuronidation is catalyzed primarily, by UGT1A1 in studies with cloned, expressed enzymes. Iyer et al., compared the liver microsomal glucuronidation rate of SN-38 and bilirubin in 44 patients genotyped for the (TA)7TAA allele (UGT1A1*28) and found a high correlation (r ¼ 0.9) (Iyer et al., 1998). Patients with the UGT1A1*28 allele who take irinotecan have a significantly higher risk for neutropenia, and the FDA has recently recommended that patients should be genotyped prior to use of irinotecan. Evidence for drug-drug or herb-drug interactions involving UGT1A1 and irinotecan are limited (Toffoli et al., 2006).
The inheritable unconjugated hyperbilirubinemias are all the result of either mutant UGT1A1 alleles (Wilson et al., 2004; Veenendaal et al., 1981; Macdonald et al., 1995; Upton et al., 1980; Yu and Perel, 1980; Bannier et al., 1985) or UGT1A1 promoter polymorphisms (Smith et al., 1985; de Miranda et al., 1989). To date, 33 mutant UGT1A1 alleles have been identified (Costa et al., 2006b; Yu and Perel, 1980; Meffin et al., 1983) (Table 2.2). Nine of these mutations have been located within the unique first exon of the UGT1A1 gene (Veenendaal et al., 1981; Bannier et al., 1985).

Table 2.2 Allelic polymorphism of human UGT1A1 gene and association with unconjugated hyperbilirubinemia (Robert et al., 2000)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Nucleotide changes</th>
<th>Protein changes</th>
<th>Type</th>
<th>Exon</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1*1</td>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>UGT1A1*2</td>
<td>879 del 13</td>
<td>Truncation</td>
<td>Deletion</td>
<td>2</td>
<td>CN1</td>
<td></td>
</tr>
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<td>UGT1A1*5</td>
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<td>S375F</td>
<td>Missense</td>
<td>4</td>
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<td>124</td>
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<td>Q357X</td>
<td>Nonsense</td>
<td>3</td>
<td>CN1</td>
<td>126</td>
</tr>
<tr>
<td>UGT1A1*5</td>
<td>991 C→T</td>
<td>Q331del 44</td>
<td>Missense</td>
<td>2</td>
<td>CN1</td>
<td>124</td>
</tr>
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<td>CN2</td>
<td>130</td>
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<td>1</td>
<td>CN2</td>
<td>114</td>
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<td>2</td>
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<td>129</td>
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<td>CN1</td>
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<td>F170del</td>
<td>Deletion</td>
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<td>Frameshift</td>
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<td>CN2</td>
<td>127</td>
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<tr>
<td>UGT1A1*25</td>
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<td>Missene</td>
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<td>CN1</td>
<td>127</td>
</tr>
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<td>CN1</td>
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<td>Frameshift</td>
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<td>119</td>
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<td>Insertion</td>
<td>Promoter</td>
<td>Gilbert</td>
<td>121</td>
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<td>R367G</td>
<td>Missene</td>
<td>4</td>
<td>Gilbert</td>
<td>119</td>
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<td>Missene</td>
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<td>R334W</td>
<td>Missene</td>
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<td>CN1</td>
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<td>E294T</td>
<td>Missene</td>
<td>2</td>
<td>CN2</td>
<td>123</td>
</tr>
</tbody>
</table>
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All the other polymorphic alleles have differences located in exons 2–5 (Mackenzie et al., 1997; Upton et al., 1980; Barbier et al., 2000; Rajanarison et al., 1991; Sim et al., 1991; Trapnell et al., 1998; Rowland et al., 2007; Boase, 2002; Soars et al., 2003; Howell et al., 1986). It is debatable whether the distinction of Crigler-Najjar syndrome type I and type II and of Gilbert’s disease, which is based on serum bilirubin levels and the clinical course of the metabolic error, should be regarded as a single disease entity with a combination of functionally relevant or silent allelic polymorphisms of the UGT1A1 gene. Studies have shown that homozygous or compound heterozygous mutations can lead to Crigler-Najjar syndrome type I (Wilson et al., 2004) and type II (Soars et al., 2003). Promoter polymorphisms plus mutant coding region alleles can lead to Crigler-Najjar syndrome type I and type II (Meffin et al., 1983) and to Gilbert’s disease (Smith et al., 1985) as well as to no detectable disease at all (Smith et al., 1985; Meffin et al., 1983; Fabre et al., 1990). Given the theoretically unpredictable impact of an individual mutant allele or of the combination of itself with a different mutant allele or a promoter polymorphism, a database of all identified allelic variants is likely to serve as a decision tool to predict the course and management of patients with unconjugated hyperbilirubinemia, in addition to gaining insight into the functional properties of the UGT1A1 protein. However, for a clinically apparent hyperbilirubinemic state, homozygous or compound heterozygous mutant alleles are required. The presence of a single mutant allele without other abnormalities does not result in clinically detectable disease.

2.8.5 A(TA)7TAA promoter polymorphism and neonatal hyperbilirubinemia

It had been suggested for many years that some infants with indirect hyperbilirubinemia could be manifesting the effects of Gilbert’s syndrome. However, this concept could not be fully studied until the association between the condition and its genetic mechanism had been established. Studies showing significant associations Bancroft et al were the first to confirm an association between Gilbert’s syndrome, as indexed by the variant promoter, and neonatal jaundice (Bancroft et al., 1998). 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) (Roy Chowdhury N et al., 2002). 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) (Laforgia et al., 2002). However, in this study peak STB values did not differ according to promoter genotype.

2.8.6 A(TA)7TAA promoter polymorphism and prolonged neonatal jaundice

Studies of the relationship of UGT promoter polymorphism to prolonged jaundice have not yielded consistent results. In Scotland (Monaghan et al., 1998), 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) (Monaghan G et al., 1996). On the other hand, in the two studies on Turkish population (Ulgenalp et al., 2003; Babaoglu et al., 2006), 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 (Ulgenalp et al., 2003; Babaoglu et al., 2006).

2.9 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 diverse chemical characteristics. Since the first expression of OATP1A1 in 1944, organic anion transporting polypeptide family membrane has been isolated from a variety of tissues in vertebrate animal species (Schoenwetter, 2000).

OATPs are multispecific sodium-independent transport proteins. They comprise at least 36 members of human, rat, mouse and some nonmammalian species. On the basis of
their phylogenetic relationship, all rodent and human Oatps/ OATPs so far identified within the OATP/ SLCO superfamily of solute carriers have been classified into 6 families and 13 subfamilies.

2.9.1 Nomenclature and classification

In the beginning, the Oatps/OATPs were given gene symbol of solute carrier family 21 (SLC) by HUGO gene Nomenclature Committee, but the naming of each transporter was according to the group that isolated it. Since the traditional SLC21 gene classification does not permit an unequivocal and species-independent identification of genes and gene production, all Oatps / OATPs were later classified into OATP/ SLCO superfamily (Table 2.3).

Figure 2.10 Phylogenetic tree of the Oatps/OATP family. Multiple alignments of amino acid sequences and phylogenetic were construction were carried out using CLUSTAL (http://www.ddbj.nic.ac.in/welcome.j.html)

Recently, the HUGO gene Nomenclature Committee adopted a new nomenclature, the SLCO (character “O” is from the head letter of OATP). It is subdivided into families
(≥40% amino acid sequence identity), and individual genes and gene products according to their phylogenetic relationships and chronology of identification (Honig et al., 1992).

The phylogenetic relationship was proposed as the basis of a new classification system provides an unambiguous and species-independent nomenclature for all members of Oats/OATP superfamily.

For a comprehensive understanding of Oats/OATPs. Such a nomenclature may be in part useful in promoting this field (Table 2.3 and figure 2.10) (Monahan, 1990; Michalets, 1998). Throughout the novel protein names have been used in accordance with the new nomenclature system.

Table 2.3 Oatp/OATP gene classification as implemented by the human and mouse gene nomenclature committees (Yarim and Koksal, 2010)

<table>
<thead>
<tr>
<th>Novel protein name</th>
<th>Novel gene symbol</th>
<th>Former protein name</th>
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2.9.2 Characteristics of OATP Transporters

Considerably less is known about OATP transporters. The OATP family of transporters is involved in the transport of endogenous substances such as bile acids, where they have been most widely studied as mediators of bile acid enterohepatic circulation (Kullak-Ublick GA et al., 1994; Jacquemin E et al., 1994) In addition, OATP plays a key role in the uptake of drugs into cells, in contrast to the role of P-gp, which mediates drug efflux (Cvetkovic et al., 1999). These transporters have been studied in animal models (oatpl and oatp2 in the rat) and have been evaluated in vivo and in cell lines derived from hepatic and renal tissues (Noe et al., 1997; Ishizuka et al., 1998; Pang et al., 1998; Shi et al., 1995). The first identified human OATP transporter has 67% amino acid identity to rat oatpl (Kullak-Ublick et al., 1995). Recently, several human OATP proteins have been identified, and the tissue-specific expression of these proteins is being studied (Kullak-Ublick et al., 2001).

2.9.3 Structure, Localisation and Function of OATP Transporters

This gene encodes a liver-specific member of the organic anion transporter family. The encoded protein is a transmembrane receptor that mediates the sodium-independent uptake of numerous endogenous compounds including bilirubin, 17-beta-glucuronosyl estradiol and leukotriene C4. This protein is also involved in the removal of drug compounds such as statins, bromosulfophthalein and rifampin from the blood into the hepatocytes. Polymorphisms in the gene encoding this protein are associated with impaired transporter function. Although some important members of this transporter family are selectively expressed in rodent and human livers, where they are involved in the hepatic clearance of albumin-bound compounds from portal blood plasma (Markham and Wagstaff, 1998). Most Oatps/OATPs are expressed in multiple tissues, including the blood brain barrier, choroid plexus, lung, heart, intestine, kidney, placenta and testis (Banfield et al., 2001). Tissue distribution of Oatps/OATPs has been studied using different technique. Consistent with their potential role in detoxification process, Oatps/OATPs are expressed in various tissues as demonstrated, for example, by RT-PCR technique (Tanigawara, 2000) human tissues as well as in human cancer lines (Banfield et al., 2001). Some transporter shows a more restricted tissue expression pattern (e.g., OATP1A1, OATP1A5, Oatp1b1, OATP1A2, OATP1B1, OATP1B3 and OATP1C1), while others can be detected in almost every tissue that has been investigated (e.g.,
Oatp2bl, OATP2B1, OATP3A1, and OATP4A1). This indicates that some Oatps/OATPs have been specific functions, while others might be involved in more housekeeping functions (Schoenwetter, 2000).

Oatps/OATPs are key membrane transporters for which crystal structures are not available. 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 (Kullak-Ublick et al., 2001).

Only a few of the Oatps/OATPs identified so far have been characterized in detail on the functional, structural and genomic levels. Despite the fact that a larger number of endogenous compounds are known to be transported by the Oatps/OATPs, little is known about in vivo physiological importance of these transporters. The exact transport mechanism(s) of the OATPs, has not yet been worked out. However, studies with rat Oatps suggest that they act as organic anion exchanger (Schoenwetter, 2000).

OATP2 gene, located at chromosome 12p12 (Kullak-Ublick et al., 1996). It consists of 14 exons (Figure 2.11). (König et al., 2000). The GenEMBL EST data base 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 and encodes a polypeptide of 691 amino acids (Hsiang et al., 1999) (Fig 2.12). Oatp1 and oatp2 are glycoproteins with 12 putative transmembrane domains. OATP transporters are expressed in the rat on the liver sinusoidal membrane (Bergwerk et al., 1996). The apical membrane of the kidney, (Bergwerk et al., 1996) and the choroid plexus of the brain (Angeletti et al., 1997). Oatp1-mediated transport requires ATP, is Na+ -independent, and is bidirectional (Shi et al., 1995).

Mediates the Na(+)-independent uptake of organic anions such as pravastatin, taurocholate, methotrexate, dehydroepiandrosterone sulfate, 17-beta-glucuronosyl estradiol, estrone sulfate, prostaglandin E2, thromboxane B2, leukotriene C3, leukotriene E4, thyroxine and triiodothyronine. It May play an important role in the clearance of bile acids and organic anions from the liver.

| 52 |
The nucleotide sequence of the longest OATP2 cDNA clone is shown with the predicted amino acid sequence underneath. Nucleotide numbering and amino acid numbering are indicated on the left and right sides, respectively. Potential N-linked glycosylation sites are indicated by an asterisk below the residue. Underlined letters indicate a putative alternative polyadenylation signal utilized in a shorter cDNA that was also cloned from the same library.

### 2.9.4 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, (Eckhardt et al., 1996) glucuronides (Noe et al., 1997; Eckhardt et al., 1996; Bossuyt et al., 1996; Kanai et al., 1996) and anionic estrogen conjugates (Noe et al., 1997, Pang et al., 1998, Bossuyt et al., 1996; Kanai et al., 1996). Oatp1 is capable of transporting endogenous organic anions such as the bile acid taurocholate (Jacquemin et al., 1994; Noe et al., 1997; Satlin et al., 1997) estradiol 17-glucuronide, (Noe et al., 1997; Kanai et al., 1996) the steroid hormone estrone-3-sulfate (Bossuyt et al., 1996) and enalapril (Pang et al., 1998). Oatp2 is similar to oatp1 in that it transports many of the same substrates as oatp1, (Noe et al., 1997) but demonstrates different specificities for some drugs such as digoxin (Reichel et al., 1999).
Figure 2.12 Nucleotide and amino acid sequence of human OATP2 (http://www.ncbi.nlm.nih.gov/nuccore/BC070172.1)
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2.9.5 Role of Organic Anion Transporter Proteins in hyperbilirubinemia

The OATPs are a family of multispecific pumps mediating the sodium-independent uptake of bile salts and a broad range of amphipathic organic compounds. In humans, three liver specific OATPs have been identified: OATP-A, OATP-2, and OATP-8 (Trauner and Boyer, 2002). OATP-2 is selectively expressed at the basolateral membrane of hepatocytes (Ritter et al., 1991), where it plays a role in the hepatic uptake of various substrates including taurocholate, conjugated steroids, thyroid hormones and peptides. The hepato-specific expression of OATP-2 is unique amongst the OATP transporters, all others of which are found in multiple tissues in addition to the liver (Hsiang et al., 1999).

Of particular interest in relation to the work of Huang and associates is evidence that unconjugated bilirubin may be a substrate for human OATP-2. Cui and colleagues reported high affinity uptake of unconjugated [3H]-bilirubin by OATP-2 in the presence of albumin in human embryonic kidney cells (HEK293) permanently expressing human recombinant OATP-2 (Cui et al., 2001). There in vitro results suggest carrier mediated hepatocyte bilirubin uptake occurs in vivo. Presumably, mutations in the gene encoding OATP-2 could enhance unconjugated hyperbilirubinemia by impairing its hepatic uptake. A distinction between carrier mediated hepatic bilirubin uptake and that attributed to passive diffusion would be supported by the identification of specific functional consequences of OATP-2 mutations. In this regard, several OATP-2 gene mutations have characterized that affect both protein maturation and organic anion transport [although bilirubin transport was not examined (Tirona et al., 2001 and Michalski et al., 2002). Complicating this picture, however, is a recent report that failed to confirm unconjugated Bilirubin transport by OATP-2 in either OATP-2 stably transfected HeLa or HEK293 cells (Wang et al., 2003), challenging the notion of OATP-2 mediated hepatic bilirubin transport. Nevertheless, one cannot discount the results of the current study by Huang and colleagues demonstrating an associated 3-fold increased risk of marked neonatal hyperbilirubinemia in infants with an OATP-2 gene polymorphism at position 388 (Huang et al., 2004).

Clearly, this area of investigation merits further study to more completely define the functional role of OATP-2 in hepatocytes and clarify how mutations in OATP-2 increase ones risk for neonatal.
2.9.6 Clinical syndromes associated with mutations in the OATP2 gene

A suspicious analog [organic anion transporter 2 (OATP 2) gene] on severe hyperbilirubinemia in Taiwanese neonates (Huang et al., 2004). 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 (Cui et al., 2001). 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. Studies also suggest that polymorphisms in the gene for glutathione-S-transferase (ligandin) may contribute to higher levels of total serum bilirubin. (Thor WR Hansen, MD, PhD, MHA, FAAP Professor, Department of Neonatology, Women and Children's Division, Chair, Clinical Ethics Committee, Oslo University Hospital HC, Rikshospitalet, Norway). Organic anion transporter polypeptide 2 (OATP2), also known as OATP-C, OATP1B1, and LST -1, is responsible for the transportation of organic anions into hepatocytes. This mechanism may also be involved in the transportation of unconjugated bilirubin (Cui et al., 2001). 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 and a study from Taiwan showed that variation at c.388A>G was a risk factor associated with unconjugated hyperbilirubinemia in newborns (Xu et al, 2007). Besides these mutations, Jada et al and Nishizato et al have reported high frequency of two other silent mutations in OATP2 gene in Asian population (Jada et al, 2007; Nishizato et al, 2003). 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 (Nishizato et al., 2003). In Malaysia, neonatal hyperbilirubinemia is a common problem. The prevalence of OATP2 variants and its possible association with severe neonatal hyperbilirubinemia has not been studied in Malaysian Chinese populations previously. This study aimed to determine the prevalence of these variations and whether they were significantly associated with severe hyperbilirubinemia in the Malaysian infants of Chinese descent (Wong, 2009).
2.10 Glucose 6 phosphate dehydrogenase (G6PD) gene

Thirty-five years ago Dr. William Dameshek, the first editor of the emerging journal Blood, invited me to write a review on “The Hemolytic Effect of Primaquine.” At the time, primaquine sensitivity, which had just recently been shown to be caused by a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD); represented a unique example of an inherited deficiency of an enzyme that caused hemolytic anemia (Beutler, 1994).

G6PD gene consists of 13 exons, which are the regions of the DNA that code for the enzyme, and 12 introns, which are intervening sequences (Scriver et al., 1995). The intervening sequences are “junk” DNA which serve no purpose in the enzyme's function. The enzyme's function is determined, however, by the sequence and size of the G6PD gene and the mRNA encoded by the gene, which is 1638 base pairs in length, and encodes a polypeptide of 545 amino acids. Respectively (Takizawa et al., 1987.). The entire genomic sequence of the G6PD gene can be found in the original research article by Chen and associates (1991).

G6PD, in its active enzyme form, is made up of either two or four identical subunits, each having a molecular mass of about 59 kilo-daltons; this is more than three times as large as the hemoglobin molecule, which is the principal oxygen carrying molecule in humans (Scriver et al., 1995).

Most individuals with G6PD deficiency have a qualitative abnormality in the structure of the G6PD enzyme (Scriver et al., 1995). Several models have been proposed suggesting possible reasons for why an abnormal enzyme is not fully active. Sharff has suggested that the decreased stability of a mutated enzyme results either from a change in the conformation of the G6PD molecule or from an increase in its susceptibility to proteolytic enzymes (Scriver et al., 1995). In either case, the G6PD enzyme is not fully active when it is mutated.

2.10.1 Genetics 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 what sex an individual will be, among other things. 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 (Scriver et al., 1995). 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 (Figure 2.13) (Szabo et al., 1984; Trask et al., 1991). The gene was cloned in 1986, (Persico et al., 1986) and consists of 13 exons and 12 introns, spanning nearly 20 kb in total (Figure 2.14); 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 (Reynolds et al., 1984; Toniolo et al., 1991; Pai et al., 1980).

Any gene located on the X-chromosome is called an X-linked gene (Verrelli et al, 2002). 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 a called a recessive trait. In males, however, where there is only one X-chromosome, one defective G6PD gene is sufficient to cause G6PD deficiency. Figure 2.15 shows the coding sequence of the human G6PD gene and the deduced amino acid sequence of the human G6PD protein.
Figure 2.13: Representation of X-chromosome and the position of the G6PD gene at the Xq28 locus (http://wiki.medpedia.com/Glucose-6-phosphatedehydrogenase (G6PD))

Figure 2.14: Diagram of the G6PD locus, spanning ~18 kb. Exons are shown as blackened boxes, and introns and noncoding regions are shown as unblackened boxes. Intron 2 is nearly 10 kb in length. The 5.2-kb region that was sequenced for this study is shown, as well as the location of the common A-, A, and Med replacement SNPs (Verrelli et al., 2002).
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M G R R G S A P G H G R E R G G

ggc cgg cgg ggc tca gcc ccc cga aac gtt cct ctt cgg ggc tcc gag cgc gga ggg
R R R R S A D S V M A E Q V A L S R T Q V
cga cga cga cga gca gac agc gtc atg gca gag cag ctg gcc cag cag cag gtt
cg gil r e l f q g d a f h q s d t h i
tgc ggg atc ctg cgg gga aag ctt ttc ttc cag gcc gac ggc gtt ctg cag tgg gag aac ccc atat
F I I M G A S D L A K K K I Y P T I W W
ttc atc atc atg gct gca tgg gat gac ggc aag aag aag atc tcc gcc acc atc tgg tgg
L F R D G L L P E N T F I V G Y A R S R L
tcg ttc cgg gat ggc ctg ccc gaa aaa acc acc ttc atc ctg ggc tct tat gcc cgt tcc cgc atc
tv a d i r k q s e f f k a t p e e k l
aca gtt gct gac atc cgc aas cag sat gag ccc ttc ttc ttc aag gcc acc cca gag gag aag ctc
k l ed p f y a r n s y v a g q y d d a a s
aag ctt ggg cgc ttc ttt gcc cgc aac acc tat tct gtt ggc cag tac tag gat gcc gca	y q r l n s h m n a l h l g s q a n r l f
tac cag cgc ctc aac ggc cac atg sat gcc ctc ccc cgc tgg gta gcc cag gcc cac ctc	y l a l p p t v y e a v t k n i h e s c m
tac ctg gcc tgg ccc cgc acc gtc tcc gag gcc ggc tcc aag gcc acc cca cac gac gcc ttc atg
s q i g n w n r i i v e k f g r d l o s s
acg cag ata gcc tgg aac cgc atc atc gtc ggg aag aag gcc tag caa ggc ttc
d r l s n h i i s l f r e d q i y r i d h
gac cgg ctg tcc aac cac atc tcc tcc tgg ttc gct gcc cag atc tac tcc gcc acc ccc
y l g k e m v q n l m v l r f a n r i f g
tac ctg gcc aag gag atg tgt cag aac ctc atg tgt ctg cag tgg ctc gcc aag aag aac atc ttc ggc
p i w n r d n i a c u l i t t k e r f g t
coc atg ctc ggc aag aac atc gcc gac tgt atc atc ccc aag gag ccc aag ttc
g e r g g y f d e f g i i r d v m q n h l
gag gtt cgc ggg ggc tat ttc gat gaa ttt gcc atc cgg gcc ggg gat ag aag ccc gcc tcc acc aac tca gaa gcc gtt
d e r v k v l k c i s e v q a a n n v v l g
gat gaa gag gtc aag tgt tgt atc gga gcc acc sat tgt gtc gtt gcc
q y v g n p d g e g e a t k g y l d d o p t
cag tac gtt ggg aac ccc gat gga gag gag gac goc acc aea ggg tac ctg gac gcc gca
e v r f g s t t a t f a a v s v l v e n e r
gtt ccc cgc ggg tcc acc acc gcc acc act ttt gcc gcc gcc gtc gcc tcc ctc tat ggt gat gat gaa gag aag w g d v p f i l r c g k a l n e r k a e v
tggt tat ggg tgt ccc ttc atc ctg ccc tgt gcc aag gcc ctc aac gag cgc aag ggc gag tgt
tggt tat ggg tgt ccc ttc atc ctg ccc tgt gcc aag gcc ctc aac gag cgc aag ggc gag tgt
r l o f h d v a g d i f h q c k r n e l
tgg ccg cag cac gtt cag gtt cag gcc gcc cag cac ccc aag gcc ggc atg
v i r v q p n e a v y t k m m t k k p g n
gtt atc cgc ctg ctg ccc aac gag ggc ggt tac acc aag atg acc aag aac cgc gcc atg
f f n p e e s e l d l t y g n r y k n v
ctc ttc aac ccc gag gac gca gcc ctg gcc gcc aac gcc tgg gac gcc acc aag gcc gcc gcc gca gac gcc
l p d a y e r l l d l v f e c g s q m t n v
cgc tgc gac gcc tcc gag gcc ctc atc ctg gcc gcc gtt tgg ggc aag cag atg ccc atc tgg
ts d e l r e a w r i f t p l l h q i e l
coc aag gcc gag ctc ctg ggt cgc tgg cc ttt ttc ccc ctc cgg ccc cag cag gcc cag ggg
e k p k p i p i y i y g s r g p t e a d e l
gag aag ccc aag gcc atc ccc tat att tat gcc aag cga cgc gcc ccc aag gag gcc gca gcc ggg
t m k r v g f q y e g t y k w v n f h k l x
gag aag cga tgt gat ttg cag tgc gat gag gcc acc tcc gag tgg gtt gag ccc cag cag ctc tag

Figure 2.15 cdna sequence of human G6PD gene with corresponding deduced amino acids (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=NUCID&DATA=10877379)
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2.10.2 Physiology of G6PD

Not only is it important to learn about the molecular biology of G6PD, but also the physiology of G6PD is important as well. The function of the normal G6PD enzyme is critical to human survival.

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 figure 2.16, 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 loses 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 (Yoshida & Beutler, 1986).

* NADP+= Nicotinamide adenine dinucleotide phosphate
* NADPH= reduced Nicotinamide adenine dinucleotide phosphate
* GS-SG= oxidized glutathione
* G-SH= reduced glutathione

Fig 2.16 The pentose phosphate pathway. Note the importance of G6PD in the production of reduced G-SH, ribose and NADPH (adapted from Yoshida and Beutler, 1986).
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 (Figure 2.16). 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 (Yoshida & Beutler, 1986). There are other metabolic pathways that can generate NADPH in all cells, except in red blood cells where other NADPH-producing enzymes are lacking (Scrive et al., 1995). 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.

G6PD deficiency is known to have over 400 variant alleles, or different forms of the same gene (Beutler et al., 1990). 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 (Scrive et al., 1995). 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 (Scrive et al., 1995).

![Figure 2.17](image_url)  
**Figure 2.17** World distribution of G6PD deficiency. The values shown by the different shading are gene frequencies in the different population. (Scrive et al., 1995)
With regards to the demographics of G6PD deficiency, figure 2.17 shows that most of the affected individuals reside in Africa, the Middle East, and Southeast Asia. African Americans and some isolated tribes in Africa and Southeast Asia exhibit the highest frequency of incidence for any given population; a defective enzyme can be found in as many as one in four people among these populations (Scriven et al., 1995).

NADPH enables cells to counterbalance oxidative stress that can be triggered by several oxidant agents, and to preserve the reduced form of glutathione. Since red blood cells do not contain mitochondria, the pentose phosphate pathway is their only source of NADPH; therefore, defence against oxidative damage is dependent on G6PD (Luzzatto et al., 2001). G6PD deficiency is an X-linked, hereditary genetic defect caused by mutations in the G6PD gene, resulting in protein variants with different levels of enzyme activity, that are associated with a wide range of biochemical and clinical phenotypes. The most common clinical manifestations are neonatal jaundice and acute haemolytic anaemia, which in most patients is triggered by an exogenous agent (Luzzatto et al., 2001). The striking similarity between the areas where G6PD deficiency is common and Plasmodium falciparum malaria is endemic provides circumstantial evidence that G6PD deficiency confers resistance against malaria (Ruwende and Hill, 1998). The highest frequencies are detected in Africa, Asia, the Mediterranean region, and in the middle east; owing to recent migrations, however, the disorder is also found in North and South America and in northern European countries (Beutler, 1996). A pathological disorder linked to ingestion of fava beans (Vicia faba), later identified as G6PD deficiency, has been recognised for centuries. The Greek philosopher and mathematician, Pythagoras, forbade his followers from eating fava beans, possibly because of their pathological effects (Russel, 1965). At the beginning of the 20th century, several doctors in southern Italy and Sardinia drew a clinical picture of so-called favism (Fermi and Martinetti, 1941). However, because the response to fava bean ingestion is inconsistent, popular theories on the pathogenesis of favism were related to toxic effects or allergy (Sansone and Segni, 1958). In 1956, Carson and colleagues discovered that individuals developing haemolytic anaemia caused by the antimalarial drug primaquine had a very low level of G6PD activity in their red blood cells (Carson et al., 1956; Beutler, 1995). After a trip to Sardinia, Crosby noted a similarity between the severe haemolytic anaemia associated with ingestion of fava beans, or even inhalation of the plant’s pollen, and the haemolytic anaemia induced by primaquine (Crosby, 1956). A low activity of G6PD in people with
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a history of favism was subsequently reported in Italy and Germany (Waller et al., 1957; Sansone and Segni, 1958).

The causes of neonatal hyperbilirubinemia are multifactorial and comprise increased hemolysis on the one hand, and diminished bilirubin conjugation on the other. In recent years, many of these etiologies have been found to have a genetic origin. The genetic conditions with increasing bilirubin production are blood group incompatibilities, glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, pyruvate kinase deficiency, hereditary RBC membrane defects and defects in the enzymes heme oxygenase and biliverdin reductase. Among these G-6-PD deficiency is a common condition with a worldwide distribution that has the potential for causing severe hyperbilirubinemia with bilirubin encephalopathy.

2.10.3 Structure and function of G6PD

G6PD catalyses the first reaction in the pentose phosphate pathway, in which glucose is converted into the pentose sugars required for glycolysis and for various biosynthetic reactions. The pentose phosphate pathway also provides reducing power in the form of NADPH (Figure 2.18), by the action of G6PD and 6-phosphogluconate dehydrogenase. NADPH serves as an electron donor for many enzymatic reactions essential in biosynthetic pathways, and its production is crucial to the protection of cells from oxidative stress. G6PD is also necessary to regenerate the reduced form of glutathione that is produced with one molecule of NADPH (Luzzatto, 1995; Tsai et al., 1998). The reduced form of glutathione is essential for the reduction of hydrogen peroxide and oxygen radicals and the maintenance of haemoglobin and other red-blood-cell proteins in the reduced state (Luzzatto et al., 2001). The monomer of G6PD consists of 515 aminoacids, with a molecular weight of about 59 kDa (Luzzatto et al., 2001). A model of the three-dimensional structure of G6PD was published in 1996, (Naylor et al., 1996) and subsequently the crystal structure of human G6PD has been elucidated (Au et al., 2000). The enzyme is active as a tetramer or dimer, in a pH-dependent equilibrium. Every monomer consists of two domains: the N-terminal domain (aminoacids 27–200), with a β-α-β dinucleotide binding site (aminoacids 38–44); and a second, larger, β+α domain, consisting of an antiparallel nine-stranded sheet. The dimer interface lies in a barrel arrangement, in this second part of the G6PD molecule. The two domains are linked by an α helix, containing the totally conserved eight-residue peptide that acts as
the substrate binding site (aminoacids 198–206) (Mason, 1996; Naylor et al., 1996; Au et al., 2000). Viewing the structure, at 3 Å (0.3 nm) resolution, reveals an NADP+ (a coenzyme) molecule in every subunit of the tetramer, distant from the active site but close to the dimer interface (Au et al., 2000). Stability of the active quaternary structures is crucial for normal G6PD activity.

Figure 2.18 Structure and function of G6PD. NADPH is produced by the action of G6PD and 6-phosphogluconae dehydrogenase. It serves as a proton donor for the regeneration of reduced glutathione and as a ligand for catalase. NADPH also acts as an electron donor for many other enzymatic reactions essential in reductive biosynthesis. Cat=catalase. GPX=glutathione peroxidase. GR=glutathione reductase. G6PDD=glucose-6-phosphate dehydrogenase. 6PDG=6-phosphogluconate dehydrogenase. GSH=reduced guthione. GSSG=oxidised glutathione (Cappellini MD et al., 2008).

G6PD is present in all cells; however, its concentration varies in different tissues (Battistuzzi et al., 1985). In healthy red blood cells, the enzyme operates at only 1–2% of its maximum potential (even under oxidative stress generated by methylene blue): a large reserve of reductive potential exists, which is substantially decreased in G6PD-deficient
red-blood cells, leading to pathophysiological features (Gaetani et al., 1974). After G6PD deficiency was established as a clinical disorder, its phenotypic expression was noted to be heterogeneous. More than 140 mutations of the G6PD gene have been identified, suggesting genetic heterogeneity (Beutler, 1990a) before G6PD deficiency was identified as the cause. Males are hemizygous for the G6PD gene and can, therefore, have normal gene expression or be G6PD deficient. Females, who have two copies of the G6PD gene on each X chromosome, can have normal gene expression or be heterozygous; in some populations, in which the frequency of the G6PD-deficient allele is very high, homozygous females are not rare. Heterozygous females are genetic mosaics as a result of X-chromosome inactivation (in any cell, one X chromosome is inactive, but different cells randomly inactivate one chromosome or the other) and the abnormal cells of a heterozygous female can be as deficient for G6PD as those of a G6PD-deficient male: therefore, such females can be susceptible to the same pathophysiological phenotype (Beutler et al., 1962). Although heterozygous women, on average, have less severe clinical manifestations than G6PD-deficient males, some develop severe acute haemolytic anaemia (Lim et al., 2005).

2.10.4 Glucose-6-phosphate dehydrogenase deficiency

G-6-PD deficiency is one of the most common enzyme deficiencies known; affecting hundreds of millions of people (Beutler, 1990b). Although originally it was distributed in Africa, Southern Europe, the Middle East, and Asia, migration of population groups and ease of travel in modern times have resulted in a virtually worldwide distribution. The enzyme G-6-PD plays a major role in the protection of cells against oxidative damage by participating in the role played by reduced glutathione in this process. In order for glutathione to be effective as an antioxidant, it must be maintained in its reduced form, and be continually regenerated from its oxidized form. This regeneration utilizes hydrogen ions obtained from the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), which is formed from NADP, the reaction being catalyzed by G-6-PD in the first step of the hexose monophosphate pathway. In situations of G-6-PD deficiency, NADPH will not be available for the regeneration of reduced glutathione and the oxidative process will not be counteracted. The red blood cell especially is at high risk for this oxidative damage, as, unlike other cells, the hexose monophosphate pathway is the only source of NADPH. G-6-PD deficiency and neonatal hyperbilirubinemia one
of the most potentially 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 (Betke et al., 1967). The mechanism by which G6PD deficiency causes neonatal hyperbilirubinemia is not completely understood. Although hemolysis may be observed in neonates who have G6PD deficiency and are jaundiced (WHO working group, 1989), other mechanisms appear to play a more important role in the development of hyperbilirubinemia as in some studies increased hemolysis has not correlated with serum total bilirubin values (Beutler, 1984). 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 (Beutler et al., 1962).

2.10.5 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., (Lim et al., 2005). The prevalence rate varies between 0-28% in different castes, tribes and ethnic groups (Szabo et al., 1984). The prevalence reported in neonates born at a tertiary hospital of Ludhiana in North India was 3.9% (Trask et al., 1991). 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 vary rare variants in the Indian population (Persico et al., 1986).

2.10.6 Clinical syndromes associated with mutations in the G6PD gene

When the red blood cell can no longer transport oxygen effectively throughout the body, a condition called hemolytic anemia arises. In addition to hemolytic anemia, G6PD deficient individuals can expect several other clinical manifestations of their condition. These include neonatal jaundice, abdominal and/or back pain, dizziness, headache,
dyspnea (irregular breathing), and palpitations (Cecil, 1992). Only neonatal jaundice and hemolytic anemia will be discussed here, since these are the two major pathologies associated with G6PD deficiency. One of the problems experienced by G6PD deficient individuals presents itself immediately after birth. Neonatal jaundice is a common condition in all newborns, but when it persists, G6PD deficiency is suspected. Neonatal jaundice is a yellowish discoloration of the whites of the eyes, skin, and mucous membranes caused by deposition of bile salts in these tissues. This is a direct result of insufficient activity of the G6PD enzyme in the liver. In some cases, the neonatal jaundice is severe enough to cause death or permanent neurologic damage (Beutler, 1994). It is just as important to learn about the molecular biology of G6PD as it is to learn about the genetics of this condition. G6PD gene consists of 13 exons, which are the regions of the DNA that code for the enzyme, and 12 introns, which are intervening sequences (Scriver et al., 1995). The intervening sequences are "junk" DNA which serve no purpose in the enzyme's function. The enzyme's function is determined, however, by the sequence and size of the G6PD gene and the mRNA encoded by the gene, which are 18,500 and 2,269 base pairs in length, respectively (Scriver et al., 1995). The entire genomic sequence of the G6PD gene can be found in the original research article by Chen and associates (1991). However, the complete 3-dimensional structure of the enzyme, which determines the active enzyme's functional properties, has not yet been determined.

G6PD, in its active enzyme form, is made up of either two or four identical subunits, each having a molecular mass of about 59 kilo-daltons; this is more than three times as large as the hemoglobin molecule, which is the principal oxygen carrying molecule in humans (Scriver et al., 1995).

This molecular data has led researchers to ask, "What is causing G6PD deficiency on the molecular level?" Most individuals with G6PD deficiency have a qualitative abnormality in the structure of the G6PD enzyme (Scriver et al., 1995). Several models have been proposed suggesting possible reasons for why an abnormal enzyme is not fully active. Sharff has suggested that the decreased stability of a mutated enzyme results either from a change in the conformation of the G6PD molecule or from an increase in its susceptibility to proteolytic enzymes (Sharff, 1991, cited in Scriver et al., 1995). In either case, the G6PD enzyme is not fully active when it is mutated.
Glucose-6-phosphate-dehydrogenase (G6PD) deficiency is the most common disease-producing enzymopathy in humans. Inherited as an X-linked disorder, G6PD deficiency affects 400 million people worldwide. The disease is highly polymorphic, with more than 300 reported variants. Most patients are asymptomatic. Some patients present with or report a history of neonatal jaundice, often requiring exchange transfusion.

G6PD deficiency has been associated with 5.1-18.2% of cases with severe indirect neonatal hyperbilirubinemia in northern India (Narang et al., 1997; Singhal et al., 1992; Manorama et al., 1988). However, no cause is identified in a large majority, the proportion of which ranges from 8.8% to 57.6% in various reports from different parts of our country (Mohapatra et al., 1984; Merchant et al., 1975). Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common genetic defect, affecting 400 million individuals worldwide (Beutler, 1994). Furthermore, G6PD deficiency was the main risk factor for development of severe hyperbilirubinemia in Taiwanese neonates in the past (Hsieh et al., 2001). 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? Since these mutations may be one of the determining factors whether or not a baby with G-6-PD deficiency will develop severe jaundice, it was felt that it is important to know in the Indian population whether mutations in this gene are common and whether they are related to the occurrence of jaundice.