CHAPTER 1

General Introduction

1. UNCONJUGATED NEONATAL HYPERBILIRUBINEMIA

Neonatal unconjugated hyperbilirubinemia, or neonatal jaundice, is a common condition in infants that primarily occurs during the first 2 weeks of life [1]. In most cases, neonatal jaundice is physiological, and mild hyperbilirubinemia in term infants does not require treatment. However, severe unconjugated hyperbilirubinemia is associated with the development of bilirubin-induced neurotoxicity. This neurotoxicity is caused by deposition of bilirubin in the brain, leading to kernicterus, or yellow core, referring to the yellowish appearance of brains of infants that died from this disease. Severe hyperbilirubinemia is associated with a variety of clinical manifestations, nowadays called kernicterus spectrum disorders (KSD) [2]. KSD consist of both acute, potentially life threatening symptoms, and long term consequences. Acute symptoms include decreased alertness, hypotonia and poor feeding, which can eventually progress to strong backward arching of neck and back, caused by hypertonia of extensor muscles. These severe symptoms are associated with permanent neurological problems, including cerebral palsy, sensorineural deafness, gaze abnormalities and potential cognitive deficits [2,3]. Due to early recognition and appropriate treatment, KSD and their consequences have become relatively rare in high income countries, with a reported incidence between 1:40.000 (USA) and 1:100.000 (UK) [4]. However, incidences in low and middle income countries are poorly reported and might be higher due to the predisposition of e.g. the Asian race to hyperbilirubinemia [5,6]. Furthermore, incidences in preterm infants are likely to be higher, as will be explained in the subsequent paragraphs.

2. BILIRUBIN METABOLISM

2.1 Bilirubin origin

Unconjugated bilirubin (UCB) is the degradation product of heme in the reticuloendothelial system (RES) and mainly originates from hemoglobin in erythrocytes [7] and to a lesser extent from myoglobin from muscle [8] and mitochondrial heme components [9]. The primary site of erythrocyte degradation is the spleen, followed by the liver. In neonates, the liver is the main bilirubin production site [10]. In the RES, heme is converted to biliverdin by heme oxygenase (HO) enzyme and subsequently converted to bilirubin by the enzyme biliverdin reductase (fig. 1A) [11,12]. In contrast to UCB, biliverdin is not toxic and in most non-mammalian species, biliverdin is the end product of heme degradation [13,14]. From an evolutionary perspective, this production of a non-toxic metabolite seems logical. However, biliverdin is not able to pass the mammalian placenta, whereas bilirubin does [15], which partially explains the evolutionary necessity of this potentially toxic metabolite. In neonates, UCB production is higher compared to adults, due to their relatively larger hemoglobin mass and the enhanced degradation of fetal hemoglobin. UCB levels are further increased in presence of large hematoma's or hemolysis, in e.g. blood group incompatibility or sepsis [16].

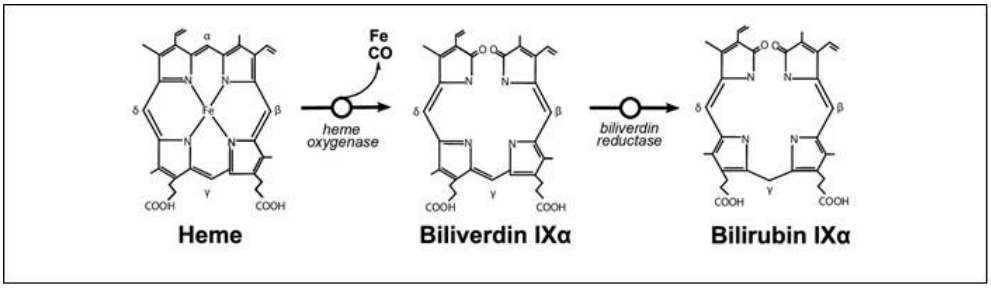

Figure 1: Enzymatic conversion of heme (erythrocyte-derived) to biliverdin and bilirubin, as occurs in the reticuloendothelial system.

2.2 Albumin-binding and free bilirubin

UCB is very hydrophobic, and therefore hardly soluble in water or blood. Upon release in the bloodstream, it is bound to albumin as its carrier. Once it arrives at the liver, UCB dissociates from albumin and passes the basolateral hepatocyte membrane [17-19]. However, under certain circumstances, UCB levels exceed the bilirubin-albumin binding capacity. This occurs at extremely high UCB levels, hypoalbuminemia or when the bilirubin-albumin binding affinity is decreased [20]. Although the binding affinity cannot be completely quantitated yet, it is known to decrease in conditions of e.g. acidosis, sepsis or by certain medications [21]. When the bilirubin-albumin binding capacity is exceeded, a small fraction of UCB can occur in the blood as free, unbound bilirubin (UCBfree). This is problematic, since in contrast to albumin-bound UCB, UCBfree can diffuse across the blood brain barrier (BBB) and deposit in the brain [22]. BBB permeability to UCBfree is inversely related to gestational and postnatal age [23]. Preterm infants have an immature BBB, causing them to be more sensitive to bilirubin-induced neurotoxicity. Therefore, UCB treatment thresholds are lower and treatment is started earlier in younger infants [24]. Apart from BBB permeability, brain UCB concentrations are also determined by export from the brain, which is known to be, at least partially, mediated by ATP Binding Cassette (ABC) Transporter B1 (MDR1/P-glycoprotein) [25]. ABCB1 expression is positively correlated with postnatal age and this is another factor that makes preterm neonates more vulnerable for bilirubin toxicity [26].

2.3 Hepatic metabolism

The exact mechanism of UCB transport across the basolateral hepatocyte membrane is not fully elucidated [27]. Partially, UCB is transported by organic anion transporting polypeptides (OATP)1B1/1B3 in humans or Oatp1a/1b in mice [28-30] (fig. 2). A deficiency in these transporters results in Rotor syndrome, a disease characterized by a mild, mixed unconjugated and conjugated

hyperbilirubinemia [31]. Although plasma UCB increases 2-fold in *Oatp1a/1b* knock-out mice, significant amounts of conjugated bilirubin (CB) are still present in bile and plasma [32]. This indicates that independent of OATP1B1/1B3, UCB is still able to be taken up by the liver where it can be conjugated.

In the hepatocyte, UCB is conjugated by UDP-glucuronosyltransferase 1A1 (UGT1A1)[33] . The addition of a glucuronyl group by UGT1A1 makes it water-soluble and easily excretable into the bile. In infants, UGT1A1 expression is modulated in a developmental manner. Between 17 and 30 weeks of gestation its expression is only 0.1% of adult levels, which increases to 1% between 30 and 40 weeks of gestation. Adult levels are only reached after postnatal day 14 [34,35]. Therefore, during the first postnatal weeks, UGT1A1 is considered to be the rate limiting enzyme in bilirubin metabolism and this conjugation deficiency, in presence of increased bilirubin production, is one of the main causes of neonatal hyperbilirubinemia [16].

A complete or partial lack of UGT1A1 results in a disease called Crigler-Najjar type I or II, respectively [36]. Crigler-Najjar type I patients need daily phototherapy (PT) to prevent otherwise inevitable KSD [37]. A mild UGT1A1-deficiency resulting from a TATA-box mutation in the UGT1A1 promoter, occurs in Gilbert-Syndrome, resulting in mostly subclinical hyperbilirubinemia. The prevalence of Gilbert syndrome in the general population is estimated to be 3-7%, but many patients live undiagnosed [38].

After conjugation, CB is transported over the canalicular hepatocyte membrane by ATP-Binding Cassette transporter 2 (ABCC2, MRP2) [39,40]. Alternatively, when CB cannot be excreted in the bile, e.g. in the case of bile duct obstruction, CB can be transported back into the blood by the basolateral transporter ABCC3 (fig. 2) [41-43]. A genetic deficiency in ABCC2 results in a clinical syndrome called Dubin-Johnson syndrome. Affected individuals display a recessively inherited conjugated hyperbilirubinemia, which can result in clinically apparent jaundice

and itch [44], especially in females in pregnancy or during oral contraceptive use [45]. However, Dubin-Johnson patients are mostly asymptomatic.

Under physiologic conditions, ABCC3 is not abundantly present in liver and otherwise healthy *Abcc3* knock-out mice do not have elevated TB levels [42,46,47]. A strong upregulation of ABCC3 is observed however, when ABCC2 function is decreased or absent, as in Dubin-Johnson syndrome [43]. ABCC3 is also upregulated in cholestatic livers of humans and rats [41,48]. Although the relative contribution of ABCC3 to normal bilirubin metabolism has not been established, ABCC3 can provide an alternative bilirubin detoxification pathway by transporting conjugated bilirubin (CB) from hepatocytes back into the blood, after which it can be either excreted into urine or transported back into liver by OATP1B1/3 [47]. The capacity of OATP1B1 and 1B3 to transport both UCB and CB explains the mixed hyperbilirubinemia in Rotor syndrome, but CB forms the main fraction. In Oatp1a/1b knock-out mice, plasma UCB increases 2-fold whereas CB increases >50 fold. Also, biliary CB decreases with >50% in these mice, highlighting the importance of the collaboration between OATP1B1/1B3 and ABCC3 in CB excretion [32]. Both ABCC2 and 3 are abundantly expressed in intestine [49,50], but their potential role as CB transporters into the intestinal lumen or back into the blood, is not established.

Another alternative hepatic UCB catabolic pathway has been identified in absence of UGT1A1. UGT1A1-deficient Gunn rats express higher levels of hepatic cytochrome P450 proteins (CYP)1A1 and 1A2 compared to wild-type controls[51]. In vitro, these enzymes have shown to be able to oxidize UCB to yet unidentified compounds [52]. However, the *in vivo* contribution of this UCB disposal pathway remains to be determined.

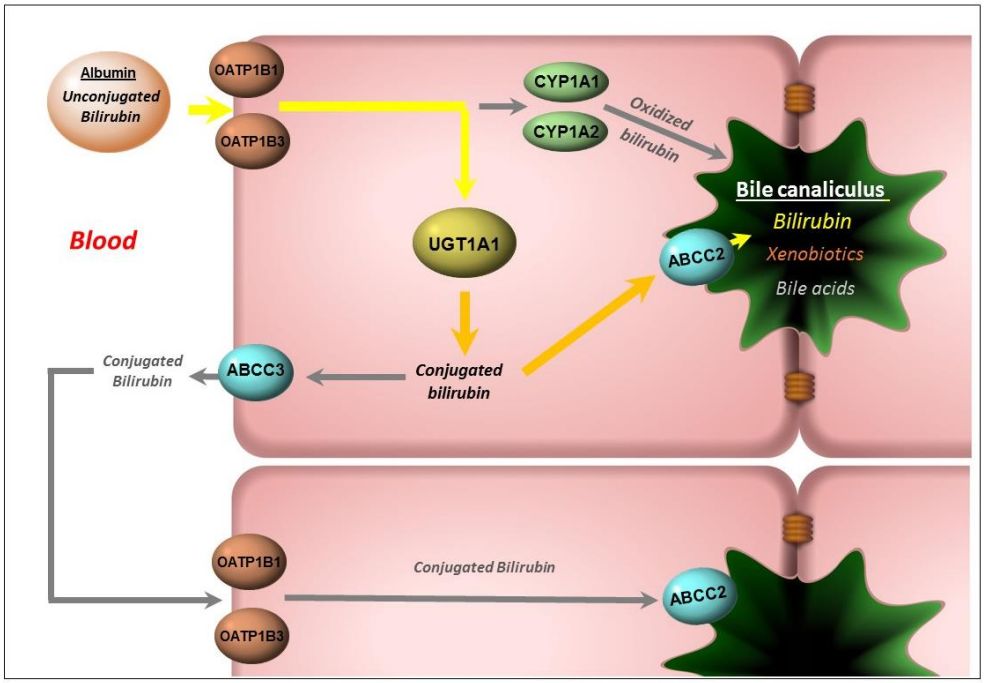

Figure 2: Schematic overview of hepatic bilirubin metabolism. *UCB is transported in the blood bound to albumin. At the basolateral membrane, UCB is released from albumin and transported in the hepatocytes (partially) by OATP1B1 and OATP1B3. Subsequently, UCB is conjugated by UGT1A1 and transported into the bile via canalicular membrane transporter ABCC2. Alternatively, CB can be transported back into the blood via ABCC3 and transported back into downstream hepatocytes via OATP1B1 and 1B and subsequently transported into the bile. In absence of UGT1A1, bilirubin can be oxidized by CYP1A1 and CYP1A2, and the oxidation product is released into the bile.*

2.4 Intestinal metabolism

CB is excreted in the intestine via the bile. In the intestinal lumen, neonatal mucosa has a high glucuronidase activity, which leads to an almost complete hydrolysis of CB to UCB [53]. After deconjugation, UCB is converted to urobilinogens and their oxidized derivatives [54,55]. These urobilinoids are believed to be nontoxic due to their increased polarity [56]. They can either leave the intestinal lumen via the feces, or are reabsorbed by the intestine to be excreted by the kidneys [57]. A small fraction of urobilinoids undergoes

enterohepatic circulation, and due to their hydrophilicity, they are easily excreted by the liver without conjugation [56-58]. Urobilinoid production is highly efficient and therefore only small amounts of bilirubin can be found in the feces of human adults, whereas urobilinoids are the predominant fecal bile pigments [55,59]. In infants however, this mechanism is believed to only play a minor role, since their still undeveloped intestinal microbiota is not capable of UCB conversion[60,61]. In a study by Vitek et al. [56], fecal samples of 60 neonates were examined for urobilinoids and bilirubin. In 57% of infants, urobilinoids only became detectable in the feces at postnatal day five and the increase in fecal urobilinoids was paralleled by a decrease in fecal bilirubin concentration. In other work by Vitek et al. [62], hyperbilirubinemic rats were treated with clindamicin/neomycin, antibiotics against the anaerobic intestinal flora that converts UCB to urobilinoids. This treatment caused fecal urobilinoids to disappear almost completely and caused a marked increase in plasma UCB. It is therefore believed that in absence of the appropriate microbiota, UCB becomes available for reabsorption in infants (fig. 3A).

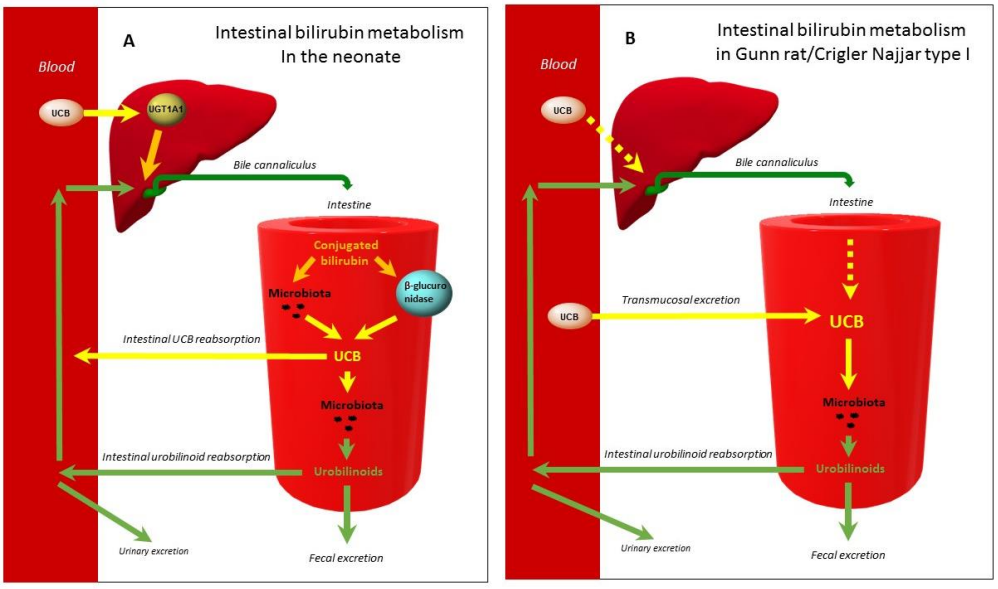

Figure 3: Schematic overview of intestinal bilirubin metabolism. A) *Intestinal bilirubin metabolism in the (term) neonate. After hepatic conjugation, CB is released via the bile into the intestine, where it can be deconjugated by either mucosal β-glucuronidases or intestinal microbiota. Following deconjugation, UCB can either be reabsorbed back into the blood or further converted to urobilinoids by intestinal microbiota. Urobilinoids are either fecally excreted, or reabsorbed and subsequently excreted via the kidney or bile.* ***B)*** *Intestinal bilirubin metabolism in UGT1A1-deficient Gunn rat or Crigler Najjar type I. In severe hyperbilirubinemia, UCB is either released into the bile by unknown mechanisms, or transmucosally excreted over the intestinal wall. In the intestine, UCB can be converted to urobilinoids, which are fecally excreted or reabsorbed, and subsequently excreted via the kidney or bile.*

2.5 Alternative intestinal metabolism: transintestinal bilirubin excretion

In patients with Crigler-Najjar type I and in the rat model for this disease, the Gunn rat, UCB levels rise until a plateau phase [36,51]. This indicates that these individuals are partially able to dispose of their UCB, independent of conjugation . A small amount of UCB can be detected in the bile [63,64]. No transporter has been identified for this biliary excretion, but it is possible that UCB enters the bile by simple diffusion, caused by the sheer overload of UCB in the hepatocyte (fig. 3B).

The UCB from the bile ends up in the intestinal lumen. However, in Gunn rats, a larger amount of UCB can be detected in the intestinal lumen that cannot be accounted for by biliary excretion. This indicates that UCB can be excreted transintestinally from the blood into the intestinal lumen [64] (fig. 3B). Although the underlying mechanism of this transport has not been elucidated so far and any responsible transporters have been not been identified, transmucosal transport can be targeted therapeutically in Gunn rats [58,65-68].

In addition to hepatic UCB conjugation, intestinal UGT1A1 could potentially also play a significant role in neonatal bilirubin metabolism, as is discussed under ‘Animal models – Humanized UGT1A mice’.

3. ANIMAL MODELS

To study neonatal hyperbilirubinemia *in vivo*, three animal models are currently in use; the Gunn rat, UGT1A1 knock-out mice and humanized UGT1A1 (*hUGT1*1*) mice.

3.1 Gunn rats

Gunn rats suffer from a spontaneous UGT1A1 mutation, leading to a complete UGT1A1 deficiency and were discovered in 1934. They are the rat equivalent of Crigler-Najjar type 1 and experience lifelong severe unconjugated hyperbilirubinemia, along with varying but relatively mild neurotoxic signs, including delayed motor development, stunting and cerebellar hypoplasia [69]. Like human neonates, Gunn rat neonates exhibit increased UCB levels when compared to adults (*unpublished observations*). After this initial peak, UCB levels decrease to young adult values and gradually increase again during ageing [70]. The advantage of this model lies in its long existence and the broad experience with the Gunn rat in the hyperbilirubinemia research field. Also, Gunn rats can be studied both as neonates and adults and their size (larger than mice) allows for collection of considerable volumes of e.g. bile, blood and feces, which is sufficient for numerous scientific analyses.

3.2 Ugt1a1 knock-out mice

The mouse equivalent of the Gunn rat was developed in Italy in 2012, by the group of prof. Andrés Muro [71]. These genetically engineered knock-outs have a similar mutation as Gunn rats. However, in contrast to Gunn rats, their postnatal UCB levels are higher and, if untreated, their phenotype is lethal within the first 5-11 postnatal days [71]. These mice thus need continuous rescue treatment in the form of phototherapy or albumin administration to survive and they die as soon as these treatments are stopped [72]. Although the course of disease is in several aspects more similar to the human course of Crigler-Najjar type I [36], the therapy requirement can complicate the testing of new treatment

strategies. Since these animals die without rescue therapies, insufficiently effective experimental therapies will quickly lead to death of the animal, which comes with ethical concerns. In addition, new therapies have to be either tested in neonatal animals or in adults that are previously treated with other rescue therapies. Neonatal mice are vulnerable and provide little tissue material and in adults, the previously required rescue therapies can interfere with subsequent experimental therapies. Nevertheless, this model has proven very valuable in studying bilirubin-induced brain toxicity and development effects [25,73-76].

3.3 Humanized UGT1A mice

The *hUGT1*1* mice are knock-outs for the entire mouse Ugt1a family (7 UGT1A members) in which the respective locus has been replaced by a human UGT1A locus (9 members) [77,78]. Although the Ugt1a family knock-out was lethal, like the *Ugt1a1*-knock-out mice described above, the humanized mice exhibit a milder hyperbilirubinemia. Interestingly, this hyperbilirubinemia is largely present during the first 3 postnatal weeks, with a peak around day 14. This is similar to the course of breast milk jaundice; a prolonged unconjugated hyperbilirubinemia observed in breastfed neonates. After this period, the *hUGT1*1* mice become normobilirubinemic adults. This specific postnatal course is explained by the course of hUGT1A1 expression, which is significantly induced between postnatal day 14 and 21 in both liver and intestine. Before day 14, hUGT1A1 in this model is hardly detectable in liver, but its intestinal expression is clearly present [78].

The hUGT1A1 expression profile seems counter-intuitive at first instance, since in adult humans and rodents, UGTs are predominantly expressed in liver [79]. However, in preterm human neonates, UGT1A1 is also expressed at very low levels in liver during the first postnatal days [35,80]. Already in the 90's, McDonnel et al.[81] established intestinal UGT1A1 expression and bilirubin conjugation

activity in the human intestinal tract. In adults human tissues, the intestinal conjugation of estradiol, an UGT1A1 substrate, even exceeds the liver conjugation capacity [82], indicating its potential importance. Also, this model demonstrates that intestinal hUGT1A1 expression is enough to rescue the kernicterus phenotype, confirming the impact of intestinal UGT1A1. However, the intestinal UGT1A1 expression and its contribution to neonatal bilirubin metabolism in human neonates has never been studied. Therefore, we do not know for sure whether this model is an accurate reflection of the human neonatal situation.

In contrast to Gunn rats and *Ugt1a1*-knock-out mice, the *hUGT1*1* model allows the study of UGT1A1 regulation. Along with the UGT1A locus, the human UGT1A1 promoter has been inserted in these mice, including the distally located phenobarbital-response element (PBREM). Consequently, the known transcriptional regulation of hUGT1A1 by the pregnane X-receptor (PXR), constitutive androstane receptor (CAR), Aryl hydrocarbon receptor (AhR) and Glucocorticoid receptor (GR) agonists is intact [83,84]. Since the UGT1A1 promoter is poorly conserved between rodents and humans [85], this is the only model in which the transcriptional regulation of UGT1A1 can be accurately tested *in vivo*.

4. THERAPEUTIC STRATEGIES

Over the years, various treatment strategies in neonatal hyperbilirubinemia have been developed and investigated. Below, the currently available strategies are discussed in detail. In the various chapters of this thesis, we address several steps in neonatal hyperbilirubinemia management, each targeting different steps in bilirubin metabolism (fig. 4)

4.1 Decreasing total serum bilirubin – Phototherapy

Phototherapy, blue light therapy, has been the gold standard treatment for neonatal hyperbilirubinemia since 1968 [86]. Through the skin, phototherapy

exposes UCB that is present in the superficial capillaries and interstitial spaces. Upon exposure to blue light, UCB undergoes photochemical conversion e.g. photo-oxidation, configuration and structural isomerization, which result in non-toxic isomers (fig. 5) [87]. In contrast to UCB, these isomers are water-soluble can be excreted in the bile without the need for conjugation[88]. Since the evolution of phototherapy, the use of exchange transfusions has been dramatically reduced [89]. Currently, phototherapy is used in over 80% of preterm infants admitted at a neonatal intensive care unit (NICU) [90,91].

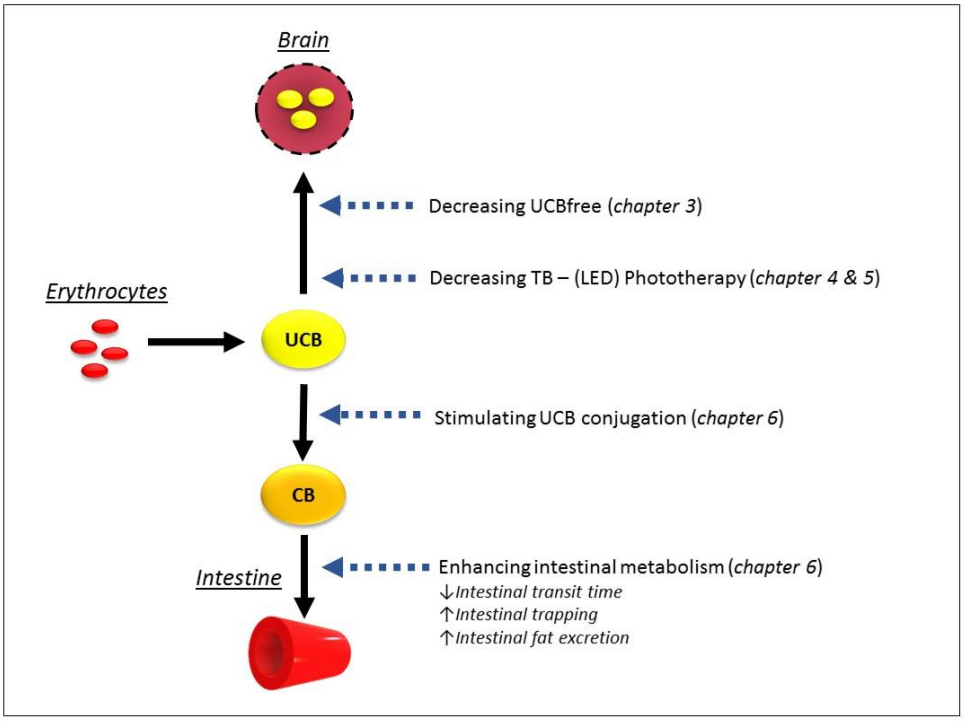

Figure 4: Treatment strategies in neonatal hyperbilirubinemia and their underlying mechanism. *Chapter 3 describes the postnatal course and risk factors of UCBfree in preterm neonates. Chapter 4 & 5 evaluate a recently introduced form of phototherapy: LED phototherapy. Chapter 6 investigates two novel drug strategies targeting both UCB conjugation and intestinal metabolism.*

Although generally safe and effective, phototherapy has some disadvantages. Firstly, it is not sufficiently effective in all infants and therefore does not completely eliminate the need for exchange transfusions. In addition, it can only decrease already accumulated UCB and does not prevent its accumulation. Furthermore, phototherapy has been associated with an increased mortality risk in the smallest preterm infants [92-94] and more recently, with the development of oxidative stress and infantile cancer [95-101] and with diabetes, asthma and epilepsy during childhood [102-105]. Another downside of phototherapy is that it is not suitable for places with unreliable power supply and consequently, is hardly usable or affordable in low resource countries. Therefore, significant effort has been put into the development of alternative or complementary treatments strategies [106]. A selection of these strategies will be discussed below.

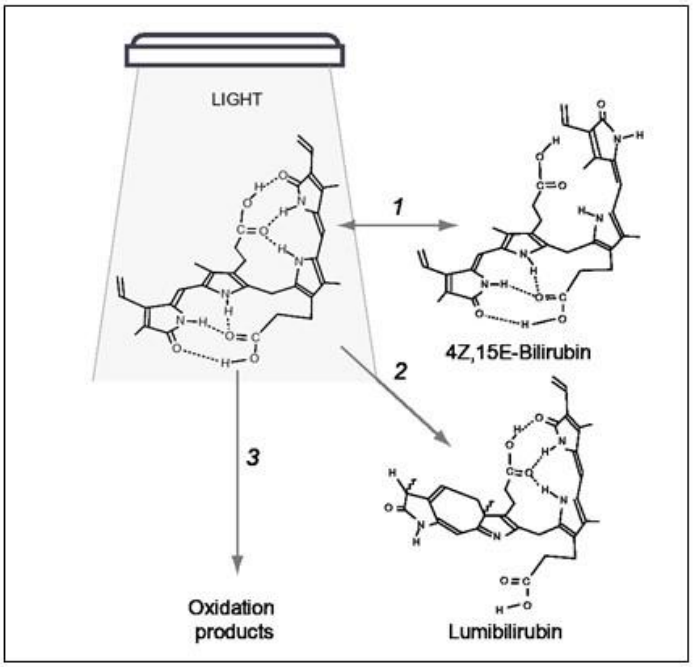

Figure 5: Conversion of UCB into oxidation products, configurational and structural isomers by phototherapy.

4.2 Decreasing free bilirubin

Given that non-albumin bound UCBfree is the neurotoxic fraction of UCB, any UCBfree reduction is a rational strategy to prevent bilirubin-induced neurotoxicity. This has been successfully achieved in Gunn rats and *Ugt1a1* knock-out mice, in which infusion of human serum albumin (HSA) decreased both the UCBfree fraction in blood and UCB level in brain [72,107,108]. More importantly, HSA had an additional therapeutic advantage on both parameters when used in parallel with phototherapy. In *Ugt1a1* knock-out mice, regular HSA infusions, could even rescue them from kernicterus-induced death [72]. Counterintuitively however, HSA alone increased TSB in both Gunn rats and *Ugt1a1* knock-out mice [72,107,108]. These data support a model first proposed by Cuperus et al.[108], in which UCBfree is able to move between the vascular (blood) and extravascular (tissue) compartments. HSA infusions increase the vascular binding capacity of albumin in the blood. Thereby, UCBfree is drawn from the tissues to the blood. Once in the blood, UCBfree becomes available for e.g. phototherapy, which explains the additional therapeutic advantage of HSA when used in combination with phototherapy.

Although these data are both intriguing and promising, the lack of TSB decrease upon HSA treatment, makes it hard to prove its efficacy in human neonates. Brain UCB levels cannot be determined in human neonates and reliable clinical markers for bilirubin-induced toxicity are lacking. Only two studies investigated the combined effect of albumin and phototherapy in neonates. Caldera et al. [109] combined phototherapy with two hours albumin infusion at the start of phototherapy. Albumin infusion with phototherapy caused a significant additional decrease of UCB and UCBfree compared to phototherapy alone. Later, Hosono et al.[110,111] showed the same with a similar setup and also showed that the addition of albumin infusion to phototherapy and caused a significantly better auditory brainstem response (ABR) at 6 months follow-up. Other studies primarily focused on the use of albumin infusions before

exchange transfusion, but the results on post-exchange UCB levels are inconsistent [112-116].

Other studies targeting UCBfree primarily focus on factors influencing the bilirubin-albumin binding affinity [21]. So far, it is known that low gestational and postnatal age, birth weight, illness and acidosis predispose to a lower albumin-bilirubin binding affinity. Furthermore, several endogenous and exogenous substances are known to act as bilirubin replacers from albumin, at least *in vitro*, including commonly used drugs such as ibuprofen, ceftriaxone and sulfonamide antibiotics [117]. Recently, intravenously administered free fatty acids in the blood have been shown to competitively decrease bilirubin-albumin binding, and increase UCBfree levels in neonates, but this has not led to clinical management alterations [118,119]. Attempts to actively increase the bilirubin-albumin binding affinity have so far not been described.

4.3 Stimulating hepatic bilirubin conjugation

In addition to exchange transfusion, phenobarbital has been used in the past to treat neonatal hyperbilirubinemia before the introduction of phototherapy. Phenobarbital, currently used as an anti-epileptic drug, is a Constitutive Androstane Receptor (CAR) agonist, that binds to the promoter of UGT1A1, induces its transcription [120] and thereby increases bilirubin conjugation [121-123]. Several clinical trials have shown that phenobarbital administration to neonates limits the severity of unconjugated hyperbilirubinemia and the need for exchange transfusion [124,125]. Also, phenobarbital administration to pregnant mothers in the last week before delivery decreased the incidence of hyperbilirubinemia and exchange transfusions [126,127]. In addition to inducing bilirubin conjugation, phenobarbital also increased the hepatic uptake and storage of UCB, which lowers its plasma levels [128]. However, phenobarbital was abandoned because of its adverse sedative effect and limited and slow effect compared to phototherapy.

In addition to CAR, three other transcription factors are known to induce *hUGT1A1* transcription: PXR, GR and AhR. Like CAR, these transcription factors bind to PBREM. *In vitro*, UGT1A1 is transcriptionally induced by the PXR-agonist rifampicin, the GR-agonist cortisol and the AhR agonist benzo(a)pyrene [120,129-132]. Rifampicin is a broad spectrum antibiotic that is used for a variety of infections in children, including tuberculosis [133]. However, it is not suitable for treatment of neonatal hyperbilirubinemia due to its potentially serious adverse effects, including hepatotoxicity, renal failure and thrombocytopenia [133,134]. Dexamethasone, a GR-agonist appears to induce UGT1A1 mainly via PXR-dependent mechanisms [120,135]. Dexamethasone is not routinely used in neonates, but a trial studying the effect of early neonatal dexamethasone on chronic lung disease did not show any effect on hyperbilirubinemia [136]. Many foetuses are antenatally exposed to corticosteroids, since corticosteroids are prescribed to promote lung maturation in the preparation for preterm labour. However, studies on the effect of antenatal steroids on neonatal bilirubin levels are largely conflicting [136]. For AhR, no pharmaceutical ligands suitable for human use are currently available.

4.4 Decreasing intestinal transit time

The intestinal transit time affects the window of intestinal UCB absorption. When infants receive insufficient feeding during the first postnatal week, this causes their UCB levels to rise [137,138]. Conversely, early and frequent feedings reduce UCB levels [139]. In rats, fasting increases UCB in the intestinal lumen, but decreases fecal UCB excretion. This intestinal UCB is reabsorbed from the intestine, which increases plasma UCB. This intestinal reabsorption is attributed to decreased intestinal motility and associated increased intestinal transit time [140,141]. In line with this, infants with decreased gastrointestinal transit, as is the case in e.g. pyloric stenosis or Hirschprung disease, are known to have higher UCB levels [142,143]. Correspondingly, the anti-diarrhea drug loperamide, decreases intestinal motility and increases plasma UCB while the

laxative polyethylene glycol (PEG) accelerates intestinal transit and decreases plasma UCB in Gunn rats [144]. In neonates however, PEG is not registered for infants < 1 month.

In addition to intestinal transit time, the amount of stool production could theoretically affect fecal bilirubin excretion, which has been postulated as potential mechanism for fasting and breastfeeding jaundice [145-147]. Breastfeeding is associated with higher neonatal TSB levels, due to enhanced intestinal bilirubin reabsorption [148]. Studies by Carvalho and Gourley et al. showed lower postnatal stool production in breastfed infants and a negative relationship between stool production and bilirubin levels [145,146]. However, later studies by Bertini et al. and Buiter et al. did not find a difference i n bilirubin levels between breast- and formula fed infants, nor a difference in stool production [147,149]. The discrepancy between studies could potentially be explained by different formula feeding compositions, including the fat content (see paragraph 4.6).

4.5 Intestinal trapping

Besides affecting intestinal motility, intestinal UCB could also be affected by intestinal entrapment. Several compounds have been shown to bind to UCB in the intestinal lumen, thereby preventing its reabsorption and promoting its fecal excretion. These compounds include agar, cholestyramine, charcoal, amorphous calcium phosphate and zinc salts. Of these compounds, both agar [150-152] and cholestyramine [153-156] have been tested in neonates, with inconsistent results. Charcoal has some proven additional value over phototherapy when used in combination in neonates, but only when used directly postpartum [157,158]. Calcium-phosphate has a mild beneficial effect in patients with Crigler-Najjar type I, but has not been tested in neonates [159]. Zinc sulphate is known to increase the fecal UCB excretion in Gunn rats and Gilbert patients [160,161], but is not clinically used because of zinc-induced toxicity risks.

4.6 Increasing intestinal fat content

In Gunn rats, bilirubin levels are inversely correlated to fecal fat excretion. On a low fat diet, their UCB levels were shown to increase twofold when compared to high fat diet, and a completely fat-free diet increases UCB threefold [67,162]. This effect of dietary fats was hypothesized to be mediated by fecal fat excretion [163], as increased dietary fat increased both fecal fat and fecal bilirubin excretion in Gunn rats. Since UCB is highly hydrophobic, it is hypothesized to associate with unabsorbed fat in the intestinal lumen. This fat 'entrapment' is thought to prevent intestinal reabsorption and thereby decrease plasma UCB.

Treatment with orlistat, which blocks intestinal fat absorption, had the same effect. Orlistat decreased plasma UCB levels to a similar extent as phototherapy and had additional bilirubin-lowering capacity when used in combination with phototherapy in Gunn rats [67,68]. In Crigler-Najjar type-I patients, orlistat decreased UCB by an additional 9% when used in combination with phototherapy [164]. Orlistat inhibits gastrointestinal lipases and thereby prevents the conversion of triglycerides into absorbable fat, e.g. free fatty acids or monoglycerides [165]. Thereby it increased the concentration of unabsorbable fat in feces, which again increased the fecal UCB excretion. Theoretically, the inverse could be true for breastfeeding. Mother's milk is known to increase fecal fat absorption compared to formula feeding [148,166]. The consequent lower fecal fat excretion could promote intestinal UCB reabsorption and thereby contribute to higher plasma UCB levels.

Although this hypothesis is attractive, the actual concept has never been proven *in vitro*, and it is not known how the association between unabsorbed fat and UCB works. Theoretically, both dietary fats and orlistat could also work by modulating intestinal motility, intestinal microbiota or bile acid metabolism or a combination of these [58] . Although orlistat has not been described to alter total fecal bile acids, it does alter the bile acid composition [68,165]. From previous work

by Cuperus et al.[68], it is known that bile acid composition can significantly affect intestinal bilirubin metabolism and that treatment with bile acids cholic acid (CA) and ursodeoxycholic acid (UDCA) can significantly decrease plasma UCB in Gunn rats. Although the mechanism of this effect has not been elucidated, both bile acids are known to enhance fecal, but not biliary bilirubin excretion, indicating that they act via decreasing intestinal reabsorption and/or promoting transmucosal excretion. In human neonates, UDCA in combination with phototherapy has been shown to cause a larger bilirubin decrease and shorten PT duration [167].

5. OXIDATIVE STRESS AND DNA DAMAGE IN NEONATAL HYPERBILIRUBINEMIA

5.1 Bilirubin and oxidative stress

The role of UCB in oxidative stress has the nature of a double-edged sword; on the one hand, UCB is an endogenous anti-oxidant that at mild levels may protect against oxidative stress. On the other hand, severe hyperbilirubinemia is associated with increased oxidative stress and neurotoxicity. However, since no clear threshold UCB value for oxidative stress has been described and in neonates, it is possible that both protective and damaging effects are present at the same time. In 1954, Bernhard et al. showed that low levels of UCB could prevent the oxidation of vitamin A and linoleic acid *in vitro* [168]. Years later in 1987, Stocker et al. performed a landmark study in which they showed UCB to exceed the anti-oxidative power of α-tocopherol, which was until then regarded as the best anti-oxidant for lipid peroxidation [169]. In following years, the protective effects of UCB have been investigated in many research fields, in particular cardiovascular and metabolic diseases. In 1994, a negative correlation was shown betwe en TSB and ischemic heart disease [170] and the incidence of ischemic heart disease was shown to be lower in patients with

Gilbert syndrome [171]. The nature of the correlation between bilirubin and decreased cardiovascular risk is still not fully elucidated, but is hypothesized to be partially explained by reduction of oxidative stress upon mild hyperbilirubinemia [172]. Vitek et al.[173], showed an inverse relationship in Gilbert syndrome patients between TSB and urinary biopyrrins, an oxidative stress marker which is generated upon the reaction of bilirubin with reactive oxygen species (ROS) [174]. Furthermore, in studies with Gilbert syndrome patients and healthy individuals, a positive association between total serum antioxidant capacity (TAC) and TSB was reported [171,175]. Although these findings point towards oxidative stress as a mediator, the causality between oxidative stress, bilirubin and cardiovascular risk in Gilbert patients has never been shown. Gilbert patients have a generally leaner phenotype and a more favourable lipid profile compared with age-matched controls [176]. In addition, UCB is known to decrease platelet activation and thereby the risk of thrombotic events, independent of oxidative stress [177]. All these factors could theoretically explain the decreased cardiovascular risk in Gilbert patients and do not necessarily depend on reduction of oxidative stress. Lastly, increased TSB levels due to liver dysfunction in humans did not protect against cardiovascular events [178].

Regarding neonatal hyperbilirubinemia, one study by Dennery et al.[179] describes the protective effects of hyperbilirubinemia against hyperoxia-induced oxidative stress in Gunn rat pups. Most studies, however, focussed on bilirubin-induced neurotoxicity in brain, where part of the neurotoxic effects are known to be mediated by oxidative stress [76,180]. In human neonates, studies on the bilirubin-oxidative stress relationship are largely conflicting and rather heterogeneous in terms of gestational age of the studied infants, TSB levels and interventions during the study period (Table I) (*reviewed by Dani et al.*[181]*)*.

Table I: Effect of total serum bilirubin increase on oxidative stress parameters in neonates*

Study	Infants	TSB (mg/dL)**	Treatment	Effect of TSB increase
Yigit et al. [182]	58 term infants	23.9 ± 5.7	Phototherapy and/or exchange transfusion	↑ MDA
Gopinathan et al. [183]	16 term, 31 preterm infants	—	12 preterm infants treated with phototherapy	↑ TPAC in term infants but not in preterm infants
Belanger et al. [184]	Term infants	14.5–25.0	Exchange transfusion	↑ TPAC
Kumar et al. [185]	70 term infants	<5.0–25.0	Phototherapy	↓ MDA ↑ TPAC
Basu et al. [186]	64 term infants	>12.0	Phototherapy	↑ MDA ↓ TPAC
Dogan et al. [187]	36 term infants	20.9 ± 5.1	Phototherapy	↑ MDA ↑ TPAC
Hammerman et al. [188]	41 preterm infants	<10.0	—	↑ TPAC
Dani et al. [189]	21 preterm infants	12.7 ± 1.5	Phototherapy	↓ TPAC
Dani et al. [190]	12 preterm infants	13.7 ± 0.9	Phototherapy	↓ TPAC = NTBI

*TSB: total serum bilirubin; MDA: malondialdehyde; TPAC: total plasma antioxidant capacity; HO: heme oxygenase; NTBI: nontransferrin-bound iron. *Adapted from Dani et al. (2018): Bilirubin and oxidative stress in term and preterm infants. ** To convert mg/dL to µmol/L, multiply by 17.1.*

5.2 Phototherapy, oxidative stress and DNA damage

In almost all studies describing the relationship between oxidative stress and hyperbilirubinemia infants are treated with phototherapy, exchange transfusion or both (Table II). These therapies can potentially interfere with the relationship between bilirubin and oxidative stress; especially phototherapy has been associated with adverse effects, including oxidative stress and DNA damage [95-100]. Phototherapy has also been associated with increased mortality in very low birth weight infants [92-94] and with an increased incidence of infantile cancer [101]. However, the causality between phototherapy and these adverse outcomes has never been shown and is hard to prove in humans, since the phototherapy-induced bilirubin decrease could also account for changes in oxidative stress or antioxidant capacity. Furthermore, especially preterm neonates are also exposed to various diseases and interventions associated

with oxidative stress, such as sepsis, inflammation, respiratory distress, supplemental oxygen, or mechanical ventilation, which could also interfere [191,192].

Table II shows all studies on the effect of phototherapy on oxidative stress and DNA damage and their conclusions. Noteworthy, the vast majority of studies was performed using fluorescent tube (FT)-phototherapy, and all FT phototherapy studies show induced oxidative stress after PT. Currently, however, FTs are gradually being replaced by LED phototherapy, since LED phototherapy is able to produce a higher irradiance without significant heat production. Only three studies have so far described the effect of LED phototherapy on oxidative stress and compared LED with FT phototherapy. The results are inconsistent, since Demirel et al.[193] concluded that FT- but not LED phototherapy induced oxidative stress, whereas Kale et al.[194] concluded that both FT and LED induce oxidative stress. El-Farrash et al.[195] also reported a significant induction of lipid peroxidation marker malondialdehyde (MDA) after both types of phototherapy, but this induction was smaller in LED phototherapy when compared to FT phototherapy. However, the results are difficult to interpret since Demirel and Kale et al. compared rather low light irradiances of FT phototherapy with substantially higher doses of LED phototherapy. El-Farrash et al. also included intensive FT phototherapy, but unfortunately did not accurately report the irradiances used (only >60 $\mu W/cm^2/nm$).

Table II: Effect of phototherapy on oxidative stress and DNA damage parameters in neonates

Study	Infants	Phototherapy (PT)	Effect of Phototherapy on Oxidative stress/DNA damage markers			
			TOS	**TAC**	**OSI**	**Miscellaneous**
Gathwala et al [95].	30 preterm infants	-Duration: 96h -Type: FT PT -Irradiance: not measured				↑ TBRS
Aycicek et al. [96]	34 term infants	-Duration: 24h -Type: FT PT -Irradiance 12-16 µW/cm2/nm	↑TOS		↑OSI	↓MDA
Tatli et al [97].	47 term infants	-Duration: 72h -Type: FT PT -Irradiance: 12 µW/cm²/nm				↑ lymphocyte DNA damage (Comet assay)
Aycicek et al [98].	65 term infants	-Duration: max. 72h -Type: FT PT -Irradiance: 12-16 or 30-34 µW/cm2/nm	↑TOS	=TAC	↑OSI	↑leukocyte DNA damage (Comet assay)
Kahveci et al [99].	22 term infants	-Duration: 24-96h -Type: FT PT -Irradiance: 15 µW/cm²/nm				↑SCE in blood
Yahia et al. [196]	45 term infants	-Duration: till normal bilirubin was reachted -Type: FT PT -Irradiance: 10 16 µW/cm²/nm				↑leukocyte DNA damage (Comet assay)
Demirel et al [193].	60 term and late preterm (≥35 wk) infants	-Duration: till normobilirubinemic levels were reached -Type: FT and LED -Irradiance FT PT : 12-16 µW/cm²/nm -Irradiance LED PT 30 µW/cm2/nm	FT PT ↑TOS LED-PT =TOS	FT PT =TAC LED PT =TAC	FT PT ↑OSI LED PT =OSI	
Kale et al [194].	90 term and late preterm (≥35 wk) infants	-Duration 24h -Type: FT, LED, LED fibre optic blanket -Irradiance FT PT 10-15 µW/cm2/nm -Irradiance LED PT	FT PT ↑TOS LED-PT ↑TOS LED blanke	FT PT ↓TAC LED-PT ↓TAC LED blanket ↓TAC	FT PT ↑OSI LED-PT ↑OSI LED blanket ↑OSI	

		60-90 µW/cm2/nm -Irradiance LED, fibre-optic blanket 35 µW/cm2/nm	t =TOS			
El-Farrash et al [195].	120 term and late preterm (≥35 wk) infants	-Duration 24h -Type: conventional FT PT, intensive FT PT, LED PT -Irradiance conventional FT PT 12-16 µW/cm²/nm -Irradiance intensive FT PT >60 µW/cm²/nm -Intensity LED PT 30-120 µW/cm²/nm		Conventi-onal FTPT ↓TAC Intensive FT PT ↓TAC LED PT ↓TAC		Conventional FT PT ↑MDA ↑NO Intensive FT PT ↑MDA ↑NO LED PT ↑MDA* ↑NO

*TBRS thiobarbituric acid reactive substances; SCE sister chromatid exchange; TOS total oxidant status; OSI oxidative stress index; TAC total antioxidant capacity; MDA malondialdehyde; NO nitric oxide. *The MDA increase after LED PT was lower compared to the increase after FTPT.*

There are several arguments why FT phototherapy could be expected to induce more oxidative stress than LED phototherapy. First, the heat produced by FTs can induce hyperthermia, which is known to enhance oxidative stress [197]. Second, FTs primarily produce ultraviolet (UV)-light, which is subsequently converted to visible light by the internal phosphor coating. Several studies have described leakage of UV-light from FTs, especially when the coating wears off and becomes damaged over time [198]. Although this was never shown for FT phototherapy devices, UV-leakage could theoretically lead to a clinically significant UV-exposure, especially when the newborn infant is placed close to the lamp. Compared to LEDs, FTs need to be placed closer, due to the relatively lower irradiance of the emitted light, which could aggravate the exposure to UV [198]. To protect from UV exposure, FT-based phototherapy devices need to be used with a protective screen to absorb UV-radiation. However, most studies do not report on the use of such a screen and in practice, the screens may be removed after a certain time because they get dirty or damaged, or because the

protective role of the screens is not realized. Finally, FTs emit a broader wavelength of light compared to LEDs, and FTs emit several high irradiance peaks at different wavelengths. Although these are not visible by eye, these different wavelength peaks can potentially cause side effects, including the previously reported induction of oxidative stress or DNA damage. The blue LED spectrum on the other hand is narrower and does not have high irradiance peaks at other wavelengths [199].

5 SCOPE OF THIS THESIS

The aim of this thesis is ***to evaluate current developments in the management of neonatal hyperbilirubinemia and to explore new treatment possibilities***. In the various chapters, we address several steps in neonatal hyperbilirubinemia management (fig. 4), including the evaluation of UCBfree and the UCBfree/TSB ratio in preterm neonates, an evaluation of recently introduced LED phototherapy and two new potential therapies for neonatal hyperbilirubinemia. In all studies we aim to use a translational approach, by connecting established molecular knowledge with clinical applicability, using specific hyperbilirubinemia animal models, both adult and neonatal, and by testing clinically available compounds. Hereby, we aim to make a valuable contribution to both biomedical science and pediatric clinical care.

In **Chapter 2** we review the role of CB transporters ABCC2 and ABCC3 in bilirubin metabolism, cholestasis and drug disposition and their (post)transcriptional regulation.

Chapter 3 describes the postnatal course of UCBfree and the UC Bfree/TSB ratio and studies their correlation with gestational age, postnatal age, birth weight and hyperbilirubinemia risk factors.

Whereas current treatment guidelines of neonatal hyperbilirubinemia determine their thresholds for starting phototherapy on TSB levels, it is known that TSB poorly correlates with brain bilirubin levels [200,201]. Not surprising, TSB is a poor predictor of bilirubin-induced neurologic dysfunction (BIND) and various cases have been described of BIND under low TSB levels [202]. UCBfree is the neurotoxic fraction of UCB, is able to pass the BBB and more closely correlates with BIND [22]. In addition, the UCBfree/TSB ratio could be used to assess BIND risk, since it is a diagnostic marker that not only represents the magnitude of the bilirubin pool (represented by TSB), but combines it with UCBfree, which represents the tissue distribution of the pool. Both UCBfree and the UCBfree/TSB ratio are not routinely determined in clinical care, and their postnatal course was not accurately described.

Chapter 4 and 5 evaluate the potential of LED phototherapy to induce oxidative stress and DNA damage in rats and preterm infants, respectively. Phototherapy has been associated with oxidative stress, increased incidence of infantile cancer and increased mortality in extremely low birth weight infants. However, these associations are reported in studies using conventional phototherapy devices, using FT light. However, FT phototherapy is currently being replaced by LED phototherapy, which allows higher irradiance treatment without significant heat production. However, the implementation of this high in phototherapy has been impeded by concerns about detrimental side-effects. In chapter 4, we investigated whether LED phototherapy induced oxidative DNA damage. We performed studies using super-intensive LED phototherapy (dosage up to 100 μW/cm^2/nm) in Gunn rats, using a urinary oxidative DNA-damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG). Subsequently, in chapter 5, a translation step is made towards the pediatric clinic and the same marker is determined in preterm infants under LED phototherapy.

In **Chapter 6**, we studied the potential of therapeutic bile acids to treat and prevent neonatal hyperbilirubinemia, that could be used as prevention or in adjunct to phototherapy. We studied treatment of two anticholestatic drugs: UDCA and Obeticholic acid (OCA), in two animal models for neonatal hyperbilirubinemia; neonatal *hUGT1*1* mice and neonatal Gunn rats. In this chapter we show the effect of these drugs on plasma and brain bilirubin and investigate the underlying mechanisms of these compounds.

In **Chapter 7**, we discuss the findings of the different studies in relation to current literature on neonatal hyperbilirubinemia and its management and conclude with the implications of the present findings on current clinical care of neonatal hyperbilirubinemia and future research.

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