Chapter 7

General Discussion, Conclusions, and Future Directions

7.1 Introduction

In this thesis we used the Gunn rat model to develop and refine treatment strategies for severe unconjugated hyperbilirubinemia, as occurs in Crigler-Najjar disease and neonatal hemolytic jaundice. Forty years ago, severe unconjugated hyperbilirubinemia often led to irreversible brain damage in these patients. The only available therapy at that time, exchange transfusion, was associated with significant morbidity and mortality.[1-3] Phototherapy, clinically introduced in the late 1960s, proved a safer treatment for unconjugated hyperbilirubinemia.[4,5] Its widespread use decreased the need for exchange transfusions and enabled long-term treatment for Crigler-Najjar patients.[5,6] Although generally effective, phototherapy had (and still has) some disadvantages. The lifelong phototherapy needed in Crigler-Najjar disease may last >16 hours per day, and nevertheless fails to prevent brain damage in ~25% of the patients.[6,7] Neonatal phototherapy is more effective, but may still require additional, and potentially dangerous, exchange transfusions.[1,2] These considerations prompted us to explore alternative treatment strategies for unconjugated hyperbilirubinemia. To provide a basis for this undertaking, we first reviewed the existing treatment strategies, both experimental and conventional (Chapters 1 and 2). Subsequently, we performed several experiments using Gunn rats, the established model for severe unconjugated hyperbilirubinemia.[8-10] This animal model enabled us to develop two novel oral treatment strategies, as described in Chapters 3, 4, and 5. In the final chapter, we investigated the impact of phototherapy and albumin infusion on brain bilirubin levels during acute and permanent jaundice in Gunn rats.

7.2 Oral treatment strategies for unconjugated hyperbilirubinemia

The first part of this thesis focused on oral treatment strategies for unconjugated hyperbilirubinemia. We aimed for these strategies to be non-invasive, safe, and at least as effective as phototherapy.

Existing hypobilirubinemic treatments (**chapters 1 and 2**) are usually based on inhibition of bilirubin production, or on the stimulation of bilirubin disposal via the bile. In **chapter 3**, however, we decided to focus on an alternative route for bilirubin disposal, namely transmucosal excretion. Transmucosal bilirubin excretion involves the translocation of non-protein bound UCB (UCB_{free}) from the blood into the intestinal lumen.[11-14] This translocation is caused by the ability

of UCB_{free} to passively (and possibly also actively) cross lipid bilayers.[15-17] In hyperbilirubinemic Gunn rats most bilirubin enters the intestinal lumen via this pathway, rather than via the bile.[12] Transmucosal bilirubin excretion, in short, is an important excretory pathway during severe unconjugated hyperbilirubinemia. Its efficacy in actually disposing bilirubin from the body, however, is limited by the intestines' ability to reabsorb UCB_{free} from its lumen.[13,14] We, consequently, aimed to decrease this reabsorption. This had been attempted before by binding UCB_{free} to intestinal compounds, thereby trapping bilirubin within the gut lumen. Most trapping agents, however, including agar,[18] cholestyramine,[19] charcoal,[20] amorphous calcium phosphate,[21] zinc salts,[22] and fat,[23,24] have been clinically unsatisfactory, due to side-effects and inconsistent results. Since bile salts do not only bind UCB_{free} in vitro, but also increase biliary UCB excretion in vivo, we reasoned that they might be used to treat unconjugated hyperbilirubinemia.[25-27] In chapter 3 we demonstrated that the bile salt ursodeoxycholate (UDCA) did indeed treat severe unconjugated hyperbilirubinemia in Gunn rats. UDCA is a well-established and well-tolerated treatment for various hepatobiliary diseases in neonatal, pediatric and adult patients.[28,29] Our results, therefore, can readily be verified in randomized clinical trials. The observation that even low (and clinically relevant) UDCA dosages decreased plasma UCB concentrations further underlined its clinical applicability. Treatment with cholic acid (CA), a hydrophobic bile salt, yielded similar results as UDCA treatment. Although clinical CA treatment is not feasible due to its toxicity, this did demonstrate that the main therapeutic effects were bile salt type independent.

One of the more important principles of this thesis is that "hypobilirubinemic treatment" is not a goal in itself. Far more important than its hypobilirubinemic effect is a treatments' ability to prevent neurotoxicity. Neurotoxicity, however, may occur in the presence of relatively low plasma UCB levels. In the 1950s, several sulfisoxazole-treated neonates developed kernicterus in the presence of unusually low plasma bilirubin levels.[30,31] Odell *et al.* soon discovered that sulfasoxizole displaced UCB form albumin, a discovery that first highlighted the importance of non-protein bound UCB (UCB_{free}).[32] Since then, many studies have underscored the poor correlation between plasma bilirubin and the individual risk for bilirubin-induced brain damage.[33-36] The reason for this poor correlation lies in the inability of protein-bound bilirubin (>99% of total plasma bilirubin) to leave the circulation. Only its small (<1%) unbound fraction, *i.e.* UCB_{free}, is able to translocate across the blood-brain barrier (figure 1A).[37,38] The inability of bilirubin to leave the circulation does, naturally, undermine its potential to predict neurotoxicity.[33,36] This prompted us to extend our evaluation of bile salt treatment beyond plasma bilirubin levels. A radiolabelled ^{3}H-UCB kinetic study demonstrated that bile salt treatment decreased not only plasma, but also tissue bilirubin levels. This decrease was accompanied by an

increase in fecal bilirubin excretion, mostly (~80%) derived from the transmucosal excretory pathway. Taken together, our data demonstrated that bile salts effectively treat unconjugated hyperbilirubinemia in Gunn rats. The underlying mechanism involved a stimulation of transmucosal and fecal bilirubin excretion, which induced a decrease in the total (plasma + tissue) bilirubin pool size.

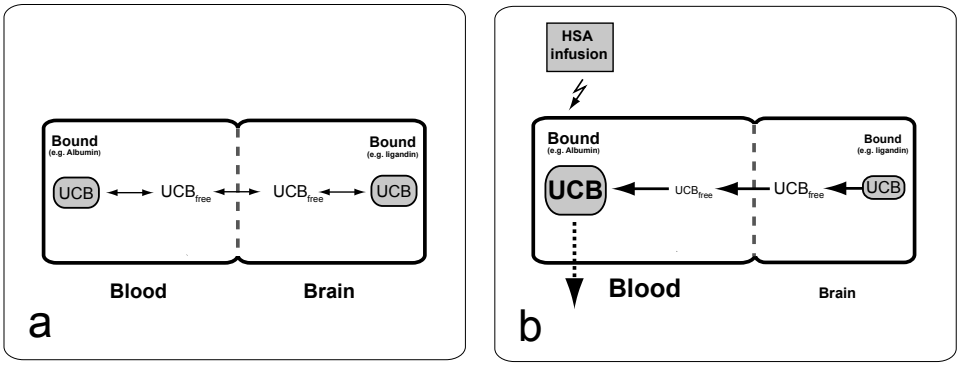

Figure 1. Human serum albumin (HSA) treatment during unconjugated hyperbilirubinemia: (a) Unconjugated hyperbilirubinemia may result in the accumulation of unconjugated bilirubin (UCB) within the brain. Only non-protein bound UCB (UCB_{free}) is able to move between the blood (e.g. vascular compartment) and the brain (e.g. extravascular compartment). (b) Treatment with HSA decreases UCB_{free} levels within the blood. This promotes an UCB_{free}-induced shift of bilirubin from the brain into the circulation. Additional phototherapy subsequently converts this bilirubin into photo-isomers that can readily be excreted with the bile (dashed arrow).

The results of chapter 3 confirmed the important contribution of transmucosal bilirubin excretion to its overall turnover. Accordingly, we continued our pursuit of lowering the bilirubin pool via this excretory pathway. This led to an investigation into the role of the gastrointestinal transit in **Chapter 4**. Several studies had suggested that an acceleration of the gastrointestinal transit, such as prebiotic supplementation of infant formula, seemed to mitigate neonatal jaundice.[39,40] Other studies had linked conditions that delay transit, such as fasting, to an increase in plasma bilirubin levels.[41-43] None of these studies, however, had *directly* investigated the relationship between transit time and plasma bilirubin levels. We explored this relationship by treating Gunn rats with the laxative polyethylene glycol (PEG), or with loperamide, which delays transit. This approach revealed a linear relationship between gastrointestinal transit and

plasma bilirubin levels. The strength of this relationship had two major implications. First, it identified the gastrointestinal transit as an important regulator for plasma bilirubin levels in hyperbilirubinemic Gunn rats. Secondly, it suggested that pharmacological manipulation of the transit time might well be used to treat unconjugated hyperbilirubinemia. Acceleration of the gastrointestinal transit by PEG did effectively decrease plasma bilirubin levels in Gunn rats. Simultaneously with the decrease in plasma bilirubin, which occurred within hours, we observed a marked increase in its fecal disposal. This disposal did not originate from an enhanced biliary UCB excretion. Since bilirubin can only enter the intestinal lumen via the bile or via transmucosal excretion, this showed, by inference, that the PEG-induced acceleration in gastrointestinal transit enhanced transmucosal UCB_{free} excretion. We hypothesized that this enhanced excretion occurred because the PEG-induced acceleration had decreased the intestinal UCB_{free} concentration. This claim is indirectly supported by the impressive additive effect of phototherapy in PEG-treated animals. The final plasma bilirubin decrease in phototherapy + PEG treated animals was 65%, which was higher than either treatment alone. This decrease might well be due to the distinct hypobilirubinemic mechanisms of phototherapy (enhanced biliary bilirubin excretion) and PEG (enhanced transmucosal bilirubin excretion). These two mechanisms had been previously described to complement each other in Gunn rats.[9,44]

Oral treatment of unconjugated hyperbilirubinemia, as stated previously, is not a novel concept. In 1962 Lester *et al.* already treated unconjugated hyperbilirubinemia by feeding Gunn rats cholestyramine, which binds UCB_{free} within the intestinal lumen.[19] Since then many oral treatments have been evaluated. These treatments, however, tended to lower plasma bilirubin levels within days.[18,19,21-23] This, naturally, had limited their therapeutic potential in acutely jaundiced patients. For example, the recently developed oral treatment with orlistat only decreased plasma UCB levels by a modest 10% after 36h of treatment in our study.[24] PEG treatment, however, lowered plasma bilirubin within hours, which sets it apart from other oral treatment strategies. Another major benefit of PEG treatment, namely its additive effect to standard phototherapy, has already been discussed. A (novel) treatment that complements routine phototherapy has an evident clinical advantage. Combining PEG administration with phototherapy resulted in a hypobilirubinemic effect that was not only superior to single PEG treatment, but also to any conventional or experimental treatment combination previously explored in Gunn rat studies.[18,19,21-23] Taken together, our data thus clearly supported the clinical feasibility of PEG treatment, with or without phototherapy, in hyperbilirubinemic patients. PEG is currently widely used as a laxative, without any serious side effects.[45,46] A randomized clinical trial, perhaps initially in phototherapy-

treated Crigler-Najjar patients, thus seems an appropriate continuation of this line of research.

As chapter 4 had identified the gastrointestinal transit time as a major player in bilirubin metabolism, we next sought to evaluate its role in other treatments than PEG or loperamide. In **chapter 5** we determined whether routine phototherapy or experimental oral treatments (*e.g.* orlistat, UDCA, or amorphous calcium phosphate) also partly rely on the transit time for their hypobilirubinemic effects. This notion may seem somewhat far-fetched, since each of these treatments had already been associated with a well-defined mechanism of action. Upon careful examination, however, we noticed that each of these treatments had been troubled with side effects that do affect the gastrointestinal transit.[47-49] Diarrhea, for example, often occurs during orlistat or UDCA administration.[50] This could imply that these side effects are actually beneficial, because they could lower plasma bilirubin by accelerating the gastrointestinal transit. We clearly demonstrated, however, that each of the aforementioned treatments decreased plasma bilirubin levels *without* affecting the gastrointestinal transit time. These results prompted us to investigate the value of PEG co-treatment, which might complement the underlying hypobilirubinemic mechanisms of phototherapy and the experimental oral treatments. PEG co-treatment accelerated the gastrointestinal transit time in all treatment groups. A complementary therapeutic effect of this acceleration, however, was only observed in orlistat and phototherapy treated Gunn rats. The complementary effect of adding PEG to phototherapy was reassuringly similar to that of adding phototherapy to PEG. This fits with our previous argument (see: discussion on Chapter 4, above), namely that these treatments truly complement each other. The lack of an additive effect during PEG and UDCA or orlistat treatment, although somewhat unexpected, might have several explanations. First, PEG, orlistat, and UDCA all increased the *transmucosal* excretion of bilirubin. Combining treatments that use a similar route for bilirubin disposal will usually tend to oversaturate their common pathway, whereas treatments that use distinct routes (*e.g.* biliary and transmucosal bilirubin disposal) will tend to complement each other (see: discussion on Chapter 4, above). An alternative explanation for the lack of effect is that PEG decreased the intraluminal bile salt concentration, which naturally counteracts additional bile salt therapy. Finally, PEG might have also decreased the precipitation of amorphous calcium phosphate (and thus the capture of intestinal bilirubin) by accelerating the transit. Whatever the explanation for these observations might be, the most striking result of Chapters 4 and 5 remains the profound hypobilirubinemic effect of combined PEG and phototherapy treatment. This truly complementary combination deserves an extensive clinical evaluation in the near future.

7.3 Phototherapy and bilirubin disposition in the brain

Plasma bilirubin levels are, at best, poor predictors for neurological damage. At worst, however, they can provide a false sense of security that may lead to inertia when intervention is needed.[33,36,51] Only UCB_{free}, and not protein-bound UCB, can cross the blood brain barrier (§ 7.2). We must thus conclude that the small UCB_{free} fraction (<1% of total plasma bilirubin) plays a major role in the pathogenesis of bilirubin-induced neurotoxicity. We consequently aimed to lower this fraction with human serum albumin (HSA), in order to prevent bilirubin-induced neurotoxicity (Figure 1). The experiment in **chapter 6** was primarily designed to evaluate the effect of HSA treatment on brain bilirubin levels of phototherapy-treated Gunn rats. Our experiments were performed in either permanently or acutely (*e.g.* hemolytic) jaundiced Gunn rats, which served as model for Crigler-Najjar's disease or neonatal hemolytic jaundice.[52] In non-hemolytic Gunn rats, long-term phototherapy decreased plasma UCB, plasma UCB_{free}, and brain bilirubin concentrations. Adjunct HSA treatment, however, increased phototherapy's efficacy by 32-35%. In hemolytic Gunn rats, phototherapy + HSA decreased plasma UCB_{free} levels, and completely prevented bilirubin accumulation within the brain. A striking finding was the inability of phototherapy alone to protect these animals from bilirubin accumulation within their brains. Taken together, our data showed that HSA provides a synergistic effect to phototherapy in Gunn rats. We speculate that HSA and phototherapy act *in tandem*. First, HSA decreases UCB_{free} within the plasma, which promotes a bilirubin shift from the brain into the blood. Phototherapy then converts this bilirubin into photo-isomers that can readily be excreted with the bile (Figure 1). Interestingly, our results also question the efficacy of single phototherapy and, indirectly, the use of the total plasma bilirubin concentration as a marker for bilirubin-induced brain damage. Although HSA treatment has (infrequently) been applied in neonatal jaundice, its effects have remained controversial. This controversy might be partly due to HSA itself, which usually contains preservatives that were described to interfere with the HSA-bilirubin binding.[53] We, however, found no such interference in our FDA-approved HSA solution. We consequently feel that large-scale clinical trials are the next step towards the routine application of HSA in a clinical setting. These trials should incorporate UCB_{free} measurements and, ideally, a functional marker of brain function, such as auditory brain stem response measurements. These measurements would allow non-invasive monitoring of bilirubin-induced brain damage, and have been well described in neonates.[51,52,54]

7.4 Conclusions and future directions

In this thesis we demonstrated that administration of UDCA, PEG, and PEG combined with phototherapy effectively treats unconjugated hyperbilirubinemia in Gunn rats. In addition we showed that HSA infusion synergizes the therapeutic effect of phototherapy in these animals.

The effects of UDCA, PEG, and HSA administration were studied in Gunn rats, the well-established model for unconjugated hyperbilirubinemia. The use of Gunn rats enabled us to study the therapeutic potential and the underlying mechanism of these new treatments. We exclusively investigated FDA-approved compounds, since this should facilitate their future use in a clinical setting. UDCA, PEG, and HSA have all been routinely applied in patients and their use appeared to be safe, well tolerated, and devoid of significant side effects. Consequently, a logical next step would be to determine the effects of UDCA, PEG, and HSA in clinical trials. To our opinion, trials with UDCA and with PEG should first be performed in patients with Crigler-Najjar disease. These patients require life-long phototherapy, and would thus benefit directly if UDCA or PEG proved to be effective. Clinical trials in Crigler-Najjar patients can be difficult, however, due to the low prevalence of this disease.[6] This could be addressed by using a crossover design, and by including both type I and type II patients.[21,23] A third clinical trial should evaluate adjunct HSA infusion in phototherapy-treated neonates. It will be interesting to see whether adjunct HSA will, as we observed in Gunn rats, synergize the therapeutic effect of routine phototherapy in hyperbilirubinemic neonates. In order to study this efficacy, however, it is necessary to evaluate reliable predictors for bilirubin-induced neurological damage, such as UCB_{free} and auditory brain stem response measurements. These markers are essential, since it is evidently impossible to measure UCB in human tissue.

Continuing our line of research in a more clinical setting could shed more light on the potential of UDCA, PEG, and HSA as alternative treatment strategies in severely hyperbilirubinemic patients. Simultaneously, we should continue to use animal and in vitro studies to increase our knowledge with regard to the underlying mechanisms of these treatments. Our original experiments were not only designed to evaluate the efficacy of UDCA, PEG, and HSA, but also to tried to elucidate their mechanisms. This “mechanistic” approach increased our understanding of bilirubin metabolism during UDCA, PEG, and HSA treatment. Such understanding is not merely a goal on its own, but will also provide a template for the development of future treatments.

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