Treatment of neonatal hyperbilirubinemia

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APPENDICES

ENGLISH SUMMARY

Neonatal jaundice, characterized by a yellow colorization of the skin, mucosa and sclerae, is a common disease in newborn infants. Especially in preterm infants it occurs frequently; up to 80% experiences a certain level of jaundice. The yellow colorization is caused by elevated levels of the yellow pigment bilirubin, leading to the name hyperbilirubinemia. Bilirubin is a breakdown product of heme in red blood cells. Premature newborns have a temporary high erythrocyte turnover during the first days of life, which causes a higher bilirubin production. When hyperbilirubinemia is severe, bilirubin can deposit in the brain (especially in the so-called basal nuclei), where it can cause damage. This damage can cause a variety of problems described as kernicterus spectrum disorders (KSD). The KSD can include irreversible cerebral palsy (spasticity) and/or hearing and sight problems. Severe untreated hyperbilirubinemia has even been described to cause death. In order to prevent this neurological damage, it is essential to timely diagnose and treat neonatal hyperbilirubinemia. This thesis describes studies on treatment options of this disease, using both animal models and preterm infants.

Free unbound bilirubin

To diagnose hyperbilirubinemia, clinicians currently measure total bilirubin levels in blood. However, KSD is not caused by bilirubin in blood, but rather by bilirubin deposited in the brain. From previous studies we know that brain bilirubin levels are not linearly correlated with blood bilirubin levels. Cases of KSD have been described in infants with low blood bilirubin levels. Likewise, some infants with high blood bilirubin levels may never develop KSD. One of the explanations of this phenomenon includes the degree of blood-to-brain movement and brain deposition of bilirubin. Under physiological circumstances, the bilirubin concentration in the brain is minimal, since bilirubin in the blood is bound to a large carrier protein (albumin). This

bilirubin-protein complex cannot cross the blood brain barrier and therefore does not deposit in the brain. Under certain circumstances however, a small fraction of the bilirubin occurs in the blood in an non-protein bound form, for example in case of a low blood albumin concentration. This 'free' bilirubin can readily cross the blood brain barrier and cause neurological damage. Since it is not possible to measure brain bilirubin levels in a reasonable manner, free bilirubin in blood has been described as a good surrogate marker for bilirubin-induced neurotoxicity risk. Alternatively, the ratio between 'free' and total bilirubin (so the sum of both protein-bound and free bilirubin) in blood, has been suggested to be even better, since it represents both the neurotoxic fraction (free bilirubin) and the total bilirubin pool that the neurotoxic free bilirubin is derived from. However, free bilirubin is not being routinely measured and has only been used in research settings. Therefore, its postnatal course in infants has not been accurately described. Therefore, in **chapter 2**, we measured the level of both free bilirubin and the free bilirubin/total bilirubin ratio in a cohort of preterm infants and we described the postnatal course of both diagnostic markers, as well as risk factors that predispose to high levels. These data could be the first step towards eventual application of free bilirubin and the free bilirubin/total bilirubin ratio in treatment guidelines of neonatal hyperbilirubinemia.

Phototherapy

During the past 60 years, the treatment of neonatal hyperbilirubinemia has mainly consisted of blue light phototherapy. By exposing the skin to blue light, the bilirubin in the subcutaneous blood vessels is converted to a non-toxic isomer (a substance very similar to bilirubin). This isomer can be readily excreted via the liver into the bile and leave the body mainly via the feces. Until recently, phototherapy was emitted by fluorescent tube-based devices. The disadvantage of fluorescent tubes is that in addition to the blue light, they emit a lot of heat. Fluorescent tube phototherapy has been associated with harmful

side effects, including so-called increased oxidative stress and DNA damage. In addition fluorescent-tube phototherapy has been associated with the development of epilepsy and even infantile cancer. In recent years, fluorescent tube devices are gradually being replaced by LED light-based devices. The advantage of these devices is that they emit less heat, and that they can produce a significantly higher light irradiance. Previous research has shown that high irradiance is highly effective in the treatment of neonatal hyperbilirubinemia. However, questions and concerns about the safety of this high irradiance LED phototherapy have impeded its wide-spread clinical implementation. In **chapter 3 and 4**, we tested the effect of LED phototherapy on oxidative DNA damage in both rats and preterm infants. In **chapter 3**, we tested the effect of LED phototherapy on DNA oxidation and DNA damage in jaundiced rats. We show that even on high irradiance, LED phototherapy does not affect the markers 8-hydroxy-2'deoxyguanosine (8-OHdG) and gamma-H2AX. Gamma-H2AX occurs upon the reparation of DNA strand breaks and therefore is a marker for DNA damage in cells and tissues. This marker was not increased in rat skin after phototherapy exposure. 8-OHdG is a breakdown product of DNA that occurs upon DNA oxidation. As clearance, it is almost completely excreted in urine, which makes it a non-invasive test to estimate DNA oxidation. In rats, 8-OHdG did not increase during of after LED phototherapy. In **chapter 4**, we measured 8-OHdG in urine of premature infants on the neonatology intensive care to establish the effect of LED phototherapy on DNA oxidation. In this chapter we show that LED phototherapy with an irradiance up to 35 $\mu W/cm^2/nm$ does not induce 8-OHdG excretion. These results suggest that in contrast to fluorescent tube phototherapy, LED phototherapy does not cause oxidative DNA damage.

Therapeutic bile acids

Phototherapy is not always sufficiently effective. If despite phototherapy, blood bilirubin levels remain dangerously high, the only currently available treatment

option is exchange transfusion. During exchange transfusion, the infants blood with high bilirubin levels, needs to be replaced by donor blood with low bilirubin levels. However, this procedure carries the risk of severe complications, including sometimes death. Besides the fact that phototherapy cannot completely eliminate the need for exchange transfusion, phototherapy can only decrease already accumulated bilirubin and therefore is not used to prevent its accumulation. Therefore, in **chapter 5**, we describe two therapeutic bile acids that could potentially serve as adjunct or preventative therapy for neonatal hyperbilirubinemia. We tested these two compounds; ursodeoxycholic acid and obeticholic acid as treatment for hyperbilirubinemia in a mouse model for neonatal hyperbilirubinemia in humans. In this mouse model, both compounds significantly decreased bilirubin levels in both blood and brain. Our results suggest that both bile acids partially work via increasing bilirubin detoxification in intestine, by stimulating the bilirubin conjugation enzyme UGT1A1. Hereby, bilirubin can be excreted in the intestine and leave the body faster.

Conjugated bilirubin transporters

Under normal circumstances, unconjugated bilirubin is taken up by the liver and subsequently detoxified (conjugated) by the bilirubin conjugation enzyme UGT1A1. After detoxification, bilirubin is transported into the bile by two transporter proteins; ATP Binding Cassette (ABC)-transporters C2 and C3. **In chapter 6**, we review the role these transporters bilirubin metabolism, cholestasis and drug disposition and their expression regulation.

In the final **chapter 7**, we put our results in clinical and scientific perspective. We discuss the meaning and relevance of our results as well as future research options. In addition, we speculate on a new hypothesis on intestinal bilirubin detoxification.

Taken together, this thesis aimed to evaluate current developments in the management of neonatal hyperbilirubinemia and to explore new treatment possibilities. Hereto, we are using a translational approach in which we aim to translate the results from fundamental experimental (animal) studies to clinically relevant applications for patient care. Thereby, our results could provide a valuable contribution to both biomedical hyperbilirubinemia research and pediatric clinical care for infants with neonatal hyperbilirubinemia.

NEDERLANDSE SAMENVATTING

Neonatale geelzucht is zichtbaar door een gele verkleuring van de huid, slijmvliezen en sclerae en komt veel voor bij pasgeborenen. Het komt vooral vaak voor bij te vroeg of prematuur geboren kinderen; 80% van de prematuren heeft deze aandoening in meer of mindere mate. De gele kleur wordt veroorzaakt door een hoge concentratie bilirubine in het bloed. Neonatale geelzucht wordt daarom ook wel neonatale hyperbilirubinemie genoemd. Bilirubine is een gelig pigment dat ontstaat als afbraakproduct van heem dat zich o.a. in rode bloedcellen bevindt. Bij premature pasgeborenen worden in de eerste levensfase tijdelijk meer rode bloedcellen afgebroken, waardoor uiteraard ook meer bilirubine ontstaat en in het bloed belandt. Bij ernstige hyperbilirubinemie kan bilirubine terecht komen in de hersenen (met name in de zogenaamde basale kernen) en daar schade veroorzaken. Deze schade kan verschillende problemen veroorzaken, die worden beschreven als kernicterus spectrum aandoeningen (KSA). Deze KSA kunnen bestaan uit permanente cerebrale parese (spasticiteit) en/of problemen met het zien en het gehoor. Ook is overlijden beschreven als gevolg van ernstige onbehandelde hyperbilirubinemie. Om deze neurologische schade te voorkomen is het van essentieel belang om neonatale hyperbilirubinemie op tijd te diagnosticeren en te behandelen. Dit proefschrift bevat verschillende studies naar nieuwe behandelopties voor dit ziektebeeld, die zowel in diermodellen als in prematuur geboren kinderen zijn uitgevoerd.

Vrij ongebonden bilirubine

Op dit moment wordt hyperbilirubinemie in de kliniek gediagnosticeerd door het meten van de zogenaamde 'totale' bilirubine concentratie in bloed. KSA wordt echter niet veroorzaakt door het bilirubine in het bloed, maar door het bilirubine dat in de hersenen terechtkomt. We weten uit eerder onderzoek dat er geen lineair verband bestaat tussen de concentratie van bilirubine in de

hersenen en in het bloed. Zo zijn er kinderen met KSA beschreven, die een relatief lage bilirubineconcentratie in het bloed hadden, terwijl er ook gezonde kinderen zijn, die ondanks een ernstige hyperbilirubinemie geen KSA ontwikkelden. Een van de oorzaken van deze discrepantie is de wisselende mate waarin bilirubine zich over de bloedhersenbarrière beweegt en in de hersenen terecht komt. Onder normale omstandigheden is de bilirubineconcentratie in de hersenen minimaal, omdat bilirubine in het bloed is gebonden aan een groot transporteiwit (het albumine). Dit grote bilirubine-eiwitcomplex kan de bloedhersenbarrière niet passeren en hierdoor kan bilirubine dus niet in de hersenen terechtkomen. Onder bepaalde omstandigheden kan er echter toch een kleine hoeveelheid bilirubine in het bloed voorkomen die niet eiwitgebonden is. Dit kan bijvoorbeeld als er te weinig albumine aanwezig is door welke oorzaak dan ook. Dit 'vrije' bilirubine kan wel gemakkelijk door de bloedhersenbarrière en schade veroorzaken. Omdat het niet mogelijk is om bilirubine in de hersenen te meten, kan het 'vrije' bilirubine in het bloed gebruikt worden als een surrogaattest om het risico op neurologische schade door bilirubine te voorspellen. De ratio tussen het 'vrije' bilirubine en het totale bilirubine (dus de som van zowel eiwit-gebonden als vrij bilirubine) in het bloed zou mogelijk een nog betere test zijn, omdat in deze test zowel de neurotoxische fractie van bilirubine, als de totale bilirubinehoeveelheid in het lichaam vertegenwoordigd wordt. Het vrije bilirubine wordt echter op dit moment niet routinematig gemeten in de kliniek en wordt alleen gebruikt in onderzoeksetting. Het postnatale verloop van het vrije bilirubine in neonaten is dan ook nog niet tot in detail bekend. Om hier verandering in te brengen, hebben we in hoofdstuk 2 de concentraties van zowel het vrije bilirubine als de ratio tussen het vrije en het totale bilirubine gemeten in een groep van premature zuigelingen. In dit hoofdstuk beschrijven we het postnatale verloop van het vrije bilirubine en van de ratio tussen het vrije en het totale bilirubine en de risicofactoren die gerelateerd zijn aan elk van

beide. Deze gegevens vormen een eerste stap naar de uiteindelijke toepassing van vrij bilirubine en de vrij bilirubine/totaal bilirubine ratio in de behandelrichtlijnen van neonatale hyperbilirubinemie.

Fototherapie

In de afgelopen 60 jaar heeft de behandeling van neonatale hyperbilirubinemie voornamelijk bestaan uit fototherapie met blauw licht. Doordat het blauwe licht op de huid schijnt, wordt het bilirubine in de onderhuidse bloedvaten omgezet naar een niet-schadelijke isomeer (een stof die erg lijkt op bilirubine). Deze isomeer kan gemakkelijk worden uitgescheiden via de lever in de gal en verlaat het lichaam voornamelijk via de ontlasting. Tot voor kort werd fototherapie gegeven met TL-buizen. Deze TL-buizen hebben als nadeel dat ze naast het blauwe licht ook veel warmte uitstralen. Daarnaast is TL-fototherapie in verband gebracht met schadelijke effecten, waaronder zogenaamde 'oxidatieve stress' en DNA schade en wordt het geassocieerd met het ontstaan van epilepsie en zelfs kanker tijdens het eerste levensjaar. In de afgelopen jaren worden de TL-lampen geleidelijk vervangen door apparaten met LED lampen. Het voordeel van deze lampen is dat ze minder warmte uitstralen en dat ze een veel hogere stralingssterkte kunnen halen. Eerder onderzoek heeft aangetoond dat een hogere stralingssterkte erg effectief is voor de behandeling van neonatale hyperbilirubinemie, maar er bestaan nog steeds onzekerheid over de veiligheid van fototherapie met een heel hoge intensiteit. Mede op grond hiervan is deze therapie nog niet breed geïmplementeerd. In **hoofdstuk 3 en 4** hebben we het effect van LED fototherapie getest op het ontstaan van oxidatieve DNA schade in ratten en in premature kinderen. In **hoofdstuk 3** hebben we het effect van fototherapie getest op DNA oxidatie en DNA schade in ratten met geelzucht. Hierbij hebben we tot hoge stralingssterktes getest en aangetoond dat dergelijk sterke fototherapie geen effect heeft op de markers 8-hydroxy-2'deoxyguanosine (8-OHdG) en gamma-H2AX. Gamma-H2AX ontstaat bij de reparatie van DNA-breuken en is daardoor een marker voor DNA schade in

cellen en weefsels. Deze marker was niet verhoogd in de huid van ratten na blootstelling aan fototherapie. 8-OHdG is een afbraakproduct van DNA dat ontstaat bij DNA oxidatie en wordt vrijwel volledig uitgescheiden in urine. Hierdoor kan het worden gebruikt als niet-invasieve test voor het inschatten van DNA oxidatie. In ratten was deze marker niet verhoogd tijdens of na fototherapie. In **hoofdstuk 4** hebben we 8-OHdG ook gemeten in de urine van premature zuigelingen op de neonatologie intensive care om het effect van LED fototherapie op DNA-oxidatie te bestuderen. In dit hoofdstuk laten we zien dat LED fototherapie tot een stralingssterkte van 35 μW/cm^2/nm niet gepaard gaat met verhoogde 8-OHdG uitscheiding. Deze resultaten suggereren dat in tegenstelling tot TL fototherapie, LED fototherapie geen oxidatieve DNA schade veroorzaakt.

Therapeutische galzouten

Fototherapie is niet altijd effectief genoeg. Als de bilirubineconcentratie in het bloed te hoog blijft ondanks fototherapie, dan is wisseltransfusie de enige andere optie. Hierbij wordt het bloed van de pasgeborene vervangen door donorbloed met een lage bilirubineconcentratie. Hoewel wisseltransfusie erg effectief is, is er bij deze ingreep risico op ernstige complicaties tot aan overlijden toe. Behalve dat fototherapie een wisseltransfusie niet altijd kan voorkomen, wordt het ook niet als preventieve therapie gebruikt. Het kan alleen bilirubine omzetten dat al aanwezig is maar kan bilirubinestapeling niet voorkomen. Om deze reden, hebben we in **hoofdstuk 5** twee middelen getest die als preventieve therapie zouden kunnen dienen voor neonatale hyperbilirubinemie. We hebben deze twee therapeutische galzouten, ursodeoxycholzuur en obeticholzuur, toegepast als therapie in een muismodel voor neonatale geelzucht bij de mens.. In dit muismodel blijken beide galzouten in staat om de bilirubineconcentratie te verlagen in zowel het bloed als in de hersenen. Onze resultaten suggereren dat beide galzouten gedeeltelijk werken via het verhogen van de bilirubine-ontgifting in de darm, door het stimuleren

van het bilirubineconjugatie-enzym UGT1A1, waardoor bilirubine sneller kan worden uitgescheiden.

Geconjugeerd bilirubine transporters

Onder normale omstandigheden wordt ongeconjugeerd bilirubine opgenomen door de lever, waarna het ontgift (geconjugeerd) wordt door het bilirubine conjugatie-enzym UGT1A1. Na de ontgifting wordt bilirubine naar de gal getransporteerd door twee transporteiwitten; ATP Binding Cassette (ABC)-transporters C2 and C3. In **hoofdstuk 6** geven we een overzicht van de rol van deze transporteiwitten in bilirubinemetabolisme, cholestase en farmacologie en hun expressieregulatie.

In het laatste **hoofdstuk 7** plaatsen we de verschillende verkregen resultaten in een klinisch en wetenschappelijk perspectief. Hierin bespreken we de betekenis en het belang van de resultaten en de mogelijkheden voor vervolgonderzoek. Daarnaast introduceren we een nieuwe hypothese over bilirubine-ontgiftiging door de darm.

Dit proefschrift bevat een evaluatie van huidige ontwikkelingen binnen de behandeling van neonatale hyperbilirubinemie en onderzoekt nieuwe behandelmogelijkheden. Hierin wordt gebruik gemaakt van een 'translationele' benadering: resultaten vanuit fundamenteel (proefdier)onderzoek worden zo veel mogelijk vertaald naar wat zij kunnen betekenen voor patiënten. Onze resultaten kunnen bijdragen aan zowel de biomedische wetenschap betreffende hyperbilirubinemie als aan de klinische kindergeneeskundige zorg voor pasgeborenen met neonatale hyperbilirubinemie.

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BIOGRAPHY

Lori Wilhelmina Everhild van der Schoor was born on the 21st of May 1992 in Groningen. She grew up in Vriescheloo and graduated from secondary school *cum laude* at the Dollard College Winschoten. In 2010 she started studying the International Bachelor of Medicine at the University of Groningen. She did her bachelor thesis on the role of Toll Like Receptor 4 in multiple sclerosis at the laboratory of Dr. W. Baron in Groningen. In addition, she successfully completed the Bachelor Honours Program of the University of Groningen. After her bachelor, Lori started the Master of Medicine in Groningen in 2013. She performed her master thesis in Pediatrics on the transcriptional regulation of bilirubin conjugation enzyme UGT1A1 in the laboratory of Prof. Henkjan Verkade and Prof. Hans Jonker. After finishing her thesis, she applied for the Junior Scientific Masterclass MD/PhD scholarship, which was awarded to her in 2014. After one year of clinical internships in the Martini Hospital Groningen, she started her 2-year research period in the Pediatrics department under supervision of Henkjan Verkade and Hans Jonker and the Neonatology department under supervision of Dr. Christian Hulzebos. This period was extended by a 6-months stay in the laboratory of Prof. Robert Tukey at the University of California San Diego. Afterwards, she did her senior clerkships in the Röpcke Zweers Hospital in Hardenberg, followed by an 8-week internship at the Pediatrics department at the Diakonessenhuis in Paramaribo, Surinam. Lori did her final internship in Pediatrics at the University Medical Center Groningen and the Wilhelmina Children's Hospital in Utrecht and will graduate from her Masters in November 2019.

During her PhD, Lori took part in the organization of the European MD/PhD Conference. Furthermore, she got awarded the KNAW Ter Meulen Grant, the Young Investigators Grant of ESPGHAN, a grant from the Dr. J.C. Vaillantfonds and from 'Stichting Vrienden Beatrix Kinderziekenhuis'.

BIOGRAFIE

Lori Wilhelmina Everhild van der Schoor werd geboren op 21 mei 1992 in Groningen. Ze groeide op in Vriescheloo en volgde haar middelbare school bij het Dollard College te Winschoten, waar zij het Gymnasium *cum laude* afsloot. In 2010 startte zij de International Bachelor of Medicine aan de Rijksuniversiteit Groningen. Zij deed haar bachelorthese in het laboratorium van Dr. Wia Baron in Groningen, over de rol van Toll Like Receptor 4 in multipele sclerose. Hiernaast voltooide zij tijdens haar Bachelor het Honours programma van de Rijksuniversiteit Groningen. Na haar bachelor startte Lori aan de Master Geneeskunde in Groningen in 2013. Zij deed haar master thesis in de Kindergeneeskunde over de transcriptionele regulatie van bilirubine conjugatie enzym UGT1A1 onder begeleiding van Prof. Henkjan Verkade en Prof. Hans Jonker. Na het afmaken van haar thesis, solliciteerde zij voor de Junior Scientifc Masterclass MD/PhD beurs, welke zij kreeg toegekend in 2014. Na een jaar junior co-schappen te hebben gedaan in het Martini Ziekenhuis in Groningen, startte zij haar 2-jarige onderzoeksperiode bij de afdeling Kindergeneeskunde onder supervisie van Henkjan Verkade en Hans Jonker en bij de afdeling Neonatologie onder supervisie van Dr. Christian Hulzebos. Deze periode werd verlengd met een 6-maanden lang verblijf in het laboratorium van Prof. Robert Tukey aan de University of California San Diego. Hierna deed Lori haar senior-coschappen in het Röpcke Zweers Ziekenhuis in Hardenberg, gevolgd door een 8-weken lange stage op de Kinderafdeling van het Diakonessenhuis in Paramaribo, Suriname. Lori deed haar semi-artsstage op de afdeling Kindergeneeskunde van het Universitair Medisch Centrum Groningen en het Wilhelmina Kinderziekenhuis in Utrecht en zal haar Master afronden in november 2019.

Tijdens haar PhD maakte Lori deel uit van de organisatie van de European MD/PhD Conference. Daarnaast kreeg zij de KNAW Ter Meulen Grant

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