Advanced treatment of severe hyperbilirubinemia and cholestasis

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REMCO VAN DIJK

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Introduction – Aim of this thesis
The discovery of bilirubin and bilirubin metabolism

'Nanos, gigantium humeris insidentes' (dwarfs, standing on the shoulders of giants) was conceptualised by the 12th century French philosopher Bernard of Chatres to point out that "we see more and farther than our predecessors, not because we have keener vision or greater height, but because we are lifted up and borne aloft on their gigantic stature". This introductory chapter will therefore be used to give a brief scientific summary of the most important descriptions and discoveries in the field of bilirubin research trough out the centuries. The second part of this introduction will be used to set out the aims of this thesis.

Since the ancient times of the Greeks both liver and bile have played a central role in medicine and the understanding of physiology and anatomy of the human body. It was Hippocrates (c. 460 – c. 370 BC), known as the father of medicine, who first mentioned the important role bile played in the homoeostasis of both body and mind. In his book *On the nature of man* (*De Natura Hominis*), he hypothesised that the body contains four basic substances (humours); bloods, phlegm and black and yellow bile. It was thought that the right balance of the four humours would result in a healthy state of body and mind. The Greek-Roman philosopher Galenus (September 129 AD – c. 200/c. 216), a firm believer of the humourism theory, placed the liver as one of the three principle organs next to the heart and the brain. Although he wrongly stated that the liver was the source of blood, the gallbladder for black bile and the spleen for yellow bile synthesis, his theory remained widely accepted until the late 17th century.

In 1659 the English physician Francis Glisson (1597 – 1677) wrote the first medical book exclusively devoted to the liver and its anatomy *Anatomia hepatis* which was published in Amsterdam. He was the first to accurately describe the bile ducts, the hepatic blood supply and the sphincter of the bile duct and, not perturbed by modesty, named it the *Glisson's sphincter*. The 1650's were exciting times for liver research and in 1651 the Danish anatomist Thomas Bartholin discovered that the liver did not produce blood but instead was the organ responsible for the formation of bile. In his book *Historiarum anatomicarum rariorum* published in 1651 in The Hague he declared the end of the liver as the 'ruler of the abdomen'. Due to its explicit appearance, jaundice (yellow decolourisation of the skin and sclerae) has been widely renowned for many centuries as a symptom of

illness. The relationship between the jaundiced patient and a troubled liver had already been described by Hippocrates. However, it took until the 19th century before the first experiments revealed the chemical structure and the source of the molecule responsible for jaundice. Rudolph Virchow was the first in 1849 to isolate bilirubin from old hematomas[1] and although he did not have conclusive evidence, he correctly hypothesised that bilirubin was formed from a compound in the blood. In 1864, Georg Städeler was able to purify the yellow pigment from bile and called it *bilirubin* (from *bilis*, bile and *rubidus*, dark red)[2]. It took almost a century, but in 1937 Fischer and Orth managed to determine the structure of bilirubin, a tetrapyrrole closely related to haemoglobin[3, 4]. The group of Irving London provided the final evidence in 1950 that bilirubin indeed is derived from a compound in blood known as haem[5].

Although the structure of bilirubin had not yet been discovered in 1883, Paul Ehrlich was able to determine bilirubin concentrations in serum by coupling bilirubin to a diazotized sulfanilic acid-diazo reagent[6]. The Dutch physicians Abraham Albert Hijmans van den Bergh and Peter Müller optimised this method and by adding a catalysator, alcohol, they demonstrated in 1916 that there are actually two types of bilirubin in serum, 'indirect bilirubin' which reacted only with a catalysator and 'direct bilirubin' reacting directly with the diazo reagent without the need of a catalysator. They also showed that human serum contains both forms of bilirubin, in contrary to bile were only the direct bilirubin is present[7]. In the 1950's it was demonstrated by three independent groups that direct bilirubin could be converted into indirect bilirubin by adding β-glucuronidase. Hereby they could show that direct bilirubin is conjugated with one or two glucuronide groups, making it a water soluble molecule[8-10]. A third form of bilirubin, a bilirubin covalently bound to proteins was described in the early 1980s in patients with severe and longstanding jaundice[11].

In 1934, the Canadian animal geneticist Charles Kenneth Gunn found three young Wistar rats, in a total nest of thirteen rats, to be icteric. He also noticed that their serum bilirubin levels only consisted of the indirect (unconjugated) form and that no bilirubin was found in the bile of these rats. Via cross-breeding experiments, resulting in 226 rats coming from 26 litters, he discovered that this was due to an autosomal recessive mutation[12]. After the first description of the 'Gunn rat', this particular rat was not subject to further study for almost two decades. Eventually in the late 1950's scientific interest in bilirubin research picked up again and

fortunately enough one laboratory in Boston was still breeding a small colony of the Gunn rat. Ever since, the Gunn rat has played a major role in bilirubin research all over the world, contributing to hundreds of research papers.

Lathe and Walker showed in 1957 that both human newborns and Gunn rats are deficient in glucuronyl transferase, the hepatic enzyme responsible for the formation of 'direct bilirubin', the glucuronide conjugate form of bilirubin[13]. It took until 1986 when the group of Roy Chowdhury was able to isolate the hepatic enzyme responsible for bilirubin conjugation, UDP-glucuronate glucuronosyltransferase, from normal Wistar rats[14]. Eventually in the 1990's the exact isotype responsible for bilirubin glucuronidation in man, uridine diphospho-glucuronosyl transferase 1A1 (UGT1A1), was cloned for the first time[15]. Crigler-Najjar syndrome presents, just like the Gunn rat, with a non-hemolytic, unconjugated hyperbilirubinemia from the onset of birth[16]. If left untreated Crigler-Najjar syndrome can cause brain damage (kernicterus) and can even be lethal. For long the cause of Crigler-Najjar syndrome has been enigmatic but the discovery of UGT1A1 finally demonstrated that Crigler-Najjar syndrome is due to a lack of activity of this enzyme to a deficient UGT1A1 gene[17]. Crigler-Najjar syndrome and the role of UGT1A1 will be described in further detail in chapter 1 of this thesis.

By showing that bilirubin is derived from haem, that it has an unconjugated and conjugated form and that a hepatic enzyme is responsible for bilirubin glucuronidation a great deal of the bilirubin metabolism had been unravelled in the 1950's. How many steps and by which enzymes haem is converted into bilirubin was still to be solved. The first breakthrough came in 1968 when the group of Rudi Schmid showed that the enzyme haem oxygenase is responsible for the first step in bilirubin metabolism by converting haem into the green hydrophilic biliverdin[18].

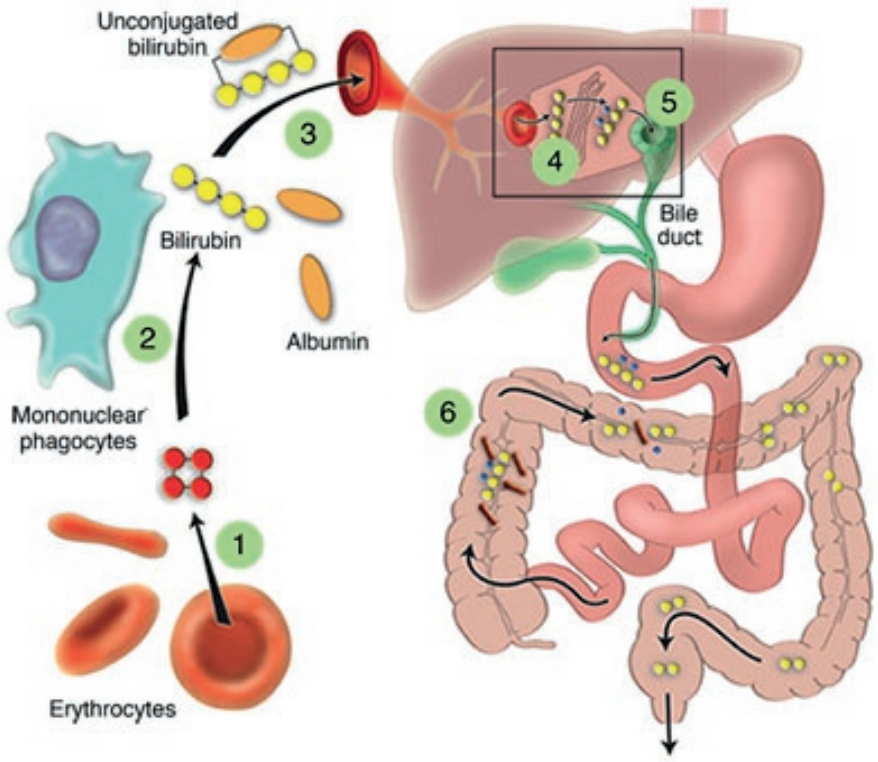


Figure 1. Bilirubine metabolism

1. *Haem, the substrate of bilirubin, is derived from senescent red blood cells.*
2. *Haem is degraded to biliverdin by haem oxygenase in the mononuclear phagocytes*
3. *Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. Circulating bilirubin (insoluble) is bound to albumin and subsequently taken up by the hepatocytes.*
4. *To make it soluble, bilirubin undergoes conjugation, a reaction catalyzed by UGT1A1*
5. *Conjugated bilirubin (soluble) is excreted into bile and reaches the bowel.*
6. *Bilirubin glucuronides are deconjugated by colonic bacteria and eliminated in the faeces*

Two years later the same group also showed that the second step consists of the conversion of biliverdin into unconjugated bilirubin by the enzyme biliverdin reductase[19]. Together with the discovery here in Amsterdam in the 1990's and 2000's of the transporter-proteins responsible for biliary secretion (MRP2) and hepatic basolateral uptake (OATP1B1 and OATP 1B3) of conjugated bilirubin the whole pathway of bilirubin metabolism and secretion is known[20-22] (figure 1).

Kernicterus

The icteric patient can be of any age, but historically the earliest detailed and most scientific observations were done in the yellow newborn infant. In his book *Traite de L'amaigrissement des enfants (*1806*)* the French pathologist Jean Baptiste Baumes devoted a specific chapter to the icteric paediatric patient, *'Traite de L'ictere ou jaunisse des enfants de naissance'*. Not only did Jean Baptiste Baumes refer to the liver in his chapter, he also describes somnolence, poor feeding and symptoms of cerebral involvement in some of the icteric newborns. He is therefore possibly the first person to describe the toxic effects of high levels of free unconjugated bilirubin on the brain, currently referred to as bilirubin encephalopathy or kernicterus.

In 1875 Johannes Orth, an assistant in Rudolph Virchow's lab found yellow pigmentation in the brain of a severely icteric neonatal patient who had died 2 days after birth. On closer examination he saw that the basal ganglia, cerebellum and hippocampus showed the highest intensity of yellow pigmentation. Under microscopic dissection it was noticed that only the nuclei of the neurons in the basal ganglia contained the yellow pigment[23]. In 1904 Christian Schmorl studied the brains of 120 icteric infants who had died shortly after birth. He noticed that the brains were diffuse icteric in 114 of the patients, however in 6 patients the yellow pigmentation was only seen in the basal ganglia and medulla oblongata. Just like Johannes Orth he found that the pigmentation was specifically present in the nuclei of the cells, and therefore named the condition 'kernicterus'[24]. Ten years later, in 1914, Guthrie was the first to suggest the direct link between neurological symptoms and hyperbilirubinemia in a 19 months old baby which had suffered from hypotonia and chorioatetosis after an episode of jaundice caused by erythroblastosis fetalis at birth. We now know that unconjugated bilirubin can damage brain tissue cells via necrosis and apoptosis, in an anatomical distribution dependent on the amount, duration, and the developmental timing of exposure of brain tissue to free unconjugated bilirubin[25].

Therapy in the icteric patient

The most urgent clinical question involving bilirubin has for long been the search for an effective and safe treatment of severe neonatal jaundice (*icterus gravis neonatorum*), often caused by erythroblastosis and very rarely by Crigler-Najjar syndrome. Although the symptoms and clinical presentation of erythroblastosis fetalis have been well described in the early 20th century[26], it took until the 1940's

and 1950's to reveal the cause of this hemolytic disease[27, 28] with the discovery of Rhesus antibodies and the different blood groups. Now the cause was known for the high bilirubin production in erythroblastosis fetalis, an effective therapy could be developed. After initially unsuccessful attempts by others, Harry Wallerstein was the first to correct anaemia and to reduce bilirubin levels in three infants with erythroblastosis fetalis by using exchange transfusion in 1946[29]. With the introduction of umbilical vein intubation exchange transfusion became the established procedure for the treatment of severe neonatal jaundice in the 1950's[30].

Arguably one of the most important observations for the development of the most effective and safest therapy for icterus neonatalis was made by Sister Jean Ward. She was an experienced nurse on the neonatal department at Rochford General Hospital in Essex, England. During sunny days in the summer sister Ward would take the infants outside to the hospital courtyard. During these nice little breaks she noticed that jaundiced babies became less yellow while lying in the sun, she also saw that parts of the skin which were not exposed to the sun remained yellow in these babies. Around the same time hospital biochemists noticed that serum bilirubin levels would decrease over time when blood samples were exposed to sunlight. Richard Cremer, a specialist registrar at Rochford General Hospital and friend of Sister Ward, heard about these observations and set up the first trial of phototherapy for infants with jaundice neonatalis[31].

Although Richard Cremer's trial clearly showed that phototherapy decreased serum bilirubin very effectively there was great resistance towards the implication of phototherapy for icterus neonatalis. The main concern at the time was that the degradation products caused by phototherapy could be as toxic as bilirubin itself, as well as the fear for possible malignant side effects of phototherapy. This led many hospitals to continue with exchange transfusions far into the 1960's, although the procedure was technically difficult and fraught with the possibility of a fatal electrolyte or fluid imbalance. Only after the landmark publication of Lucey's and colleagues randomised controlled trial in 1968[32], confirming safety and effectiveness of this method, phototherapy has been the first choice in the treatment of icterus neonatalis including lifelong therapy for Crigler-Najjar syndrome.

The impressive work by Tony McDonagh in California in the 1970-80's finally

revealed the mechanism behind phototherapy. He discarded the then popular hypothesis that the effect of phototherapy was caused by bilirubin photo-oxidation which is a slow and inefficient process. With his experiments also involving the Gunn rat McDonagh showed that phototherapy caused to change the shape, but not the constitution, of bilirubin by breaking the internal hydrogen bonds. These so-called bilirubin photo-isomers can be easily excreted in urine and bile with the use of transporters such as MRP2[33]. Further experiments showed that blue light with a wave length around 460-490nm, resulting in maximal absorption of bilirubin, is the most effective source for phototherapy[34].

Although phototherapy is very effective in temporary conditions of unconjugated hyperbilirubinemia such as in erythroblastosis fetalis, it becomes less effective over time and has a great negative impact on the quality of life in patients with Crigler-Najjar syndrome, who would need life-long phototherapy. With the introduction of liver transplantation in the 1960's by the pioneer surgeon Thomas Starzl in Chicago and Boston[35], the first curative option for Crigler-Najjar syndrome had become available. UGT1A1 function is fully restored by orthotopic liver transplantation, however it has been shown that partial recovery of the UGT1A1 activity via an auxiliary liver transplantation is sufficient enough to cure Crigler-Najjar syndrome[36, 37]. However liver transplantation has currently gross limitations, such as the lack of sufficient liver donors, the significant rate of morbidity and mortality after liver transplantation, and the risk of severe side effects with the use of lifelong immunosuppressive drugs, such as corticosteroids (metabolic derangements, infections and delirium), calcineurin inhibitors (acute kidney injury, neurotoxicity, hyperglycemia and the risk for malignancies such as post-transplant lymphoproliferative disorder) and mycophenolate mofetil (nausea, vomiting, diarrhea and bone marrow depression). Therefore, the search for new therapies for Crigler-Najjar syndrome continues and this will be the focus of the first part of this thesis.

Aims of this thesis

This thesis is divided in two parts: the first part will start with an overview of hereditary causes of hyperbilirubinemia, both conjugated and unconjugated, in **chapter 1**. It will cover the genetic and molecular background as well as the possible treatments and clinical implications of these diseases. This chapter will

focus especially on the Crigler-Najjar syndrome and it will scope out potential new curative treatments using viral gene therapy.

Chapter 2 will discuss the optimisation of adeno-associated virus (AAV) vector mediated liver gene transfer to treat Crigler-Najjar syndrome. Multiple optimised vectors expressing the human UGT1A1 transgene were developed and tested *in vitro* and *in vivo*. In doing so we reveal that transgene codon-optimization is a strategy that can improve efficacy of gene transfer. It also demonstrates that a translationally optimised AAV vector expressing the UGT1A1 transgene rescues the phenotype of Crigler-Najjar syndrome in two different animal models of the disease.

Chapter 3 will describe the role of the innate immune system in viral gene therapy preventing AAV transduction of the liver. We will show that the scavenger-receptor, which is present on the surface of macrophages and Kupffer cells, is involved in the endocytosis of AAV *in vitro* and blocking of this receptor *in vivo* resulted in increased efficacy of AAV viral gene therapy in female Gunn rats.

Chapter 4 looks into a new target for the treatment of Crigler-Najjar syndrome by inhibiting the enzyme responsible for the second step in bilirubin metabolism, biliverdin reductase. First, we'll show that via a semi high-throughput drug screen we found 26 chemicals which inhibit biliverdin reductase *in vitro*. Two of the drugs, Disulfiram and Montelukast, were further investigated and tested in the Gunn rat, the animal model for Crigler-Najjar disease.

A patient with a Crigler-Najjar syndrome type 2 phenotype but without a mutation in the UGT1A1 coding region will be the subject of discussion in **chapter 5**. Sequence analysis of the UGT1A1 promoter revealed a mutation which results in inhibition of more than 95% of the transcriptional activity of the UGT1A1 gene promoter, explaining the lack of UGT1A1 activity in this patient. We will also demonstrate that the mutation in the promoter also explains the lack of response to phenobarbital therapy, the standard treatment of Crigler-Najjar syndrome type 2.

The second part of the thesis is dedicated to the treatment of severe conjugated hyperbilirubinemia (cholestasis) and cholestatic pruritus. **Chapter 6** will serve as the introduction to the second part of the thesis. There we will discuss the

important role of the enterohepatic circulation on bile acid homeostasis and the effect of cholestasis on the liver. Possible causes and treatment for cholestasis-associated pruritus, a frequently reported symptom, will be discussed in this chapter.

Chapter 7 focuses on 13 consecutive patients with deep jaundice due to severe persistent hepatocellular secretory failure (PHSF). All patients were successfully treated with rifampicin, the most potent pregnane X receptor (PXR) agonist and improved dramatically without any side effects. Treatment with rifampicin resulted in significant decrease and normalisation of serum bilirubin levels. *In vitro* studies revealed that besides the known target genes (CYP3A4, UGT1A1 and MRP2), rifampicin also induces the expression of the basolateral bile acid export pump OSTβ in a PXR dependent manner. In three patients, a mutation in one allele of one of the PFIC (progressive familial intrahepatic cholestasis) genes ATP8B1 or *ABCC11*/BSEP was identified.

Chapter 8 will highlight that increased serum autotaxin (ATX) activity is relatively specific for pruritus of cholestasis. Serum autotaxin activity correlated to effectiveness of therapeutic interventions including rifampicin and nasobiliary drainage. It could be shown that the beneficial antipruritic action of rifampicin may be explained, at least partly, by PXR-dependent transcriptional inhibition of autotaxin expression.

Finally, **chapter 9** provides an overview of all the results obtained in the research presented in this thesis. We look into the future perspective of the use of AAV viral in gene therapy in the clinic and the 'new' role of rifampicin in the treatment of cholestatic conditions such as PHSF and cholestatic pruritus.

References

1. Virchow R. *Die Pathologischen Pigmente*. (1847) Arch. Pathol. Anat. 1:379
2. Städeler G. (1864) *Über die Farbstoffe de Galle.* Annalen d. Chemie u. Pharmacie. 132;323-354
3. Fischer H., Orth H. (1937) *Die Chemie des Pyrrols*. Akademische Verlagsgesellschaft m.b.H., Leipzig
4. Fischer H., Pleininger H.,und Weissbarth O. (1941). *Über die Konstitution des Bilirubins und über die Bilirubinoide Farbstroffe.* Hoppe-Seylers Zeitschrift f. Phys Chem. 268;197-226
5. London I. M., West R., Shemin D. et al. (1950) *On the origin of bile pigment in normal man.* J. Biol. Chem. 184:351,
6. Ehrlich P. (1883) *Sulfodiazobenzol, ein Reagens auf Bilirubin.* Centralblatt für klinische Medicin. 45;721-723
7. Hijmans van den Bergh A. A., und Müller P. (1916*) Über eine directe und eine indirecte Diazoreaction auf Bilirubin.* Biochemische Zeitschrift. 77:90-103
8. Billing B. H., Cole P. G., Lathe G. H. (1957) *The excretion of bilirubin as a diglucuronide giving the direct van den Bergh reaction.* Biochem. J. 65:774
9. Schmid R. (1956) *Direct-reacting bilirubin, bilirubin glucuronide, in serum, bile, and urine.* Science. 124;76-77
10. Talafant E. (1956) *Properties and composition of the bile pigments giving a direct diazo reaction.* Nature 178:312
11. Weiss JS, Gautam A, Lauff JJ, Sundberg MW, Jatlow P, Boyer JL, Seligson D. *The clinical importance of a protein-bound fraction of serum bilirubin in patients with hyperbilirubinemia.* N Engl J Med. 1983 Jul 21;309(3):147-50.
12. Gunn C.K. (1938) *Hereditary acholuric jaundice in a new mutant strain of rats.* J. Heredit. 29; 137-139
13. Lathe G. H., Walker M. (1957) *An enzyme defect in human neonatal jaundice and in Gunn's strain of jaundiced rats*. Biochem. J. 67:9P
14. Roy Chowdhury, J., Chowdhury, N., Falany C.N., et al (1986) *Isolation and characterisation of multiple forms of rat liver UDP-glucuronate glucuronosyltransferase. Biochem. J. 233;827-837*
15. Ritter J.K., Crawford J.M., Owens I.S., (1991) *Cloning of two human liver bilirubin UDP-glucuronosyltransferase cDNAs with expression in COS-1 cells.* J Biol Chem 266:1043–104
16. Crigler J.F. Jr, Najjar V.A., (1952) Congenital familial nonhemolytic jaundice with kernicterus. Pediatrics 10:169–180
17. Seppen J, Bosma P.J. Goldhoorn B.G. et al. (1994) *Discrimination between Crigler-Najjar type I and II by expression of mutant bilirubin uridine diphosphate-glucuronosyltransferase. J. Clin. Invest. 94;2385-2391*
18. Tenhunen R., Marver H.S., Schmid R. (1968) *The enzymatic conversion of heme to bilirubin bymicrosomal heme oxygenase.* Proc Natl Acad Sci U S A 61:748–755
19. Tenhunen R, Ross ME, Marver HS, Schmid R (1970) *Reduced nicotinamide-adenine dinucleotide phosphate dependent Biliverdin reductase: partial purification and characterization.* Biochemistry 9: 298–303, 20-1
20. Jansen P.L.M, Peters W.H.M., and Lamers W.H., (1985) *Hereditary chronic conjugated*

hyperbilirubinemia in mutant rats caused by defective organic anion transport. Hepatology 5;573-579

21. Paulusma CC, Bosma PJ, Zaman GJ, et al., (1996) *Congenital jaundice in rats with a mutation in a multidrug resistance associated protein gene.* Science 271:1126–1128
22. van de Steeg E, Stranecky V, Hartmannova H,et al. (2012) *Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver.* J Clin Invest 122:519–528
23. Orth J. (1875) *Uber das Vorkommen von Bilirubinkrystallen bei neugebornen Kindern.* Arch. Path. Anat. Phys. u. f. Klin. Med. (Virchows Arch.) 63:477
24. Schmorl C.G., (1904) *Zur Kenntnis des Ikterus neonatorum, insbesondere der dabei auftretenden Gehirnveranderungen.* Vehandl. d. Deut. Path. Gesell. 6:109
25. Shapiro S.M., (2005) *Definition of the Clinical Spectrum of Kernicterus and Bilirubin-Induced Neurologic Dysfunction (BIND).* Journal of Perinatology 25:54–59
26. Diamond L.K., Blackfan K.D., Baty J.M. (1932) *Erythroblastosis fetalis and its association with universal edema of fetus, icterus gravis neonatorum andanemia of the newborn.* J. Pediatr. 1:269
27. Landsteiner K., Wiener A.J., (1940) *An agglutinable factor in human blood recognized by immune sera for rhesus blood.* Proc. Soc. Exp. Biol. & Med. 43:223
28. Halbrecht L (1944) *Role of hemagglutinins anti-A and anti-B in pathogenesis of jaundice of newborn (icterus neonatorum precox)*. Amer. J. Dis. Child. 68:248
29. Wallerstein H. (1946) *Treatment of severe erythroblastosis fetalis by simultaneous removal and replacement of the blood of the newborn infant.* Science 103:583
30. Diamond L. K. (1947) *Erythroblastosis fetalis or hemolytic disease of the newborn.* Proc. Roy. Soc: Med. 40: 546
31. Cremer RJ, Perryman PW, Richards DH (1958). *Influence of light on the hyperbilirubinaemia of infants.* The Lancet 271 (7030): 1094–1097
32. Lucey J, Ferriero M, Hewitt J., (1968) *Prevention of hyperbilirubinemia of prematurity by phototherapy.* Pediatrics 41(6):1047–1054
33. McDonagh AF, Palma LA, Lightner DA. (1980) *Blue light and bilirubin excretion.* Science; 145:208
34. Maisels M.J. and Mc Donagh A.F., (2008) *Phototherapy for neonatal jaundice.* N. Eng. J. Med. 358:920-928
35. Starzl T.E., Groth C.G., Brettschneider L. et al. (1968) *Orthotopic homotransplantation of the human liver.* Ann. Surg. 168(3):392-415
36. Kaufman S.S., Wood R.P., Shaw Jr B.W. et al (1986) *Orthotopic liver transplantatrion for type I Crigler-Najjar syndrome.* Hepatology Vol 6: 1259-1262
37. Whittington P.F., Edmond L.C., Heffron T.G., THistelwaite J.R., (1993) *Orthotopic auxiliary liver transplantation for Crigler-Najjar syndrome type I.* Lancet 342;779-780

PART I

1

GENE ADDITION THERAPY FOR GENETIC HEPATOCELLULAR JAUNDICE

Modified from van Dijk R, Beuers U, Bosma PJ.

Clinical Review Allergy Immunology (2015) 48:243-53

Abstract

Jaundice results from the systemic accumulation of the red-yellow coloured bilirubin, the end product of the catabolism of haem. In addition to liver failure, due to viral infection, drug and/or alcohol abuse and bile duct obstruction, there are several inherited liver disorders that result in impaired bilirubin metabolism causing jaundice. In patients with Rotor syndrome bilirubin (re)uptake by the liver is impaired due to the deficiency of two basolateral/sinusoidal hepatocellular membrane proteins, organic anion-transporting polypeptide 1B1 (OATP1B1) and OATP1B3. Dubin-Johnson syndrome is caused by a defect in the ATP-dependent canalicular transporter, multidrug resistance-associated protein 2 (*ABCC2*/MRP2), which mediates the export of conjugated bilirubin into bile. Both disorders are benign, are not progressive and are characterised by elevated serum levels of conjugated bilirubin, a non-toxic metabolite. In contrast to its conjugates, unconjugated bilirubin is a neuro-toxic compound. Bilirubin glucuronidation is catalysed by uridine diphospho-glucuronosyl transferase 1A1 (UGT1A1) and deficiency of this enzyme results in unconjugated hyperbilirubinemia. Partial deficiency, as seen in Gilbert syndrome results in mildly increased serum levels of unconjugated bilirubin (UCB) that do not reach toxic levels. This common benign form of inherited unconjugated hyperbilirubinemia is mostly caused by reduced transcriptional activity due to polymorphisms in the promoter of the UGT1A1 gene. Complete and almost complete deficiency of this enzyme does result in a rare but very severe form of inherited unconjugated hyperbilirubinemia, Crigler-Najjar syndrome. In these patients unconjugated bilirubin accumulates to serum levels that when left untreated can cause irreversible and even lethal brain damage. Because of the shortcomings of the current standard treatments for Crigler-Najjar syndrome, phototherapy and liver transplantation, novel effective therapeutic strategies such as gene therapy are needed. In this chapter, the clinical features, pathophysiology and genetic background of inherited disorders of bilirubin metabolism are reviewed. In addition, the upcoming novel treatment options such as viral vector mediated gene therapy for these genetic disorders and potential immunological responses to this therapy will be discussed.

Introduction

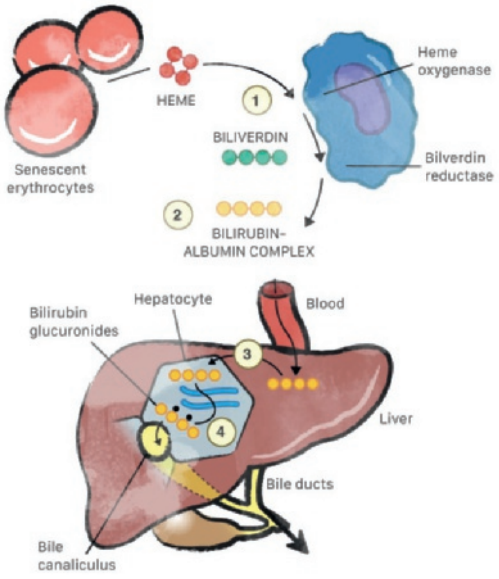
Jaundice is the key clinical feature in patients with hyperbilirubinemia and results from the accumulation of bilirubin in the systemic circulation, skin and sclera. Bilirubin is formed from the catabolism of haem[1]. Haemoglobin from erythrocytes forms the major source of haem[2]. Haem is degraded in the reticuloendothelial system by the microsomal enzyme haem oxygenase, which results in the formation of the green hydrophilic pigment biliverdin, carbon monoxide and free iron[3, 4]. Biliverdin reductase subsequently catalyses the formation of the red-yellow pigment bilirubin IXα (also known as unconjugated bilirubin)[5]. Unconjugated bilirubin (UCB) is bound to albumin in blood and transported to the liver where it dissociates from albumin and is taken up by the hepatocytes[6, 7]. In the liver, uridine diphospho-glucuronosyl transferase 1A1 (UGT1A1) catalyses the glucuronidation of bilirubin into mono- and diglucuronidated forms (figure 1) that are hydrophilic and are actively transported into bile by multidrug resistance-associated protein 2 (*ABCC2*/MRP2)[8].

Two forms of hereditary hyperbilirubinemia can be distinguished in the clinic, a form characterised by the accumulation of unconjugated hyperbilirubinemia and a mixed form accumulating predominantly conjugated bilirubin. Both impaired hepatocellular bilirubin (re)uptake and biliary secretion result in the accumulation of conjugated bilirubin, a nontoxic metabolite. In contrast, deficient conjugation causes unconjugated hyperbilirubinemia that due to its toxicity may cause brain damage and does require treatment including a liver transplantation. This review discusses all inherited forms of hyperbilirubinemia, the promising novel treatment strategies and their potential immunological risks.

Inherited Conjugated Hyperbilirubinemia: Dubin-Johnson Syndrome and Rotor Syndrome

Dubin-Johnson syndrome is an autosomal recessive liver disorder characterised by non-haemolytic chronic, predominantly conjugated, hyperbilirubinemia in the absence of biliary obstruction[9, 10]. Histomorphological findings show dense pigment formation in

A



B

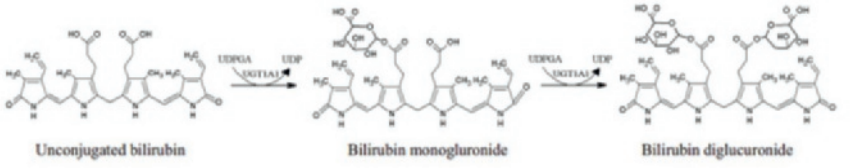


Figure 1 A. Haem catabolism and bilirubin conjugation. (1) Haem, mostly derived from old erythrocytes, is converted to biliverdin by Haem oxygenase by macrophages at the reticulo-endothelial system. (2) Biliverdin is reduced to unconjugated bilirubin by biliverdin reductase, also in the reticulo-endothelial system. (3) Unconjugated bilirubin bound to albumin in blood is transported to liver where it is taken up by hepatocytes. (4) In the hepatocyte bilirubin is conjugated with one or two glucuronides groups by the endoplasmic reticulo protein UGT1A1. Conjugated bilirubin is secreted over the apical membrane in bile by MRP2. **B**. Glucuronide conjugation of bilirubin by UDP-glucuronosyltransferase 1A1 (UGT1A1).

hepatocytes[11]. Serum bilirubin levels range between 10 and 100 µmol/L but can be higher; approximately 50 % of the serum bilirubin is conjugated. In these patients, total urinary excretion of coproporphyrin is normal, but consists of 80 % coproporphyrin I, while in healthy controls 75 % is coproporphyrin III[12, 13]. The syndrome was described in 1954, and in 1996 it was revealed that mutations in the ATP-dependent canalicular transporter ABCC2/MRP2 cause Dubin-Johnson syndrome[14, 15]. ABCC2/MRP2 is a membrane transport protein present in multiple tissues such as kidney, intestine, placenta and blood-brain barrier but is most abundantly expressed in the canalicular membrane of hepatocytes[16, 17, 18]. It mediates transport of amphipathic uncharged compounds and glutathione-, glucuronate- or sulfate-conjugated organic anions[19, 20]. Dysfunction of MRP2 results in an impaired secretion of bilirubin glucuronides into bile leading to their systemic accumulation. Multiple mutations in the MRP2 gene causing Dubin-Johnson syndrome have been reported varying from missense mutations, resulting in amino acid substitutions, to mutations resulting in exon skipping and/or those resulting in a premature stop generating a truncated protein.

Rotor syndrome (RS), first reported in 1948, also presents with increased serum conjugated bilirubin without abnormal haematological and biochemistry blood tests[21]. It can be distinguished from Dubin-Johnson syndrome by its normal liver histology, up to five times increased urinary coproporphyrin excretion, delayed clearance and absent conjugation of anionic diagnostics (e.g. bromsulphthalein)[22, 23]. Only very recently, the aetiology of RS has been clarified. It is caused by the deficiency of two organic anion transporters localised in the basolateral-sinusoidal membrane of hepatocytes, organic anion-transporting polypeptide 1B1 (OATP1B1) and OATP1B3[24]. These proteins mediate the (re)uptake of conjugated endo- and exogenous compounds[25]. The unravelling of these two very rare gene disorders also revealed that not all glucuronidated bilirubin is secreted into bile via MRP2 but that a substantial part is secreted into the circulation by the hepatic efflux pump MRP3[26]. OATP1B1 and OATP1B3 present on downstream-located hepatocytes mediate its reuptake. The authors hypothesise that this so-called hepatocyte hopping of conjugated bilirubin, and maybe other substrates, prevents saturation of the detoxification process in upstream hepatocytes.

Due to the benign course of both disorders, treatment is not indicated for Dubin-Johnson and Rotor syndrome.

Inherited Unconjugated Hyperbilirubinemia: Crigler-Najjar Syndrome and Gilbert Syndrome

Crigler-Najjar syndrome (CNS) is an autosomal recessive disorder characterised by severe unconjugated hyperbilirubinemia caused by a deficiency of bilirubin glucuronidation. It was first described as a lethal disorder in six patients in 1952[27]. In 1969, Arias found that there are two types of CN syndromes. In the most severe form, CNS type I, hepatic bilirubin glucuronidation is completely lacking and virtually no glucuronidated bilirubin is detected in bile[28]. Patients suffering from the less severe form, CNS type II, do have traces of hepatic bilirubin glucuronidation resulting in a small percentage of glucuronidated bilirubin in bile, mostly mono-glucuronide. These patients respond to phenobarbital therapy[29]. Phenobarbital reduces serum bilirubin levels by >30 % in CNS type II patients, while in type I patients little or no effect is observed. Phenobarbital is therefore a useful tool to discriminate CNS type I from type II in the clinic.

In the early 1990s, the UGT1A1 gene was cloned, and in 2001, the entire structure of the UGT1A locus was reported[30, 31]. The UGT1A gene contains 17 exons of which 13 specific aminoterminal exons encode the substrate-binding domain and four common carboxyterminal exons encode for the UDP-glucuronic acid binding site and membrane binding[32]. Ten messenger RNAs (mRNAs) are generated from the UGT1A gene, all containing one of the specific aminoterminal exons and four common carboxyterminal exons. Each mRNA encodes an UGT1A isoform. Of the ten encoded UGT1A isoforms, UGT1A1 is the only one with bilirubin glucuronidation activity in man[33]. The discovery of the UGT1A gene allowed the identification of genetic mutations causing CNS type I and II[34, 35, 36]. Since 1992, over 130 different missense and nonsense mutations, deletions and insertions of UGT1A(1) have been identified[37] (figure 2).

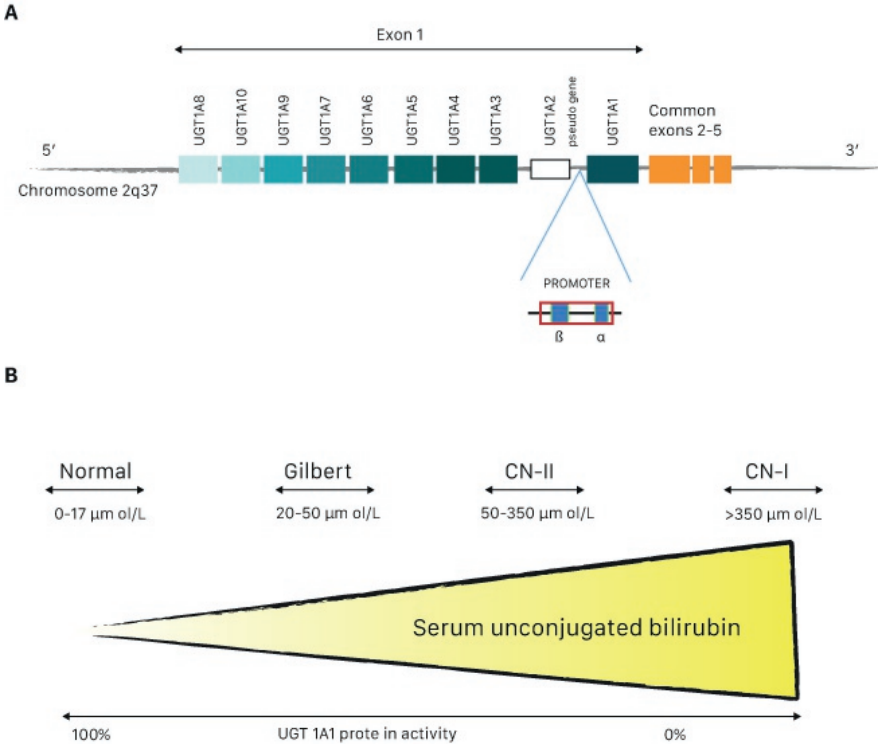


Figure 2 A. From the UGT1A locus ten different isomers are expressed. Every isomer has his own unique first exon but has exons 2-5 in common. Every isomer has its own promotor, which is located in front of the first exon. The promoter of UGT1A1 consists of two parts: α proximal promoter region (-181/-1 bases from the startcodon) containing the HNF1α binding site and the TATA box. β distal gtPBREM module (-3499/-3210 bases from the startcodon), which has binding site for secondary regulatory nuclear receptors such as PXR and CAR. **B.** UGT1A1 is the only protein capable of glucuronidation of bilirubin in humans. Reduced UGT1A1 protein activit, such as observed in Gilbert's and Crggler-Najjar syndrome (CNS), can result in increased concentrations of serum unconjugated bilirubin. *(Adjusted from P. de Sousa Montenegro Miranda, Gene Therapy with Adeno Associated Viral Vectors for Inherited Hyperbilirubinemia)*

Gilbert, or Meulengracht, syndrome is a mild form of inherited unconjugated hyperbilirubinemia with serum bilirubin levels fluctuating from normal (17 µmol/L) up to 50 µmol/L[38, 39]. Gilbert syndrome (GS) results from a homozygous insertion of an extra TA repeat in the TATAA box in the proximal promoter region of the UGT1A1 gene[40]. This mutation is in literature referred to as UGT1A1*28. Bilirubin glucuronidation activity is reduced by 35 % in heterozygous and 70 % in homozygous UGT1A1*28 carriers[41, 42, 43]. In the Caucasian and African population, GS is commonly seen and an estimated 10–15 % of all Caucasians is homozygous for UGT1A1*28 [44]. In other populations, such as those in Asia,

the UGT1A1*28 mutation is much less frequent[45]. In these populations, other mutations such as G71R (211G>A) in exon 2 and Y468D (1456T>G) in exon 5 have been reported to cause GS [34, 46, 47]. UGT1A1 also plays an important role in the glucuronidation and detoxification of additional endogenous compounds, like thyroid hormone, and exogenous compounds[48]. Several commonly used pharmaceuticals such as ezetimibe[49], ethinylestradiol[50], acetaminophen[51] and statins[52] are known substrates for UGT1A1. Reduced UGT1A1 expression and activity could therefore increase the susceptibility towards drug-induced toxicity. Severe drug toxicity related to GS has for instance been found for the antimetastatic colorectal cancer drug irinotecan, also a substrate of UGT1A1[53, 54, 55]. Besides direct toxicity, competitive binding with UGT1A1 and induced haemolysis can also deteriorate hyperbilirubinemia in GS. The anti-viral protease inhibitor indinavir, used in patients infected with the human immunodeficiency virus (HIV), has both properties and worsened jaundice in patients with decreased UGT1A1 activity[56, 57].

Although therapy is not required in GS, its pharmacological implications do render recognition of GS relevant in daily clinical practice.

Current Treatment for Crigler-Najjar Syndrome

Severe unconjugated hyperbilirubinemia can have serious consequences. Brain damage, kernicterus, that will be lethal when not treated, will eventually occur in patients with persistent high concentrations of free UCB[58, 59]. Before the introduction of effective treatments, CNS type-I patients deceased during infancy due to the development of severe kernicterus. Although they have a less severe phenotype, irreversible brain damage can also occur in CNS type II patients due to an acute increase of unconjugated bilirubin due to, e.g. haemolysis[60]. In the late 1950s, it was discovered that exposure to sunlight ameliorated hyperbilirubinemia in neonates[61]. Subsequently, it was demonstrated that this therapeutic effect is due to the formation of photo-isomers upon exposure of bilirubin to blue light. These photo-isomers are more polar and can therefore be excreted via bile and urine[62, 63, 64]. This has led to the development of phototherapy for CNS patients that allows them to reach adulthood without neurological damage[65, 66, 67].

The only curative therapy available at this moment is orthotopic liver transplantation (OLT). UGT1A1 function is fully restored after liver transplantation and serum bilirubin levels return to normal. However, due to the risk of complications,

mortality up to 10%in the first year[70], the burden of lifelong immunosuppressive therapy (increased risk of malignancies, opportunistic infection, and development of diabetes mellitus) and the lack of sufficient liver donors, the development of new alternative curative therapies is warranted. Since only about 5 to 10 % of normal UGT1A1 activity is sufficient to reduce serum bilirubin levels to levels below those causing brain damage, several patients have been treated with transplantation of donor hepatocytes[71, 72, 73]. In most patients, a partial reduction of serum bilirubin levels was seen. This effect, however, was transient. In the months following hepatocyte transplantation, the effect was gradually lost. Although much less invasive resulting in a much lower mortality of this treatment, the need for lifelong immune suppression remains a major drawback of this treatment just like OLT. Because CNS is a monogenetic disorder that does not result in liver damage, it is an attractive target for liver-directed viral vector mediated gene replacement therapy.

Viral Vector mediated Gene Therapy

Propagation of viruses depends on the introduction of their genome into host cells. The natural selection during the course of evolution, has resulted in viruses that have highly efficient gene delivery methods. In viral vector-mediated gene therapy, this characteristic is used for the transfer of a therapeutic gene to treat monogenetic disorders. During the past 25 years, different types of viral vectors have been used in clinical trials for the treatment of a variety of diseases[74]. The most commonly used viral vectors in clinical trials are retrovirus, adenovirus and adeno-associated virus (AAV) (for all registered clinical trials, see http://www.abedia.com/wiley/).

Viral vector mediated gene therapy can be applied both *ex vivo* as well as *in vivo*. For *ex vivo* gene therapy, the target cells are isolated from the patients, are then genetically modified outside the body and are re-introduced via autologous transplantation. This *ex vivo* treatment has important advantages such as avoidance of important unwanted effects like targeting of non-target organs, germ line transmission and triggering of the (innate) immune system. Cells can also be treated with a higher dose of the therapeutic vector, and positive selection of corrected cells is an option. Important limitations of *ex vivo* therapy are the limited number of cells that can be transduced and the poor grafting of the corrected cells in solid organs such as the liver. Presently it is only applicable for the correction

of haematopoietic stem cells (HSC) using integrating retroviral vectors. Highly effective ex-vivo gene therapy treatment of HSC has been developed for instance for several forms of inherited severe combined immune deficiencies.

As part of its life cycle, upon cell entry, a retrovirus converts its RNA genome into a DNA genome using reverse transcriptase. This genomic DNA is integrated into chromosomes of the host cell to induce transcription of viral RNA to produce viral proteins and RNA genomes to generate progeny. The first approved gene therapy trial was conducted in the early 1990s with a retrovirus[75]. Isolated T lymphocytes of two children suffering from the severe primary immune disease adenosine deaminase deficiency (ADA-SCID) were treated *ex vivo* with a gamma-retrovirus containing a functional copy of the adenosine deaminase gene. One patient showed a temporary response to the therapy. After this first trial, more clinical trials administering retrovirus for the treatment of both ADA- and X-linked SCID were conducted with success, resulting in significant mortality reduction in comparison to conventional allogenic HSC therapy[76]. Although these results demonstrated the clinical potential of retroviral gene therapy, a setback followed when 2–5 years after the gene therapy five X-linked SCID patients developed an acute T cell lymphoblastic leukaemia, from which four patients could be brought in remission[77]. The theoretical risk of this specific retroviral vector, the Moloney murine leukemia virus, to induce leukaemia was known from studies in mouse models[78]. In the patients, the leukaemia was caused by retroviral insertional mutagenesis and activation of the transcriptional co-factor and proto-oncogene LMO2, a protein which induces self-renewal of T cells and the prolonged expression of the encoded therapeutic gene, the Y-common chain[79]. Although no leukaemia developed in ADA-SCID patients, in other primary immune diseases such as Wiskott-Aldrich syndrome, up to 30 % of the patients developed T cell leukaemia after retroviral gene therapy[79]. Novel vectors had to be developed to overcome the adverse effects caused by oncogenic integration of the retrovirus in the host genome and especially the aberrant activation of oncogenes. Development of self-inactivating retroviral vectors and (self-inactivating lentiviral vectors which upon integration in the regulatory regions of (onco)genes do not activate transcription. In addition, DNA isolator sequences that further reduce the risk of oncogene activation were incorporated in these vectors[80]. These novel vectors are now applied for *ex vivo* gene therapy for several diseases, with Strimvelis (a Moloney murine leukemia gammaretrovirus vector carrying the ADA gene) approved by the European commission in 2016 for the use in ADA-SCID patients without a suitable HLA-matched related donor.

In *vivo* gene therapy mainly relies on the use of viral vectors containing double-stranded DNA. These vectors circumvent to a large extent the issue of genomic integration since these persist mainly in episomal form. This does render them safer but also makes them not suitable for correcting tissues with replicating cells due to vector genome loss upon cell division. One of the first DNA vectors explored for *in vivo* gene therapy was the human adenovirus serotype 5. Upon infection, the adenovirus genome escapes from the endosomal compartment and is transported into the nucleus where it is actively transcribed to produce viral proteins and genome replicates and upon the production of large numbers of novel virus particles, results in the lysis of infected cells. In case of an adenoviral vector viral genes in the E1 region, encoding proteins necessary for the expression of other adenoviral genes, have been deleted and have (partially) been replaced with a therapeutic gene resulting in the synthesis of therapeutic protein in all transduced cells. An important advantage of the adenovirus is that it has a high affinity for the liver and is capable of transducing quiescent cells such as hepatocytes providing long-term expression. A major disadvantage is its immunogenicity. Upon entry it induces a strong and rapid innate immune response, a characteristic that resulted in the first viral gene therapy-related death. A young man suffering from the inherited liver disorder ornithine transcarbamylase (OTC) deficiency died after a systemic administration of an adenovirus ferrying the OTC gene[81]. Although improvements have been accomplished, such as the helper dependent or gutless adenoviral vectors, the innate immunity limits its use for *in vivo* correction of inherited disorders[81]. At this moment, adenoviral gene therapy is mostly used to treat cancer and the induction of an immune response upon infection and adenoviral replication against cancer cell epitopes in fact enhances treatment efficiency. In fact, 60 % of all clinical gene therapy trials use adenoviral vectors to treat solid cancers. One of these is Gendicine, an adenovirus expressing the cellular tumour antigen p53, which is used to treat head and neck squamous cell carcinoma. Gendicine is the first ever licensed viral gene therapy (in China)[82, 83]. Already, over 10,000 patients have been treated without any severe adverse effects, showing an excellent safety profile for Gendicine, but the efficacy of this drug remains controversial[84].

Adeno-Associated Virus

Recently, the clinical benefit of *in vivo* gene therapy using a small (20 – 30 nm) DNA virus, Adeno-Associated Virus (AAV), has clearly been demonstrated in several phase 1 trials (see Table 1). AAV is a non-enveloped, non-pathogenic parvovirus depending on co-infection with a helper virus, such as adeno- or herpes virus, for its replication[85]. AAV was discovered in 1965 as virus-like particles in an adenovirus preparation[86, 87]. Initially three serotypes were identified, for which serotype 2 was the first to be fully sequenced and is the most extensively studied[88]. Currently twelve different AAV serotypes in (non)-human primates have been recognised.

The capsid of AAV consists out of three proteins (VP1-3). Of these, the smallest protein VP3, (62kD) makes up of almost 90% of the entire capsid proteins but the other two capsid proteins VP1 (87 kD) and VP2 (73kD) are essential capsid components. Especially VP1 has been shown to be important for successful cellular transduction[89]. Within the AAV capsid there is a single stranded DNA genome of approximately 4.7kb. Both ends of the genome consist of a 145-bp inverted terminal repeat (ITR), which function as origins of genome replication and are necessary for efficient packaging of the AAV genome. [90]. In addition to the capsid gene there are two other genes encoded by the AAV genome (*rep* and *aap)* that are necessary for viral replication, site-specific integration, and genome packaging [91]. Transcriptions of the three AAV genes are controlled by three different promoters. P5 and P19 are responsible for the transcription of rep gene and the timely expression of the encoded proteins [92]. One large RNA transcript is produced of the *cap* gene after activation of the third promoter, P40. Splicing of this RNA results in the different mRNAs encoding all three capsid proteins VP1-3. A fourth open reading frame within the *cap* gene, encodes the assembly activating protein (AAP)[93].

The first step of AAV cell entry depends on the binding to proteins present on the outside of cells. These cellular receptors differ for the various AAV serotypes. Heparan sulfate proteoglycan, terminal galactose, hepatocyte growth factor receptor and several linkage variants of sialic acid have been identified as receptors involved in binding of AAV 1-6 and AAV9[94, 95, 96]. This wide variety of receptors in combination with specific co-receptors causes the difference in tissue tropism observed for the AAV serotypes resulting in a different bio-distribution upon delivery via the systemic circulation. The tropism of an AAV serotype can be changed by a single amino-acid substitution in the AAV capsid, or by making chimeras of different AAV serotypes[97, 98]. After binding to a surface receptor, the

AAV capsid is taken up into the cell via endocytosis. Once again multiple pathways have been demonstrated to be involved in endocytosis, for which the clathrin-mediated, dynamin-dependent endocytosis is most established[99, 100]. Independent of the pathway, the endocytosis results in the formation of an acidified compartment containing an intact AAV capsid. This compartment is transported through the cytosol via microtubules and microfilaments to the nucleus where it is taken up via active transport across the nuclear pore complexes[101]. AAV vectors that escape endosome are degraded in the cytosol by proteases. In the nucleus the vector is uncoated and via DNA synthesis or direct single strand annealing double strand vector genomes are formed[102]. The conversion of single to double strand genomes is an important rate limiting step in AAV transduction, which seems less effective in (rodent) females[103]. Just like adenovirus, recombinant AAV does not actively integrate into the host genome but remains episomal. Importantly and in contrast to adenovirus, the innate immune response towards AAV is much less severe. This is most likely due to the low efficiency by which AAV transduces macrophages and other antigen presenting cells[104, 105]. These properties of being DNA carrying, non-integrating, non-pathogenic, and having wide tropism, long lasting expression and low immunogenicity makes AAV highly suitable for the clinical gene addition therapy to treat inherited recessive disorders (figure 3).

Recombinant AAV vectors are created by deleting the *cap* and *rep* genes and insert an expression cassette containing a tissue specific promoter, a cDNA encoding the therapeutic protein and a poly-A-tail, between the two ITRs. The total length of the new recombinant insertion has to be less than 5kb, the maximum packaging size of AAV. If the transgene is smaller than 2.3kb, a self-complementary vector can be generated. Self-complementary vectors are not depending on second-strand synthesis and therefore proven to be more effective in cellular transduction *in vivo*[106].

Currently different protocols are used for the production and purification of adeno-associated viral vectors. The most commonly used is the triple transfection of the human embryonic kidney cell line HEK293(T). Hereby HEK293 cells are transfected with three plasmids using calcium phosphate or poly-ethylene-imine. The first plasmid provides the capsid genes from the chosen AAV serotype and the replication genes from AAV2 in trans. The lack of the flanking ITRs prevents the generation of replicating wt AAV. The second plasmid contains the transgene flanked on both sides with ITRs to allow efficient replication and packaging. Because AAV is depending on adenovirus for its replication, the third plasmid contains the adenovirus helper genes in *trans*[107]. Although initially HEK293

were grown in cell stacks, latest development allows them to grow and produce virus in suspension, therefore expanding the vector production capacity[108]. An alternative method to produce AAV is the transduction of Sf9 insect cell line with recombinant Baculovirus encoding all components for the generation of an AAV vector[109].

Because AAV does persist for a long time in non-dividing cells, it is a good candidate for liver-, muscle-, retina-, heart and neuronal-directed gene therapy. At the end of 2012, the first AAV gene therapy was approved by the European Medicines Agency. Glybera, which is used in the treatment of familial lipoprotein lipase deficiency, is an AAV serotype 1 vector encoding a gain of function variant of the LPL transgene which is injected on multiple sites intramuscularly. Long-term follow-up after Glybera therapy showed a reduced chylomicron triglyceride to total plasma triglyceride ratio and clinical improvement of the patients[110, 111]. However due to the lack of sufficient patients, only one patient has been treated since 2012, and due to the price (with the cost of 1million dollar per patient Glybera is the most expensive drug in the world), Uniqure did not go for renewal of its marketing authorization for Glybera in Europe and Glybera has been withdrawn since October 2016.

From all the different human AAV serotypes that have been identified, AAV serotypes 2, 5, 8 and 9 show the most pronounced hepatic tropism[112]. An initial trial using AAV2 showed a transient correction of factor IX deficiency. The loss of efficacy overtime appeared to be caused by to a cellular response against the AAV2 capsid present on the transduced hepatocytes, due to a pre-existing memory response to this virus[113]. Recently, a successful phase I–II clinical trial was conducted for this disease using AAV serotype 8. A single injection into a peripheral vein of an AAV8 vector ferrying the human factor IX behind a liver-specific promoter resulted in a prolonged therapeutic expression in the liver of patients suffering from haemophilia B. At the high dose range, a transient rise of liver enzymes was seen similar to the one that accompanied the loss of efficacy in the patients treated with the AAV2 vector in the first trial. A short course of steroids proved sufficient to prevent loss of the transduced cells resulting in sustained correction of factor IX deficiency. This trial demonstrated that AAV8 is a safe and effective vector for liver-directed gene therapy, emphasising the potential use of AAV8 in other inherited disorders of the liver, and that cellular responses can be prevented by a short course of steroids covering the period of immune presentation of AAV proteins by the transduced hepatocytes[114]. Up to June 2017, 183 clinical trials are registered using an AAV vector for the treatment of inherited

and acquired diseases in a wide variety of target organs. Table 1 shows all the clinical trials involving AAV in inherited diseases.

Several clinical trials showed promising and sustained correction leading to a novel curative option especially for inherited disorders for which no other treatment options are available. Several issues need to be addressed to allow the potential use of gene therapy for disorders for which some kind of therapy, unsatisfying therapeutic option, e.g. enzyme replacement therapy, already exists. One major issue still is the long-term safety. Although several patients have been treated more than 10 years ago without any complications, their limited number makes that solid data on the potential risk of genomic toxicity are still lacking. Another issue relates to the persistence of correction provided by episomal vectors. In theory, this treatment may lose efficacy in time while reinjection is not possible due to the humoral response towards the vector induced by the first treatment. Therefore, methods that circumvent this humoral response and do allow reinjection of the same vector need to be developed.

The Promise of Liver-Directed Gene Therapy in Crigler-Najjar Syndrome

During the last decades, several viral gene therapy strategies have successfully been tested in a rat model, the Gunn rat[115]. As in CNS type I, Gunn rats lack completely the ability of hepatic glucuronidation of bilirubin and have high concentrations of unconjugated serum bilirubin which can lead to neurotoxicity[116, 117, 118]. Multiple viral vectors showed promising results *in vivo*[119, 120, 121], of which the AAV vector seems to have most potential in CNS[122]. Administration of AAV containing a liver-specific promoter and the human UGT1A1 gene resulted in a lifelong correction of serum bilirubin in the Gunn rat[123, 124].

Although liver-directed gene therapy seems to be a promising treatment option that will be available in the near future, cautiousness is warranted regarding possible unwanted side effects. In CNS patients with a null mutation, successful gene therapy will result in the expression of UGT1A1 in a liver which previously lacked this protein. This could lead to the development of an immune response against UGT1A1. Upon induction of a cellular response, lifelong immune suppression will be needed to prevent loss of 'corrected' hepatocytes, while overcoming the need of immune suppression is one of the major advantages of gene therapy versus liver transplantation. Also, a humoral response can be

induced resulting in the formation of autoantibodies (aAbs) directed against UGT1A1. These aAbs could potentially target UGT1A1 expressing hepatocytes. UDP-glucuronosyltransferase aAbs have been described previously in patients with chronic hepatitis C and D infection and in autoimmune hepatitis (AIH) type 2[125, 126, 127]. These aAbs are known as liver-kidney microsomal antibodies 3 (LKM3). LKM3 aAbs in AIH type 2 patients not only had the strongest reactivity against UGT1A1 but also cross-reacted with other UGT1A isoforms[127]. Although LKM3 aAbs inhibited UGT1A1-mediated glucuronidation *in vitro*, none of the patients presented with an unconjugated hyperbilirubinemia. The strong humoral immune response against hUGT1A1 in the Gunn rat treated with AAV-CMV-UGT1A1 also did not prevent lifelong correction of hyperbilirubinemia in this animal. Apparently, the activity of an intracellular protein like UGT1A1 is not affected by these aAbs. This is in contrast to membrane proteins like BSEP which lost transport activity in patients after OLT due to inhibition by aAbs and to exported proteins like factor IX or VIII[128, 129]. LKM3 aAbs titres could be reduced under immunosuppressive therapy with corticosteroids and azathioprine, although this was only followed up in one patient with AIH type 2[127]. However, the immune response towards UGT1A1 can result in loss of therapeutic efficacy. In neonatal Gunn rats, upon transduction with a lentiviral vector, only a transient correction of serum bilirubin levels was seen[130]. Although the liver in neonatal rats is still growing, division of hepatocytes cannot explain the loss of UGT1A1 expression from an integrating lentiviral vector. In contrast to AAV, lentiviral vectors efficiently transduce antigen presenting cells which may result in the induction of a cellular response. In these neonatal rats a strong humoral response against the expressed UGT1A1 protein was detected, cellular immune responses were not investigated. Also, in adult Gunn rats treated with an Ad5 vector with a viral promoter, the correction was only transient[131, 132]. In the treated animals a strong immunogenicity response against the adenoviral vector was observed[132]. However, no loss of vector genome was seen, indicating that silencing of the viral promoter due to an inflammatory response rather than loss of expressing cells caused the loss of efficacy. Indeed, the use of a liver-specific promoter resulted in life-long correction of hyperbilirubinemia using both adenovirus and AAV[124]. Although with respect to immune responses, AAV is the most suitable of the vectors tested, also with this vector deleterious immune responses in animal models have been reported in two different primate species injected with AAV containing the erythropoietin (EPO) transgene. Hereby immune tolerance for EPO was abolished, and a severe anaemia developed within weeks after the genetic therapeutic intervention[133, 134]. The reasons for the induced

autoimmunity are not completely understood. Ectopic expression or spreading of the vector with subsequent EPO expression in antigen-presenting cells could all contribute to the induction of an immune response. The liver is a tolerogenic organ, and it has been shown that immune tolerance can be induced via hepatocyte-specific expression of the transgene[135]. This can be achieved by using hepatotropic viral vectors and liver-specific promoters for transgene expression. Immune tolerance seems also to be induced upon reaching sufficiently high expression levels in the liver. A strategy to prevent an autoimmune response in CNS patients could therefore be to first perform a dose-finding study only in patients with a UGT1A1 missense mutation that do express the protein. Since they have endogenous expression of (non-functional) UGT1A1, the risk of developing UGT1A1 Abs is strongly reduced. Upon establishing a sufficient level of expression in the human liver, patients that lack UGT1A1 expression completely could be included aiming for tolerance induction. As mentioned above, in addition to the response to the transgene, the delivery vector also plays a role in the induction of an immune response.

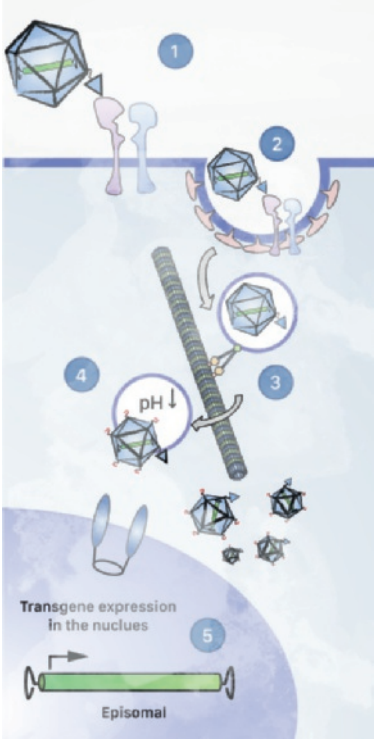


Figure 3: AAV cellular uptake
A systemic overview of cellular uptake and trafficking of AAV particles. **(1)** An AAV particle binds to its specific receptor and/or co-receptor expressed on the surface of the cell membrane. **(2)** After binding to the (co)receptor an endosome is formed which is **(3)** is transported through the cell via intracellular trafficking over microtubules. **(4)** By lowering pH within the endosome the AAV particle 'escapes' the endomsome. **(5)** AAV nuclear uptake goes via the nuclear core complex, once in the nucleas AAV releases its genetic component. *(Adjusted from Buchholz CJ et al[161]).*

Viral proteins contain signals that will alert the immune system. Therefore, the use of immunosuppressive drugs could be considered to cover the time that these viral proteins are exposed to the immune system. In the trial for haemophilia B, transient use of prednisolone proved effective to block vector-related deleterious immune responses. The choice of immune-suppressive therapy should depend on the vector used and the disease to be treated since it can interfere with vector delivery and efficacy. For instance, a frequently used drug in transplant medicine, mycophenolate mofetil, reduces hepatocyte transduction of single-stranded AAV[136] and is, therefore, not suitable in combination with this vector.

Conclusion

The liver plays a central role in the conjugation and clearance of bilirubin. A mutation of a single nucleotide may have detrimental consequences for bilirubin metabolism and transport, ranging from benign conjugated hyperbilirubinemia to potentially lethal, extensive unconjugated hyperbilirubinemia. Progress in molecular genetics allowed unravelling the pathogenesis of inherited forms of hyperbilirubinemia in the 1990s. The rapidly developing field of viral vector mediated gene therapy, e.g. adeno-associated virus mediated liver-directed gene therapy, may provide a curative therapy for potentially lethal genetic disorders like Crigler-Najjar syndrome in the near future, and will allow avoiding OLT.

Disease Serotype	Transgene product	Serotype	Route of administration	Clinical trial	Refs
α1 antitrypsin deficiency	α1 antitrypsin	AAV2 AAV1	Intramuscular	Phase I/II	[137-139]
Achromatopsia	hCNGA3	AAV2	Subretinal	Phase I/II	NA
Age-related macular degeneration	sFLT01	Not specified	Intraocular	Phase I/II	NA
Acute intermittent porphyria	5-PGBD	AAV5	Intravenous	Phase I	[161]
Aromatic L-amino acid (AADC) deficiency	AADC	AAV3		Phase I	NA
Batten's disease	CLN2	AAV2 AAV10	Direct intracranial administration	Phase I	[140]
Canavan's disease	Aspartoacylase	AAV2	Direct intracranial administration	Phase I	NA
Choroideraemia	REP1	AAV1 AAV2	Subretinal Subretinal	Phase I Phase I	NA NA
Cystic fibrosis	CFTR	AAV2	Direct instillation to maxillary sinus, bronchoscopy to right lower lobe, aerosol to whole lung	Phase I/II	[141-145]
Familial hypercholesterolaemia	LDLR	AAV8	Intravenous	Phase I/II	NA
Stage IV Gastric Cancer	Carcinoembryonic antigen (CEA)	Not specified	Ex vivo: Dendritic cells	Phase I	NA
Haemophilia A	Factor VIII	AAV8 AAVrh10	Systemic Systemic	Phase I/II Phase I/II	NA NA
Haemophilia B	Factor IX	AAV2 AAV8 AAV5	Intramuscular Hepatic Systemic Systemic	Phase I/II	[114, 146, 147]
Heart Failure	SERC2a	AAV1	antegrade epicardial coronary artery infusion	Phase I/II	NA
Leber's congenital amaurosis	RPE65 Mitochondrial NADH dehydrogenase subunit 4	AAV2 AAV2	Subretinal Intraocular	Phase I/II Phase I	[148–152] NA
LPL deficiency	LPL	AAV1	Intramuscular	Phase I-III	[110, 153, 154]

Metachromatic leukodystrophy	Arylsulfatase A	AAV10	Direct intracranial administration	Phase I/II	NA
Mucopolysaccharidosis III (Sanfilippo type A syndrome)	SGSH and SUMF1 Sulfamidase	AAV10 AAV9	Intracerebral Intracerebral	Phase I/II	NA
Mucopolysaccharidosis IIIB (Sanfilippo type B syndrome)	hNAGLU (n-acetylglucosa-minidase)	AAV9	Intravenous	Phase I/II	NA
Muscular dystrophy: Duchenne	Microdystrophin GALGT2	AAV1-AAV2 AAV2.5 rAAVrh74 rAAVrh74	Intramuscular Intramuscular Intravenous Intramuscular	Phase I Phase I Phase I/II Phase I/II	[156, 157] NA NA NA
Muscular dystrophy: limb girdle	α-Sarcoglycan	AAV1 AAV8	2 to 6 separate injections into the selected muscle Arterial delivery to an isolated limb	Phase I Phase I	[158-160] NA
Late-Onset OTC Deficiency	OTC	AAV8	Systemic	PhasI/II	NA
Parkinson's disease	GDNF Neurturin (NTN) AADC	AAV2	(Bilateral) Intraputaminal	Phase II	[162]
Pompe's disease	GAAα	AAV1 AAV2/8 AAV9	Series of intradiaphragmatic injections Intravenous Intramuscular	Phase I/II Phase I/II Phase I/III	[155] NA NA
Rheumatoid arthritis	interferon-β	AAV 5	Intra-articular	Phase I/II	NA
Tay-Sachs disease	α and β hexosaminidase	Not specified	Intracranial	Phase II	NA
X-linked Retinitis Pigmentosa	Retinitis Pigmentosa GTPase Regulator (RPGR)	Not specified	Subretinal	Phase I/II	NA

Table 1: AAV viral gene clinical trials
AADC aromatic-L-amino-acid decarboxylase, *AAV* adeno-associated virus, *CFTR* cystic fibrosis transmembrane regulator, *CLN2* also known as tripeptidyl peptidase 1 (TPP1), *GAA* acid α-glucosidase, *GAD* glutamic acid decarboxylase, *GALGT2* SdaGalNAc transferase, *LDLR* low-density lipoprotein receptor, *LPL* lipoprotein lipase, *NA* not available, *NAGLU* alpha-N-acetylglucosaminidase, *OTC* Ornithine Transcarbamylase, *PGBD* porphobilinogen deaminase, *REP1* Rab escort protein 1, *RPE65* retinal pigment epithelium-specific protein 65kDA, *SERCA2a* sarcoplasmic reticulum calcium ATPase 2a, *SGSH* Nsulfoglucosamine sulfohydrolase gene, *sFLT01* portion of the vascular endothelial growth factor natural receptor, *SUMF1* sulfatase modifying factor 1, *TNFR-Fc* tumour necrosis factor receptor-immunoglobulin Fc fragment fusion protein

References

1. Bissell DM (1975) *Formation and elimination of bilirubin*. Gastroenterology 69:519–538
2. Berk PD, Howe RB, Bloomer JR, Berlin NI (1969) *Studies of bilirubin kinetics in normal adults.* J Clin Invest 48:2176–2190
3. Tenhunen R, Marver HS, Schmid R (1968) *The enzymatic conversion of heme to bilirubin bymicrosomal heme oxygenase.* Proc Natl Acad Sci U S A 61:748–755
4. Tenhunen R, Marver HS, Schmid R (1970) *The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin.* J Lab Clin Med 75:410–421
5. Tenhunen R, Ross ME, Marver HS, Schmid R (1970) *Reduced nicotinamide-adenine dinucleotide phosphate dependent Biliverdin reductase: partial purification and characterization.* Biochemistry 9: 298–303, 20-1
6. Sorrentino D, Berk PD (1988) *Mechanistic aspects of hepatic bilirubin uptake*. Semin Liver Dis 8:119–136
7. Tiribelli C, Ostrow JD (1993*) New concepts in bilirubin chemistry, transport and metabolism: report of the Second International Bilirubin Workshop*, April 9–11, 1992, Trieste, Italy. Hepatology 17:715–736
8. Schmid R, McDonagh AF (1975) *The enzymatic formation of bilirubin.* Ann N YAcad Sci 244:533–552
9. Dubin IN, Johnson FB (1954) *Chronic idiopathic jaundice with unidentified pigment in liver cells; a new clinicopathologic entity with a report of 12 cases*. Medicine (Baltimore) 33:155–197
10. Sprinz H, Nelson RS (1954) *Persistent non-hemolytic hyperbilirubinemia associated with lipochrome-like pigment in liver cells: report of four cases.* Ann Intern Med 41:952–962
11. Swartz HM, Sarna T, Varma RR (1979) On the natural and excretion of the hepatic pigment in the Dubin-Johnson syndrome. Gastroenterology 76:958–964
12. Koskelo P, Toivonen I, Adlercreutz H (1967) *Urinary coproporphyrin isomer distribution in the Dubin-Johnson syndrome.* Clin Chem 13:1006–1009
13. Kondo T, Kuchiba K, Shimizu Y (1976) *Coproporphyrin isomers in Dubin-Johnson syndrome.* Gastroenterology 70:1117–1120
14. Paulusma CC, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RP (1996) *Congenital jaundice in rats with a mutation in a multidrug resistanceassociated protein gene.* Science 271:1126–1128
15. Paulusma CC, Kool M, Bosma PJ, Scheffer GL, Ter BF, Scheper RJ, Tytgat GN, Borst P, Baas F, Oude Elferink RP (1997*) A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome.* Hepatology 25:1539–1542
16. Keppler D, Konig J, Buchler M (1997) *The canalicular multidrug resistance protein, cMRP/MRP2, a novel conjugate export pump expressed in the apical membrane of hepatocytes*. Adv Enzyme Regul 37:321–333
17. Miller DS, Nobmann SN, Gutmann H, Toeroek M, Drewe J, Fricker G (2000*) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy.* Mol Pharmacol 58:1357–1367
18. Borst P, Zelcer N, van deWetering K (2006) MRP2 and 3 in health and disease. Cancer Lett 234:51–61
19. Jedlitschky G, Hoffmann U, Kroemer HK (2006) *Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition.* Expert Opin Drug Metab Toxicol 2:351–366

20. Dietrich CG, Geier A, Oude Elferink RP (2003) *ABC of oral bioavailability: transporters as gatekeepers in the gut.* Gut 52: 1788–1795
21. Rotor AB, Manahan L, Florentin A (1948) *Familial nonhemolytic jaundice with direct van den Bergh reaction.* Acta Med Phil 5:37–49
22. Shimizu Y, Naruto H, Ida S, Kohakura M (1981) *Urinary coproporphyrin isomers in Rotor's syndrome: a study in eight families.* Hepatology 1:173–178
23. Wolpert E, Pascasio FM, Wolkoff AW, Arias IM (1977) *Abnormal sulfobromophthalein metabolism in Rotor's syndrome and obligate heterozygotes.* N Engl J Med 296:1099–1101
24. van de Steeg E, Stranecky V, Hartmannova H, Noskova L, Hrebicek M, Wagenaar E, van Esch A, de Waart DR, Oude Elferink RP, Kenworthy KE, Sticova E, Al-Edreesi M, Knisely AS, Kmoch S, Jirsa M, Schinkel AH (2012) *Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver.* J Clin Invest 122:519–528
25. Hagenbuch B, Gui C (2008) *Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family.* Xenobiotica 38:778–801
26. Konig J, Cui Y, Nies AT, Keppler D (2000) *Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide.* J Biol Chem 275:23161–23168
27. Crigler JF Jr, Najjar VA (1952) *Congenital familial nonhemolytic jaundice with kernicterus.* Pediatrics 10:169–180
28. Sinaasappel M, Jansen PL (1991*) The differential diagnosis of Crigler-Najjar disease, types 1 and 2, by bile pigment analysis.* Gastroenterology 100:783–789
29. Arias IM, Gartner LM, Cohen M, Ezzer JB, Levi AJ (1969) *Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologicand genetic evidence for heterogeneity*. Am J Med 47: 395–409
30. Ritter JK, Crawford JM, Owens IS (1991) *Cloning of two human liver bilirubin UDP-glucuronosyltransferase cDNAs with expression in COS-1 cells*. J Biol Chem 266:1043–1047
31. Ritter JK, Chen F, Sheen YY, Tran HM, Kimura S, Yeatman MT, Owens IS (1992) *A novel complex locus UGT1 encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini.* J Biol Chem 267: 3257–3261
32. Gong QH, Cho JW, Huang T, Potter C, Gholami N, Basu NK, Kubota S, Carvalho S, Pennington MW, Owens IS, Popescu NC (2001) *Thirteen UDPglucuronosyltransferase genes are encoded at the human UGT1 gene complex locus*. Pharmacogenetics 11:357– 368
33. Bosma PJ, Seppen J, Goldhoorn B, Bakker C, Oude Elferink RP, Chowdhury JR, Chowdhury NR, Jansen PL (1994*) Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man*. J Biol Chem 269:17960–17964
34. Aono S, Yamada Y, Keino H, Hanada N, Nakagawa T, Sasaoka Y, Yazawa T, Sato H, Koiwai O (1993*) Identification of defect in the genes for bilirubin UDP-glucuronosyl-transferase in a patient with Crigler-Najjar syndrome type II.* Biochem Biophys Res Commun 197:1239–1244
35. Ritter JK, Yeatman MT, Ferreira P, Owens IS (1992) *Identification of a genetic alteration in the code for bilirubin UDPglucuronosyltransferase in the UGT1 gene complex of a Crigler- Najjar type I patient.* J Clin Invest 90:150–155
36. Bosma PJ, Chowdhury NR, Goldhoorn BG, Hofker MH, Oude Elferink RP, Jansen PL, Chowdhury JR (1992) *Sequence of exons and the flanking regions of human bilirubin-UDPglucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I*. Hepatology 15:941–947

37. Canu G, Minucci A, Zuppi C, Capoluongo E (2013) *Gilbert and Crigler Najjar syndromes: an update of the UDPglucuronosyltransferase 1A1 (UGT1A1) gene mutation database*. Blood Cells Mol Dis 50:273–280
38. Meulengracht E (1947) *A review of chronic intermittent juvenile jaundice*. Q J Med 16:83–98
39. Gilbert ALP (1901*) La cholamae simple familiale.* SemMed 21:241 40.
40. Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, Lindhout D, Tytgat GN, Jansen PL, Oude Elferink RP (1995) *The genetic basis of the reduced expression of bilirubin UDPglucuronosyltransferase 1 in Gilbert's syndrome.* N Engl J Med 333:1171–1175
41. Raijmakers MT, Jansen PL, Steegers EA, Peters WH (2000) *Association of human liver bilirubin UDP-glucuronyltransferase activity with a polymorphism in the promoter region of the UGT1A1 gene*. J Hepatol 33:348–351
42. Black M, Billing BH (1968) *'In vitro' assay of bilirubin-UDP glucuronyl transferase activity in the liver of patients with Gilbert's syndrome and a variety of hepatic disorders.* Gut 9:728–729
43. Black M, Billing BH (1969) *Hepatic bilirubin UDP-glucuronyl transferase activity in liver disease and Gilbert's syndrome*. N Engl J Med 280:1266–1271
44. Monaghan G, Ryan M, Seddon R, Hume R, Burchell B (1996) *Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome.* Lancet 347:578–581
45. Hall D, Ybazeta G, Destro-Bisol G, Petzl-Erler ML, Di RA (1999) *Variability at the uridine diphosphate glucuronosyltransferase 1A1 promoter in human populations and primates.* Pharmacogenetics 9: 591–599
46. Sato H, Adachi Y, Koiwai O (1996) *The genetic basis of Gilbert's syndrome*. Lancet 347:557–558
47. Soeda Y, Yamamoto K, Adachi Y, Hori T, Aono S, Koiwai O, Sato H (1995) *Predicted homozygous mis-sense mutation in Gilbert's syndrome.* Lancet 346:1494
48. Miners JO, Mackenzie PI (1991) *Drug glucuronidation in humans.* Pharmacol Ther 51:347–369
49. Ghosal A, Hapangama N, Yuan Y, Achanfuo-Yeboah J, Iannucci R, Chowdhury S, Alton K, Patrick JE, Zbaida S (2004) *Identification of human UDP-glucuronosyltransferase enzyme(s) responsible for the glucuronidation of ezetimibe* (Zetia). Drug Metab Dispos 32:314–320
50. Ebner T, Remmel RP, Burchell B (1993) *Human bilirubin UDPglucuronosyltransferase catalyzes the glucuronidation of ethinylestradiol.* Mol Pharmacol 43:649–654
51. McGill MR, Jaeschke H (2013) *Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis.* Pharm Res 30:2174–2187
52. Prueksaritanont T, Subramanian R, Fang X, Ma B, Qiu Y, Lin JH, Pearson PG, Baillie TA (2002) *Glucuronidation of statins in animals and humans: a novel mechanismof statin lactonization.* DrugMetab Dispos 30:505–512
53. Tukey RH, St rassburg CP, Mackenzie PI (2002) *Pharmacogenomics of human UDP-glucuronosyltransferases and irinotecan toxicity.* Mol Pharmacol 62:446–450
54. Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, Karrison T, Janisch L, Ramirez J, Rudin CM, Vokes EE, Ratain MJ (2004) *Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan*. J Clin Oncol 22:1382–1388
55. Innocenti F, Ratain MJ (2003) *Irinotecan treatment in cancer patients with UGT1A1 polymorphisms.* Oncology (Williston Park) 17:52–55
56. Zucker SD, Qin X, Rouster SD, Yu F, Green RM, Keshavan P, Feinberg J, Sherman KE (2001) *Mechanism of indinavir-induced hyperbilirubinemia*. Proc Natl Acad Sci U S A 98:12671–12676

57. Lankisch TO, Behrens G, Ehmer U, Mobius U, Rockstroh J, Wehmeier M, Kalthoff S, Freiberg N, Manns MP, Schmidt RE, Strassburg CP (2009) *Gilbert's syndrome and hyperbilirubinemia in protease inhibitor therapy—an extended haplotype of genetic variants increases risk in indinavir treatment*. J Hepatol 50:1010–1018
58. Shapiro SM, Bhutani VK, Johnson L (2006) *Hyperbilirubinemia and kernicterus*. Clin Perinatol 33:387–410 59.
59. Watchko JF, Tiribelli C (2013) *Bilirubin-induced neurologic damage—mechanisms and management approaches.* N Engl J Med 369: 2021–2030
60. Poddar B, Bharti B, Goraya J, Parmar VR (2002) *Kernicterus in a child with Crigler-Najjar syndrome type II.* Trop Gastroenterol 23: 33–34
61. Cremer RJ, Perryman PW, Richards DH (1958*) Influence of light on the hyperbilirubinaemia of infants.* Lancet 1:1094–1097
62. Ennever JF (1990) *Blue light, green light, white light, more light: treatment of neonatal jaundice.* Clin Perinatol 17:467–481
63. Zenone EA, Stoll MS, Ostrow JD (1982) *The effect of elimination of environmental light on the metabolism of unconjugated bilirubin in the Gunn rat.* Dig Dis Sci 27:1117–1120
64. Stoll MS, Zenone EA, Ostrow JD (1981) *Excretion of administered and endogenous photobilirubins in the bile of the jaundice Gunn rat*. J Clin Invest 68:134–141
65. Van Der Veere CN, Sinaasappel M, McDonagh AF, Rosenthal P, Labrune P, Odievre M, Fevery J, Otte JB, McClean P, Burk G, Masakowski V, SperlW,Mowat AP, VerganiGM, Heller K,Wilson JP, Shepherd R, Jansen PL (1996) *Current therapy for Crigler-Najjar syndrome type 1: report of a world registry.* Hepatology 24:311–315
66. Jansen PL. *Diagnosis and management of Crigler-Najjar syndrome.* Eur J Pediatr. 1999 Dec; 158 Suppl 2:S89-94.
67. Stoll MS (1986) *Phototherapy of jaundice*. In: Ostrow JD (ed) Bile pigments and jaundice. Raven, New York, pp 551–580
68. Yohannan MD, Terry HJ, Littlewood JM (1983) *Long term phototherapy in Crigler-Najjar syndrome.* Arch Dis Child 58:460–462
69. Strauss KA, Robinson DL, Vreman HJ, Puffenberger EG, Hart G, Morton DH (2006) *Management of hyperbilirubinemia and prevention of kernicterus in 20 patients with Crigler-Najjar disease. Eur* J Pediatr 165:306–319
70. Muiesan P, Vergani D,Mieli-VerganiG (2007) *Liver transplantation in children.* J Hepatol 46:340–348
71. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Saute rBV, Strom SC (1998) *Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation.* N Engl J Med 338:1422–1426
72. Ambrosino G, Varotto S, Strom SC, Guariso G, Franchin E, Miotto D, Caenazzo L, Basso S, Carraro P, Valente ML, D'Amico D, Zancan L, D'Antiga L (2005) *Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1.* Cell Transplant 14:151–157
73. Lysy PA, Najimi M, Stephenne X, Bourgois A, Smets F, Sokal EM (2008) *Liver cell transplantation for Crigler-Najjar syndrome type I: update and perspectives.* World J Gastroenterol 14:3464–3470
74. Kaufmann KB, Buning H, Galy A, Schambach A, Grez M (2013) *Gene therapy on the move.* EMBO Mol Med 5:1642–1661

75. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, Greenblatt JJ, Rosenberg SA, Klein H, Berger M, Mullen CA, Ramsey WJ, Muul L, Morgan RA, Anderson WF (1995) T *lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years.* Science 270:475–480
76. Gaspar HB, Thrasher AJ (2005) *Gene therapy for severe combined immunodeficiencies.* Expert Opin Biol Ther 5:1175–1182
77. Cavazzana-Calvo M, Fischer A, Hacein-Bey-Abina S, Aiuti A (2012) *Gene therapy for primary immunodeficiencies: part 1*. Curr Opin Immunol 24:580–584
78. van der Putten H, Quint W, van Raaij J, Maandag ER, Verma IM, Berns A (1981) M-*MuLV-induced leukemogenesis: integration and structure of recombinant proviruses in tumors.* Cell 24:729–739
79. Cavazza A, Moiani A, Mavilio F (2013) *Mechanisms of retroviral integration and mutagenesis.* Hum Gene Ther 24:119–131
80. Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicenti F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C, von Kalle C, Naldini L (2009) *The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy.* J Clin Invest 119:964–975
81. N Bessis, F J GarciaCozar and M-C Boissier (2004) *Immune responses to gene therapy vectors: influence on vector function and effector mechanisms*. Gene Therapy 11, S10–S17.
82. Peng Z (2005) *Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers.* Hum Gene Ther 16: 1016–1027
83. Wilson JM (2005) *Gendicine: the first commercial gene therapy product.* Hum Gene Ther 16:1014–1015
84. Sheridan C (2011) *Gene therapy finds its niche.* Nat Biotechnol 29: 121–128
85. Hoggan MD, Blacklow NR, Rowe WP (1966) *Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics.* Proc NatlAcad Sci USA 55:1467–1474
86. Atchison RW, Casto BC, Hammon WM. *Adenovirus-associated defective virus particles*. Science 1965;149:754–756
87. Hoggan MD, Blacklow NR, Rowe WP. *Studies of small DNA viruses found in various adenovirus preparations: Physical, biological, and immunological characteristics.* Proc Natl Acad Sci USA 1966;55:1467–1474
88. Lusby E, Fife KH, Berns KI. *Nucleotide sequence of theinverted terminal repetition in adeno-associated virus DNA*. J Virol 1980;34:402–409.
89. Rolling F, Samulski RJ. *AAV as a viral vector for human gene therapy. Generation of recombinant virus.* Mol Biotechnol. 1995; 3:9–15.
90. Srivastava, A., E. W. Lusby, and K. I. Berns. (1983*) Nucleotide sequence and organization of the adeno-associated virus 2 genome.* J. Virol. 45:555–564.
91. Salganiki M, Hirsch ML, and Samulski RJ. *Adeno-associated Virus as a Mammalian DNA Vector.* Microbiol Spectr. 2015 August ; 3(4)
92. Marcus CJ, Laughlin CA, Carter BJ. *Adeno-associatedvirus rna transcription in vivo.* Eur J Biochem 1981;121: 147–154
93. Sonntag F, Schmidt K, Kleinschmidt JA. *A viral assembly factor promotes AAV2 capsid formation in the nucleolus.* Proc Natl Acad Sci USA. 2010; 107:10220–10225

94. Summerford C, Samulski RJ. *Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions.* J Virol. 1998; 72:1438–1445.
95. Kaludov N, Brown KE, Walters RW, Zabner J, Chiorini JA. *Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity.* J Virol. 2001; 75:6884–6893.
96. Walters RW, Yi SM, Keshavjee S, Brown KE, Welsh MJ, Chiorini JA, Zabner J. *Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer.* J Biol Chem. 2001; 276:20610–20616.
97. Müller OJ, Leuchs B, Pleger ST, Grimm D, Franz W-M, Katus HA, Kleinschmidt JA. *Improved cardiac gene transfer by transcriptional and transductional targeting of adeno-associated viral vectors.* Cardiovasc Res. 2006; 70:70–78
98. Asokan A, Conway JC, Phillips JL, Li C, Hegge J, Sinnott R, Yadav S, DiPrimio N, Nam H-J, Agbandje-McKenna M, McPhee S, Wolff J, Samulski RJ. *Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle.* Nat Biotechnol. 2010; 28:79–82
99. Xiao P-J, Samulski RJ. *Cytoplasmic trafficking, endosomal escape, and perinuclear accumulation of adeno-associated virus type 2 particles are facilitated by microtubule network.* J Virol. 2012; 86:10462–10473
100. Bartlett JS, Wilcher R, Samulski RJ. *Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors.* J Virol. 2000; 74:2777–2785
101. Nicolson SC, Samulski RJ. *Recombinant adeno-associated virus utilizes host cell nuclear import machinery to enter the nucleus.* J Virol. 2014; 88:4132–4144
102. Zhong L, Zhou X, Li Y, Qing K, Xiao X, Samulski RJ, Srivastava A. *Single-polarity recombinant adeno-associated virus 2 vector-mediated transgene expression in vitro and in vivo: mechanism of transduction.* Mol Ther. 2008; 16:290–295
103. Paneda A., Vanrell L., Mauleon I. *Effect of adeno-associated virus serotype and genomic structure on liver transduction and biodistribution in mice of both genders.* Hum Gene Ther 2009; 20:908–917
104. Schnepp BC, Jensen RL, Chen CL, Johnson PR, Clark KR (2005) *Characterization of adeno-associated virus genomes isolated from human tissues.* J Virol 79:14793–14803
105. Zaiss A-K, Liu Q, Bowen GP, Wong NCW, Bartlett JS, Muruve DA. *Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors.* J Virol. 2002; 76:4580–4590.
106. Mccarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ. *Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo.* Gene Ther. 2003; 10: 2112-2118
107. Nathalie Clément and Joshua C Grieger. *Manufacturing of recombinant adeno-associated viral vectors for clinical trials.* 2016 Mar 16; 3:16002
108. Grieger JC1, Soltys SM, Samulski RJ. *Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector from the Culture Media for GMP FIX and FLT1 Clinical Vector.* Mol Ther. 2016 Feb;24(2):287-97
109. Urabe M, Ding C, and Kotin RM. *Insect cells as a factory to produce adeno-associated virus type 2 vectors.* Hum Gene Ther. 2002; 13:1935–1943

110. Gaudet D, de Wal J, Tremblay K, Dery S, van Deventer S, Freidig A, Brisson D, Methot J (2010) *Review of the clinical development of alipogene tiparvovec gene therapy for lipoprotein lipase deficiency.* Atheroscler Suppl 11:55–60
111. Buning H (2013*) Gene therapy enters the pharma market: the short story of a long journey.* EMBO Mol Med 5:1–3
112. Gao G, Vandenberghe LH, Wilson JM (2005) *New recombinant serotypes of AAV vectors.* Curr Gene Ther 5:285–297
113. Mingozzi F, High KA (2011) *Immune responses to AAV in clinical trials.* Curr Gene Ther 11:321–330
114. Nathwani AC, TuddenhamEG, Rangarajan S, Rosales C, McIntosh J, Linch DC, Chowdary P, Riddell A, Pie AJ, Harrington C, O'Beirne J, Smith K, Pasi J, Glader B, Rustagi P, Ng CY, Kay MA, Zhou J, Spence Y, Morton CL, Allay J, Coleman J, Sleep S, Cunningham JM, Srivastava D, Basner-Tschakarjan E, Mingozzi F, High KA, Gray JT, Reiss UM, Nienhuis AW, Davidoff AM (2011) *Adenovirus-associated virus vector-mediated gene transfer in haemophilia B.* N Engl J Med 365:2357–2365
115. Miranda PS, Bosma PJ (2009) *Towards liver-directed gene therapy for Crigler-Najjar syndrome.* Curr Gene Ther 9:72–82
116. Gunn CK (1944*) Hereditary acholuric jaundice in the rat.* Can Med Assoc J 50:230–237
117. Schmid R, Axelrod J, Hammaker L, Swarm RL (1958) *Congenital jaundice in rats, due to a defect in glucuronide formation.* J Clin Invest 37:1123–1130
118. Chowdhury NR, Arias IM, Lederstein M, Chowdhury JR (1986) *Substrates and products of purified rat liver bilirubin UDP-glucuronosyltransferase.* Hepatology 6:123–128
119. Tada K, Roy-Chowdhury N, Prasad V, Kim BH, Manchikalapudi P, Fox IJ, van Duijvendijk P, Bosma PJ, Roy-Chowdhury J (1998) *Long-term amelioration of bilirubin glucuronidation defect in Gunn rats by transplanting genetically modified immortalized autologous hepatocytes.* Cell Transplant 7:607–616
120. Branchereau S, Ferry N, Myara A, Sato H, Kowai O, Trivin F, Houssin D, Danos O, Heard J (1993) *Correction of bilirubin glucuronyl transferase in Gunn rats by gene transfer in the liver using retroviral vectors.* Chirurgie 119:642–648
121. van der Wegen P, Louwen R, Imam AM, Buijs-Offerman RM, SinaasappelM, Grosveld F, Scholte BJ (2006) *Successful treatment of UGT1A1 deficiency in a rat model of Crigler-Najjar disease by intravenous administration of a liver-specific lentiviral vector.* Mol Ther 13:374–381
122. Seppen J, Bakker C, de Jong B, Kunne C, van den Oever K, Vandenberghe K, de Waart R, Twisk J, Bosma P (2006) *Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats.* Mol Ther 13:1085–1092
123. Montenegro-Miranda PS, Paneda A, Ten BL, Duijst S, de Waart DR, Aseguinolaza GG, Bosma PJ (2013) *Adeno-associated viral vector serotype 5 poorly transduces liver in ratmodels.* PLoS One 8:e82597
124. Montenegro-Miranda PS, Pichard V, Aubert D, Ten BL, Duijst S, de Waart DR, Ferry N, Bosma PJ (2014) *In the rat liver, adenoviral gene transfer efficiency is comparable to AAV.* Gene Ther 21:168–174
125. Philipp T, Durazzo M, Trautwein C, Alex B, Straub P, Lamb JG, Johnson EF, Tukey RH, Manns MP (1994) *Recognition of uridine diphosphate glucuronosyl transferases by LKM-3 antibodies in chronic hepatitis D.* Lancet 344:578–581
126. Strassburg CP, Obermayer-Straub P, Alex B, Durazzo M, Rizzetto M, Tukey RH, Manns MP (1996)

Autoantibodies against glucuronosyltransferases differ between viral hepatitis and autoimmune hepatitis. Gastroenterology 111:1576–1586

127. Bachrich T, Thalhammer T, Jager W, Haslmayer P, Alihodzic B, Bakos S, Hitchman E, Senderowicz AM, Penner E (2001) *Characterization of autoantibodies against uridine-diphosphate glucuronosyltransferase in patients with inflammatory liver diseases.* Hepatology 33:1053–1059
128. Keitel V, BurdelskiM, Vojnisek Z, Schmitt L, Haussinger D, Kubitz R (2009) *De novo bile salt transporter antibodies as a possible cause of recurrent graft failure after liver transplantation: a novel mechanism of cholestasis.* Hepatology 50:510–517
129. Astermark J (2006) *Basic aspects of inhibitors to factors VIII and IX and the influence of non-genetic risk factors.* Haemophilia 12(Suppl 6):8–13
130. Seppen J, van Til NP, van der Rijt R, Hiralall JK, Kunne C, Elferink RP (2006) *Immune response to lentiviral bilirubin UDP-glucuronosyltransferase gene transfer in fetal and neonatal rats.* Gene Ther 13:672–677
131. Takahashi M, Ilan Y, Chowdhury NR, Guida J, Horwitz M, Chowdhury JR (1996) *Long term correction of bilirubin-UDPglucuronosyltransferase deficiency in Gunn rats by administration of a recombinant adenovirus during the neonatal period.* J Biol Chem 271:26536–26542
132. Askari FK, Hitomi Y, Mao M, Wilson JM (1996). *Complete correction of hyperbilirubinemia in the Gunn rat model of Crigler-Najjar syndrome type I following transient in vivo adenovirus-mediated expression of human bilirubin UDP-glucuronosyltransferase.* Gene Ther 3:381–388
133. Gao G, Lebherz C, Weiner DJ, Grant R, Calcedo R,McCullough B, Bagg A, Zhang Y, Wilson JM (2004) *Erythropoietin gene therapy leads to autoimmune anemia in macaques.* Blood 103:3300–3302
134. Chenuaud P, Larcher T, Rabinowitz JE, Provost N, Cherel Y, Casadevall N, Samulski RJ, Moullier P (2004) *Autoimmune anemia in macaques following erythropoietin gene therapy.* Blood 103: 3303–3304
135. LoDuca PA, Hoffman BE, Herzog RW(2009) *Hepatic gene transfer as a means of tolerance induction to transgene products.* Curr Gene Ther 9:104–114
136. Montenegro-Miranda PS, Ten BL, Kunne C, de Waart DR, Bosma PJ (2011) *Mycophenolate mofetil impairs transduction of singlestranded adeno-associated viral vectors.* Hum Gene Ther 22:605–612
137. Brantly ML, Chulay JD, Wang L, Mueller C, Humphries M, Spencer LT, Rouhani F, Conlon TJ, Calcedo R, Betts MR, Spencer C, Byrne BJ, Wilson JM, Flotte TR (2009) *Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy.* Proc Natl Acad Sci U S A 106: 16363–16368
138. Brantly ML, Spencer LT, Humphries M, Conlon TJ, Spencer CT, Poirier A, Garlington W, Baker D, Song S, Berns KI, Muzyczka N, Snyder RO, Byrne BJ, Flotte TR (2006) *Phase I trial of intramuscular injection of a recombinant adeno-associated virus serotype 2 alphal-antitrypsin (AAT) vector in AAT-deficient adults.* Hum Gene Ther 17:1177–1186
139. Flotte TR, Trapnell BC, Humphries M, Carey B, Calcedo R, Rouhani F, Campbell-Thompson M, Yachnis AT, Sandhaus RA, McElvaney NG, Mueller C, Messina LM, Wilson JM, Brantly M, Knop DR, Ye GJ, Chulay JD (2011) *Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing alpha1- antitrypsin: interim results.* Hum Gene Ther 22:1239–1247
140. Worgall S, Sondhi D, Hackett NR, Kosofsky B, Kekatpure MV, Neyzi N, Dyke JP, Ballon D, Heier L, Greenwald BM, Christos P, Mazumdar M, Souweidane MM, Kaplitt MG, Crystal RG (2008)

Treatment of late infantile neuronal ceroid lipofuscinosis by CNS administration of a serotype 2 adeno-associated virus expressing CLN2 cDNA. Hum Gene Ther 19:463–474

141. Wagner JA, Reynolds T, Moran ML, Moss RB, Wine JJ, Flotte TR, Gardner P (1998) *Efficient and persistsent gene transfer of AAVCFTR in maxillary sinus.* Lancet 351:1702–1703
142. Wagner JA, Messner AH, Moran ML, Daifuku R, Kouyama K, Desch JK, Manley S, Norbash AM, Conrad CK, Friborg S, Reynolds T, Guggino WB, Moss RB, Carter BJ, Wine JJ, Flotte TR, Gardner P (1999*) Safety and biological efficacy of an adenoassociated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus.* Laryngoscope 109:266–274
143. Wagner JA, Nepomuceno IB, Messner AH, Moran ML, Batson EP, Dimiceli S, Brown BW, Desch JK, Norbash AM, Conrad CK, Guggino WB, Flotte TR, Wine JJ, Carter BJ, Reynolds TC, Moss RB, Gardner P (2002*) A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies.* Hum Gene Ther 13:1349–1359
144. Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, Milla C, Brody AS, Clancy JP, Ramsey B, Hamblett N, Heald AE (2004) *Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial.* Chest 125:509–521
145. Aitken ML, Moss RB, Waltz DA, Dovey ME, Tonelli MR, McNamara SC, Gibson RL, Ramsey BW, Carter BJ, Reynolds TC (2001) *A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease.* Hum Gene Ther 12:1907–1916
146. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, OzeloMC, Hoots K, Blatt P, Konkle B, DakeM, Kaye R, Razavi M, Zajko A, Zehnder J, Rustagi PK, Nakai H, Chew A, Leonard D, Wright JF, Lessard RR, Sommer JM, Tigges M, Sabatino D, Luk A, Jiang H, Mingozzi F, Couto L, Ertl HC, High KA, Kay MA (2006*) Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response.* NatMed 12: 342–347
147. Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, Tai SJ, Ragni MV, Thompson A, Ozelo M, Couto LB, Leonard DG, Johnson FA,McClellandA, Scallan C, Skarsgard E, Flake AW, Kay MA, High KA, Glader B (2003) *AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B.* Blood 101:2963–2972
148. Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, Rossi S, Lyubarsky A, Arruda VR, Konkle B, Stone E, Sun J, Jacobs J, Dell'Osso L, Hertle R, Ma JX, Redmond TM, Zhu X, Hauck B, Zelenaia O, Shindler KS, Maguire MG, Wright JF, Volpe NJ, McDonnell JW, Auricchio A, High KA, Bennett J (2008) *Safety and efficacy of gene transfer for Leber's congenital amaurosis.* N Engl J Med 358:2240–2248
149. Testa F, Maguire AM, Rossi S, Pierce EA, Melillo P, Marshall K, Banfi S, Surace EM, Sun J, Acerra C, Wright JF, Wellman J, High KA, Auricchio A, Bennett J, Simonelli F (2013) *Three-year followup after unilateral subretinal delivery of adeno-associated virus in patients with Leber congenital amaurosis type 2.* Ophthalmology 120:1283–1291
150. Ashtari M, Cyckowski LL, Monroe JF, Marshall KA, Chung DC, Auricchio A, Simonelli F, Leroy BP, Maguire AM, Shindler KS, Bennett J (2011) *The human visual cortex responds to gene therapy-mediated recovery of retinal function.* J Clin Invest 121:2160–2168
151. Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, Rossi S, Marshall K, Banfi S, Surace EM, Sun J, Redmond TM, Zhu X, Shindler KS, Ying GS, Ziviello C, Acerra C, Wright

JF,McDonnell JW,HighKA, Bennett J,Auricchio A(2010) *Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration.* Mol Ther 18:643–650

152. Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K, Viswanathan A, Holder GE, Stockman A, Tyler N, Petersen-Jones S, Bhattacharya SS, Thrasher AJ, Fitzke FW, Carter BJ, Rubin GS, Moore AT, Ali RR (2008) *Effect of gene therapy on visual function in Leber's congenital amaurosis.* N Engl JMed 358: 2231–2239
153. Gaudet D, Methot J, Dery S, Brisson D, Essiembre C, Tremblay G, Tremblay K, de Wal J, Twisk J, van den Bulk N, Sier-FerreiraV, van Deventer S (2013) *Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial.* Gene Ther 20:361–369
154. Stroes ES, Nierman MC, Meulenberg JJ, Franssen R, Twisk J, Henny CP, Maas MM, Zwinderman AH, Ross C, Aronica E, High KA, Levi MM, Hayden MR, Kastelein JJ, Kuivenhoven JA (2008) *Intramuscular administration of AAV1-lipoprotein lipase S447X lowers triglycerides in lipoprotein lipase-deficient patients.* Arterioscler Thromb Vasc Biol 28:2303–2304
155. Smith BK, Collins SW, Conlon TJ, Mah CS, Lawson LA, Martin AD, Fuller DD, Cleaver BD, Clement N, PhillipsD, Islam S, Dobjia N, Byrne BJ (2013) *Phase I/II trial of adeno-associated virus-mediated alpha-glucosidase gene therapy to the diaphragm for chronic respiratory failure in Pompe disease: initial safety and ventilator outcomes.* Hum Gene Ther 24:630–640
156. Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, Bowles D, Gray S, Li C, Galloway G, Malik V, Coley B, Clark KR, Li J, Xiao X, Samulski J, McPhee SW, Samulski RJ, Walker CM (2010) *Dystrophin immunity in Duchenne's muscular dystrophy.* N Engl J Med 363:1429–1437
157. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, Li J,Wang B, Monahan PE, Rabinowitz JE, Grieger JC, Govindasamy L, Agbandje-McKenna M, Xiao X, Samulski RJ (2012) *Phase 1 gene therapy for Duchennemuscular dystrophy using a translational optimized AAV vector.* Mol Ther 20:443–455
158. Mendell JR, Rodino-Klapac LR, Rosales XQ, Coley BD, Galloway G, Lewis S,Malik V, Shilling C, Byrne BJ, Conlon T, Campbell KJ, Bremer WG, Taylor LE, Flanigan KM, Gastier-Foster JM, Astbury C, Kota J, Sahenk Z,WalkerCM, Clark KR (2010) *Sustained alphasarcoglycan gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D.* Ann Neurol 68:629–638
159. Mendell JR, Rodino-Klapac LR, Rosales-Quintero X, Kota J, Coley BD, Galloway G, Craenen JM, Lewis S,Malik V, Shilling C, Byrne BJ, Conlon T, Campbell KJ, Bremer WG, Viollet L, Walker CM, Sahenk Z, Clark KR (2009) *Limb-girdle muscular dystrophy type 2D gene therapy restores alpha-sarcoglycan and associated proteins.* Ann Neurol 66:290–297
160. Mendell JR, Rodino-Klapac L, Sahenk Z, Malik V, Kaspar BK, Walker CM, Clark KR (2012) *Gene therapy for muscular dystrophy: lessons learned and path forward.* Neurosci Lett 527:90–99
161. D'Avola D, López-Franco E, Sangro B, Pañeda A, Grossios N, Gil-Farina I, Benito A, Twisk J, Paz M, Ruiz J, Schmidt M, Petry H, Harper P, de Salamanca RE, Fontanellas A, Prieto J, González-Aseguinolaza G. (2016) *Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria.* J Hepatololgy 65(4):776-83
162. Bartus RT, Baumann TL, Siffert J et al (2013) *Safety/feasibility of targeting the substantia nigra with AAV2-neurin in Parkinson patients.* Neurology 80:1698-1701

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A TRANSLATIONALLY OPTIMIZED AAV-UGT1A1 VECTOR DRIVES SAFE AND LONG-LASTING CORRECTION OF CRIGLER-NAJJAR SYNDROME

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Abstract

Crigler-Najjar syndrome is a severe metabolic disease of the liver due to a reduced activity of the UDP Glucuronosyltransferase 1A1 (UGT1A1) enzyme. In an effort to translate to the clinic an adeno-associated virus vector mediated liver gene transfer approach to treat Crigler-Najjar syndrome, we developed and optimized a vector expressing the UGT1A1 transgene. For this purpose, we designed and tested in vitro and in vivo multiple codon-optimized UGT1A1 transgene cDNAs. We also optimized noncoding sequences in the transgene expression cassette. Our results indicate that transgene codon-optimization is a strategy that can improve efficacy of gene transfer but needs to be carefully tested in vitro and in vivo. Additionally, while inclusion of introns can enhance gene expression, optimization of these introns, and in particular removal of cryptic ATGs and splice sites, is an important maneuver to enhance safety and efficacy of gene transfer. Finally, using a translationally optimized adeno-associated virus vector expressing the UGT1A1 transgene, we demonstrated rescue of the phenotype of Crigler-Najjar syndrome in two animal models of the disease, Gunn rats and Ugt1a1$^{-/-}$ mice. We also showed long-term (>1 year) correction of the disease in Gunn rats. These results support further translation of the approach to humans.

Introduction

Crigler-Najjar (CN) syndrome[1] is an ultra-rare (<1 in 1,000,000 individuals at birth[2,3]) autosomal recessive disease caused by the deficiency of liver-specific UDP Glucuronosyltransferase 1A1 (UGT1A1) enzyme, resulting in the toxic, life-threatening accumulation of unconjugated bilirubin (UCB) in all body tissues, and especially in the brain.[4,5] Depending on the underlying mutation in the UGT1A1 gene, the severity of CN syndrome can vary from mild to severe.[6,7] If not promptly treated, the severe form of CN rapidly leads to bilirubin encephalopathy also known as kernicterus, an irreversible and lethal brain damage[4,8] due to the neurotoxicity of UCB. Presently, severely affected patients are treated by whole-body exposure to phototherapy for up to 10–12 hours/day.[9,10] This is a cumbersome treatment with important shortcomings like a persistent risk of life threatening spikes of UCB due, for instance, to trauma or sepsis,[11,12] and a gradual loss of efficacy over time. Orthotopic liver transplantation to restore UGT1A1 activity is the only definitive cure for the severe form of CN syndrome,[2,10] however this approach presents several risks associated with the procedure[13,14] and the need for lifelong immunosuppression.[15] In view of all the limitations of the current therapies for CN syndrome, a novel curative treatment based on gene therapy appears to be a therapeutic option for the disease.

Adeno-associated virus (AAV) vector-mediated liver gene transfer has shown promising results in preclinical animal models and, more recently, in humans.[16–18] AAV vectors are derived and essentially identical to their wild-type counterpart, a small, nonenveloped parvovirus that is nonpathogenic and naturally replication deficient.[19] AAV vectors have become one of the gene therapy vectors of choice for all *in vivo* applications due to their excellent safety profile, poor proinflammatory profile, the fact that they do not efficiently integrate into the host genome, and importantly, the fact that they can drive multiyear expression of a donated transgene in humans.[17,20] Proof-of-concept of safe, efficacious, long-term correction of a number of diseases targeting the liver with AAV vectors exist in animal models,[21] including animal models of CN syndrome,[22–25] and in humans.[16–18,20] Additionally, several gene therapy trials for inherited liver metabolic disorders have been proposed[26] and will likely reach the clinic in the near future.

In addition to the advances in AAV gene transfer to the liver, CN syndrome is an ideal disease model for AAV vector-mediated liver gene transfer for a number of reasons, including (i) the liver of affected individuals is mostly structurally normal[27]; (ii) the therapeutic window is wide, with levels of UGT1A1

enzyme activity as low as 5%, sufficient to convert the disease from severe to mild[24,28,29]; (iii) a threshold for clinical benefit is well defined: serum bilirubinlevels below 20 mg/dl in most patients will result in significantly lower risks of brain damage[10]; (iv) assessment of therapeutic efficacy is straightforward with clinically well-established assays (*e.g.*, measurement of serum total bilirubin (TB) levels); (v) relevant animal models are available, such as the Gunn rat[30] and the Ugt1a1$^{-/-}$ mouse[24]; and (vi) failure to establish correction of the disease with gene transfer for UGT1A1 would not prevent conventional phototherapy from being efficacious or orthotopic liver transplantation from being performed.

Here we developed a novel AAV vector encoding for the UGT1A1 transgene and we optimized the expression cassette for safe and long-term expression in liver. Our results indicate that codon optimization of the transgene, together with intron optimization, results in higher levels of transgene expression and are able to correct the pathological accumulation of UCB in both mice and rats affected by CN syndrome. Moreover, our data indicate that one important determinant of long-term stability of gene transfer in liver is the level of hepatocyte proliferation. Results shown here support the translation of this novel *in vivo* therapeutic approach to humans.

Materials and methods

Plasmid constructs

The transgene expression cassettes used in this study contained a wild-type (wt) or two codon-optimized cDNA sequences encoding for human UGT1A1 (v1 and v2 respectively). Codon-optimized sequences were obtained using commercially available algorithms and further modified to remove alternate open reading frames. Transgene sequences were cloned in an AAV backbone comprising the wild-type AAV2 ITRs, an enhancer derived from apolipoprotein E gene, and the hAAT promoter. The human hemoglobin beta (HBB)-derived synthetic intron (HBB2)[34] and the HBB polyadenylation signal were used in all UGT1A1 expression cassettes. The in vitro comparison of luciferase expression levels has been performed with the same expression cassette by replacing the UGT1A1 transgene with the firefly luciferase cDNA. The evaluation of the effect of different introns on the transgene expression levels has been performed by replacing the HBB2 sequence with different intron sequences, namely SV40 intron[60] and human coagulation factor IX intron 1[35]. HBB2 and coagulation factor IX intron sequences

were also modified by removing the ATGs that originated alternative open reading frames longer than 50bp. All DNA sequences used in the study were synthetized by GeneCust (Dudelange, Luxemburg).

AAV vectors

AAV vectors used in this study were produced using a slight modification of the adenovirus-free transient transfection methods as described earlier.[61,62] Briefly, adherent human embryonic kidney cells (HEK293) cells grown in roller bottles were transfected with the three plasmids containing the adenovirus helper proteins, the AAV Rep and Cap genes, and the ITR-flanked transgene expression cassette. After 72 hours of transfection, cells were harvested, lysed by sonication, and treated with benzonase (Merck-Millipore, Darmstadt, Germany). Vectors were then purified using two successive ultracentrifugation rounds in cesium chloride density gradients. Full capsids were collected, the final product was formulated in sterile phosphate buffered saline containing 0.001% of pluronic (Sigma Aldrich, Saint Louis, MO), and stored at −80°C.

Titers of AAV vector stocks were determined using quantitative realtime polymerase chain reaction (qPCR). Viral DNA was extracted using the MagNA Pure 96 DNA and viral NA small volume kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. The qPCR was performed in ABI PRISM 7900 HT Sequence Detector using Absolute ROX mix (Taqman, Thermo Fisher Scientific, Waltham, MA). Specific probe and primers were as follows: forward 5'-GGCGGGCGACTCAGATC-3', reverse 5'-GGGAGGCTGCTGGTGAATATT-3', and probe 5'-AGCCCCTGTTTGCTCCTCCGATAACTG-3'.

In vitro experiments

For plasmids transfection, plasmids were transfected using Lipofectamine (Thermo Fisher Scientific) into six-well plates containing 80% confluent Huh-7 cells accordingly to manufacturer's instructions. green fluorescent protein (GFP) expressing plasmids or untreated cells were included as control in each transfection experiment. After 48 hours of transfection, cells were harvested and frozen at −20 °C until further analysis.

RNA extraction and reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from cell lysates using Trizol (Thermo Fisher Scientific). DNA contaminants were removed using the Free DNA kit (Thermo Fisher Scientific). Total RNA was reverse-transcribed using random hexamers and the RevertAid H minus

first strand cDNA synthesis kit (Thermo Fisher Scientific). Reverse transcription-quantitative polymerase chain reaction was performed using SybrGreen (Thermo Fisher Scientific) with primers specific for the UGT1A1 transgene: forward TTCCTGCTGGCCGTGGTGCTGA and reverse AGATCTGAATTCACCCCACC. The rat albumin expression levels were evaluated using the following primers: forward GTGGAAGAGCCTCAGAAT, reverse TTGGTGTAACGAACTAATAGC, and were used to normalize the results across samples. Data were expressed as normalized number of copies of UGT1A1 per µg of RNA.

M*icrosome extraction and Western blot analysis*

Microsome extraction was performed at 4 °C. Frozen cell pellets were resuspended in 300 µl of lysis buffer (20 mmol/l Hepes, 1% Triton X-100) containing, proteases inhibitor cocktail (Sigma Aldrich) and centrifuged 5 minutes at 100×*g*. Supernatants were collected and centrifuged 60 minutes at 18,000×*g*. Pellets were resuspended in 100 µl of 20 mmol/l Hepes and the protein concentration was determined using the BCA Protein Assay kit (Thermo Fisher Scientific), following the manufacturer's instructions. Microsomal extracts were separated on a 4–12% bis-tris polyacrylamide gel (Thermo Fisher Scientific). The same amount of protein was loaded in each lane. The gel was transferred onto a nitrocellulose membrane and blotted with an anti-UGT1A1 antibody (SantaCruz biotechnology, Santa Cruz, CA) and an anti-actin antibody (Sigma Aldrich), used as loading control. Secondary antibodies and detection system were from Li-Cor Biosciences (Lincoln,NE).

Image Stream X analysis

Huh-7 cells were transfected as indicated above. After 48 hours of transfection, cells were harvested, fixed and permeabilized with the PerFix-nc kit (Beckmann Coulter, Brea, CA). Cells were then stained with a rat antihuman UGT1A1 antibody and an anti-78 KDa glucose related protein (GRP-78) as primary antibody (StressMarq, Cadboro Bay, Canada) and then with antirabbit AlexaFluor 488 and an antirat AlexaFluor 594 secondary antibodies (Thermo Fisher Scientific). Cells were analyzed at a magnification of 60× on an Image Stream X flow imaging equipment (Merck-Millipore). Image analysis was performed using the IDEAS analysis software v6.1 (Merck-Millipore). Single-color controls were used for the creation of a compensation matrix that was applied to all files to correct for spectral cross-talk. Cells in focus were gated based on the fluorescence levels observed in the two channels considering the nonspecific signal obtained with a nonspecific rat serum. Images with a bright detail similarity score >1.2 were used

to compare the endoplasmic reticulum localization of UGT1A1 expressed by the three different codon optimization constructs.

Primer elongation assay

A diagram of the primer elongation assay is shown as Supplementary Figure S2. Total RNA was extracted from Huh-7 cells using Trizol. Primer elongation has been performed as already described.[63] Briefly, 5 µg of total RNA were retrotranscribed using the RevertAid H minus first strand cDNA synthesis kit (Thermo Fisher Scientific) and a reverse IR-Dye 700 conjugated oligo (IDT technology, Coralville, IA) GTGATTCCACAGCCATGGTG specific for the firefly luciferase sequence. Samples were then separated on a 6% trisborate-EDTA-Urea gel (Thermo Fisher Scientific) and the gel was acquired using an Odissey scanner (Li-Cor Biosciences)

Animal experiments

Ugt1a1$^{-/-}$ mice have been described previously.[24] Wild type littermates were used as controls in all experiments. Mice were housed and handled according to institutional guidelines, and experimental procedures approved by International Centre for Genetic Engineering and Biotechnology board. Animals used in this study were at least 99.8 % C57BL/6 genetic background, obtained after > 9 backcrosses with wild type C57BL/6 mice. Mice were kept in a temperature-controlled environment with 12/12 hour light–dark cycle. They received a standard chow diet and water *ad libitum*.

The Gunn rat is a natural occurring model of CN syndrome that has no residual UGT1A1 enzyme activity.[64] Rats were fed *ad libitum* and were housed and handled according to institutional guidelines. All *in vivo* experimental procedures were approved by the French, Dutch, and Italian competent authorities and Ethical Committees (ref. 2013007B) according to the European Directive 2010/63/EU.

Gene transfer procedure and phototherapy treatment

For the AAV gene transfer procedure, mice pups at postnatal day 11 (P11) were i.p. injected with AAV8-UGT1A1 vectors or saline. Newborns were exposed to blue fluorescent light (Philips, Amsterdam, The Netherlands) for 12 hours per day (synchronized with the light period of the light/dark cycle) up to 12 days after birth and then maintained under normal light conditions. Intensity of the blue lamps was monitored monthly with an Olympic Mark II Bili-Meter (Olympic Medical, Port Angeles, WA). Blood samples were collected 1 month postinjection in mutant and untreated mutant littermates by facial vein exsanguination or cardiac puncture.

Rats of 6–8 weeks old, were injected i.v. via the tail vein with AAV8-UGT1A1 vectors or saline. Blood samples were collected by retro-orbital venipuncture every week after AAV injection. To determine UGT1A1 expression, liver tissues were collected 2 months post AAV-injection. For the long term-follow-up, blood samples were collected every month after AAV injection. Livers and other organs were collected 13 months post AAV-injection.

Bilirubin measurement

TB determination in mice and rats was performed in plasma as previously described. Plates were read at 560 nm on an Enspire plate reader (Perkin Elmer, Waltham, MA). Bilirubin conjugates in bile were analyzed and quantified by high performance liquid chromatography as previously described[22] using an Omnisphere column (Varian, Palo Alto, CA) for the separation of bilirubin conjugates.[65]

Virus vector genome copy number analysis

To reduce variability generated by uneven transduction of liver parenchyma by AAV vectors, whole rat livers were homogenized in 20 mmol/l Hepes, 250 mmol/l sucrose. For mouse samples, livers were harvested 1 month postinjection, pulverized in liquid nitrogen and aliquoted for further molecular analysis.

Total DNA was extracted using the MagNA Pure 96 DNA and viral NA small volume kit (Roche Diagnosis, Basel, Switzerland) according to manufacturer's instructions. VGCN measured by qPCR were normalized by the copies of titin gene measured in each sample. qPCR was performed on an ABI PRISM 7900 HT Sequence Detector (Agilent Technologies, Santa Clara, CA) using Absolute ROX mix (Thermo Fisher Scientific) and the following specific primers and probes: UGT1A1 forward 5′-GGCGGGCGACTCAGATC-3′, reverse 5′-GGGAGGCTGCTGGTGAATATT-3′, probe 5′-AGCCCCTGTTTGCTCCTCCGAT AACTG-3′; titin forward 5′-AGAGGTAGTATTGAAAACGAGCGG-3′, reverse 5′-GCTAGCGCTCCCGCTGCTGAAGCTG-3′, and probe 5′-TGCAAGGAAGCTTCTCGTCTCAGTC-3′.

VGCN in mice was quantified by qPCR using specific primers for the hAAT promoter as previously described.[25]

Western blot analysis

Microsomes extraction was performed at 4°C. The resulting material was resuspended in microsome buffer and the protein concentration was determined

by Pierce BCA Protein Assay (Thermo Fisher Scientific), following manufacturer's instructions. Western blot on microsomal extracts was performed as described above with anti-UGT1 rabbit polyclonal antibody (SantaCruz Biotechnology).
Anti-actin (Sigma Aldrich), or anti tubulin antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) were used as loading controls. For mouse samples, liver powder was homogenized in lysis buffer containing 150 mmol/l NaCl, 1% nonidet-P40, 0.5% sodium deoxicolate, 0.1% sodium dodecil sulfate, 50 mmol/l Tris HCl pH8 and protease inhibitors and analyzed by Western blot analysis as described previously.[24]

Anti-UGT1A1 antibody determination

Maxisorp 96 wells plates (Thermo Fisher Scientific) were coated with UGT1A1 protein in carbonate buffer at 4 °C overnight. A standard curve of rat recombinant IgG (Sigma Aldrich) was coated to the wells in seven twofold dilution starting from 1 µg/ml. After blocking, plasma samples were added to plates and incubated 1 hour at 37 °C. Detection was performed by adding to the wells 3,3′,5,5′-tetramethylbenzidine substrate (BD Biosciences, San Diego, CA), and color development was measured at 450 and 570 nm (for background subtraction) on an Enspire plate reader (Perkin Elmer) after blocking the reaction with H2SO4.

To detect antibodies against the human UGT1A1 protein in mice, 20 µg of total cell protein extract from HEK293 cells transfected with a plasmid expressing the human UGT1A1 cDNA or from untransfected HEK293 cells were run in 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, blotted onto a nitrocellulose membrane and blocked with 5% milk in Phosphate buffered saline with Tween 20. Plasma from individual animals was used as primary antibody (1:200).

A positive control plasma from mice immunized with recombinant human UGT1A1 was used (1:2,000). A band corresponding to the UGT1A1 protein is detected if antibodies against UGT1A1 are present in the plasma of the AAV-treated animals.

Statistics

Results are expressed as mean ± SEM or mean ± SD as described in the text. The Prism package version 7 (GraphPad Software, La Jolla, CA) or StatistiXL plugin for windows Excel were used to analyze data and prepare the graphs. Statistical analyses have been performed by *t*-test and one way or two-way analysis of variance as described in the text. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

Codon-optimization of the UGT1A1 cDNA leads to higher protein levels in a human hepatocyte cell line

Codon-optimization of the cDNA encoding for a therapeutic transgene has been used to enhance the therapeutic efficacy of AAV vectors.[31] Here we applied two codon optimization algorithms to the human UGT1A1 cDNA in order to achieve higher expression of the transgene. The two optimized sequences were significantly different from the wild-type cDNA encoding for the UGT1A1 transgene (Table 1), with the version 1 (v1) of the cDNA showing a codon adaptation index (CAI)32 of 0.76, identical to the CAI of the wild-type UGT1A1 sequence (wt). Conversely, the CAI of the version 2 (v2) of the cDNA had an enhanced CAI of 0.96, predictive of higher translational efficiency32 (Figure 1a). Additionally, v1 had similar GC content to the wt cDNA (55.30% and 50.49%, respectively), while GC content in the v2 was higher (60.59%) (Figure 1b), further confirming a higher level of sequence optimization for the v2 sequence. Next, we determined mRNA and protein expression levels of the wt and codon-optimized UGT1A1 transgenes *in vitro*. Identical expression cassettes were generated based on the liver-specific alpha 1 antitrypsin promoter and apolipoprotein E enhancer (hAAT) promoter and transfected into a Huh-7 human hepatoma cell line. Levels of mRNA and protein were measured 48 hours after transfection. Slightly higher levels of UGT1A1 mRNA were measured in triplicate testing (Figure 1c), while significantly higher levels of UGT1A1 protein were detectable by western blot in cell lysates following transfection of the v1 and v2 constructs (Figure 1d, analysis of variance P = 0.035 and 0.036, respectively).

Table 1. Characteristics of wild-type and codon optimized UGT1A1 cDNA sequences

Sequence	Similarity (% vs wt)	CAI (average)	GC content (%)
wt	100	0.76	50.5
V1	77.5	0.76	55.3
V2	78.3	0.96	60.6

CAI, codon adaptation index (CAI) and GC content of the three sequences have been calculated using an online codon analysis tool (http://www.genscript.com/cgibin/tools/rare_codon_analysis).

Next, we evaluated whether the UGT1A1 protein was correctly localized to the endoplasmic reticulum, even when overexpressed. Huh-7 cells were transfected in duplicate with the wt, v1, or v2
constructs and the colocalization of the UGT1A1 protein with the 78 kDa glucose related protein (GRP-78) marker was evaluated on an ImageStreamer X system. Representative output of the assay is shown in Figure 1 e, f. In all experiments, staining for UGT1A1 colocalized to the same extent with that for GRP-78 (Figure 1g), indicating that the overexpression of the transgene deriving from codon-optimization did not influence intracellular localization of UGT1A1.

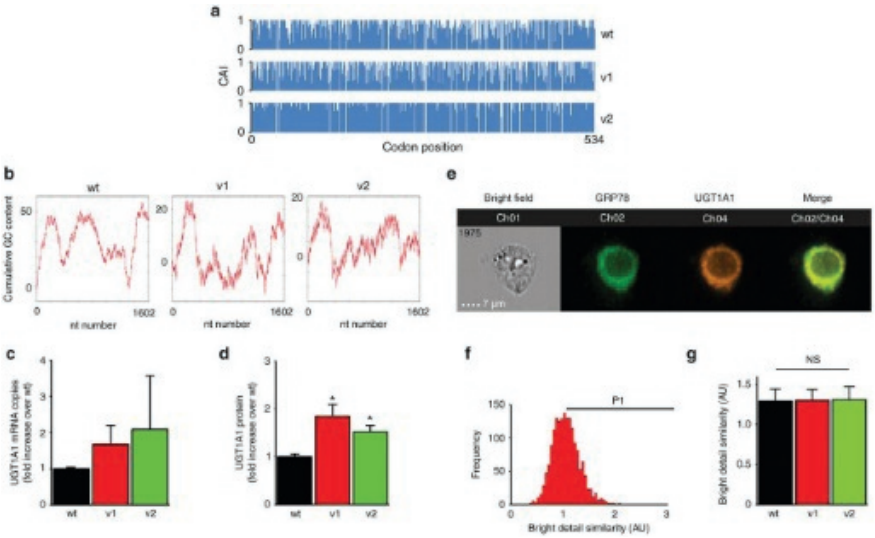

Figure 1. (**a**) Graph showing the codon adaptation index (CAI) for each codon of the wild-type (wt), codon optimized version 1 (v1), and codon optimized version 2 (v2) UGT1A1 cDNA. A CAI equal to 1 indicates that the frequency of the codons used is the same of that of the most expressed genes in humans. (**b**) Cumulative GC nucleotides content of wt, v1, and v2 UGT1A1 cDNAs. (**c,d**). The wt, v1, and v2 UGT1A1 transgenes were expressed under the control of the hAAT promoter in Huh-7 cells. 48 hours after transfection mRNA (**c**) and protein (**d**) levels were analyzed. Data are expressed as mean ± SEM of triplicate experiments. Statistical analyses in panel **c** and **d** have been performed by ANOVA (* = p<0.05). UGT1A1 mRNA levels were normalized to the expression levels of albumin and then normalized for the levels measured in cells transfected with the wtUGT1A1 plasmid. UGT1A1 protein levels were normalized on the level of calnexin and then to the levels observed in cells transfected with the wtUGT1A1 plasmid. Statistical analyses in panel **c** and **d** have been performed by ANOVA (* = p<0.05). (**e-g**) Huh-7 cells were transfected with wtUGT1A1, v1UGT1A1 or v2UGT1A1. 48 hours after transfection cells were trypsinized and stained for 78 KDa glucose-regulated protein (GRP78) and UGT1A1, as described in Materials and Methods. Cells were acquired at 60X using ImageStream®X Mark II Imaging Flow Cytometer. (**e**) Example of imaging of UGT1A1 transfected cells. Ch01, bright field; Ch02, in green, channel corresponding to the GRP78 staining; Ch04, in orange, channel corresponding to UGT1A1 staining. Channel Ch02/Ch04 shows the fluorescence overlay and in yellow the regions of the cell where the two staining colocalizes. (**f**) The graph shows the frequency of the Bright Detail similarity for GRP78 and UGT1A1 used for colocalization analysis. (**g**) Statistical analysis of the bright detail similarity obtained in cells transfected with wtUGT1A1, v1UGT1A1 or v2UGT1A1. In the graph are showed the median ± SD of the bright detail similarity of P1 population. Statistical analysis has been performed by ANOVA (* = p<0.05).

Removal of cryptic ATGs from introns in expression cassette results in higher transgene expression levels

Cryptic translation startcodons have been described as potential triggers of transgene immunogenicity.[33] While codon-optimization resulted in removal of alternative open reading frames from the UGT1A1 cDNA (not shown), we focused our attention on the synthetic intron present in the transgene expression cassette. Open reading frames analysis of the sequence of the human haemoglobin subunit beta (HBB2) synthetic intron[34] revealed the presence of three ATGs in position 128, 308, and 363, between the splicing donor and splicing acceptor of the intron at position 23 and 392, respectively (Figure 2a).

After elimination of all ATGs, the optimized HBB2 intron and its unmodified counterpart were cloned in a luciferase expressing cassette under the control of the liver-specific hAAT promoter (see Materials and Methods). As controls, luciferase expressing plasmids carrying the SV40 intron (naturally devoid of cryptic ATGs) and the intron 1 of coagulation factor IX[35] (untouched or optimized, Supplementary Figure S1) were also generated. Triplicated transient transfection experiments were performed in which the luciferase expression levels were measured and normalized to the SV40 intron construct. Interestingly, optimization of both the HBB2 and the coagulation factor introns led to significantly higher luciferase expression levels in Huh-7 cells (*t*-test, $P = 0.002$ and 0.005, respectively) (Figure 2b), indicating that cryptic translation start sites present in synthetic introns can negatively influence transgene expression levels.

To identify the mechanism by which the intron optimization influenced transgene expression levels, a primer elongation assay was performed using a reverse oligo localized at the 5′ of the luciferase transgene sequence. This oligo was annealed and elongated by reverse transcriptase, using messenger RNA as a template. The schematic representation of the assay is shown in Supplementary Figure S2. Primer elongation analysis of the mRNA isolated from cells transfected with the SV40 and HBB2 constructs revealed the presence of a prominent ~200 bp band corresponding to the predicted fully-processed mRNA, together with two other higher molecular weight (~400 bp) bands corresponding to alternative splicing forms of the mRNA (Figure 2c). These alternatively spliced mRNA forms were not detectable in the optimized HBB2 intron, possibly due to the destabilization of these transcripts. Interestingly, in another set of experiments, we tested luciferase expression levels deriving from constructs carrying HBB2 introns devoid of any splice acceptor sites (splicing incompetent), either carrying

or not cryptic ATGs (see Supplementary Figure S3). In these experiments, splicing deficient constructs showed lower levels of luciferase expression compared with the splicing competent construct, and the removal of the cryptic ATGs in the splicing deficient construct resulted in enhanced luciferase expression levels (Figure 2d).

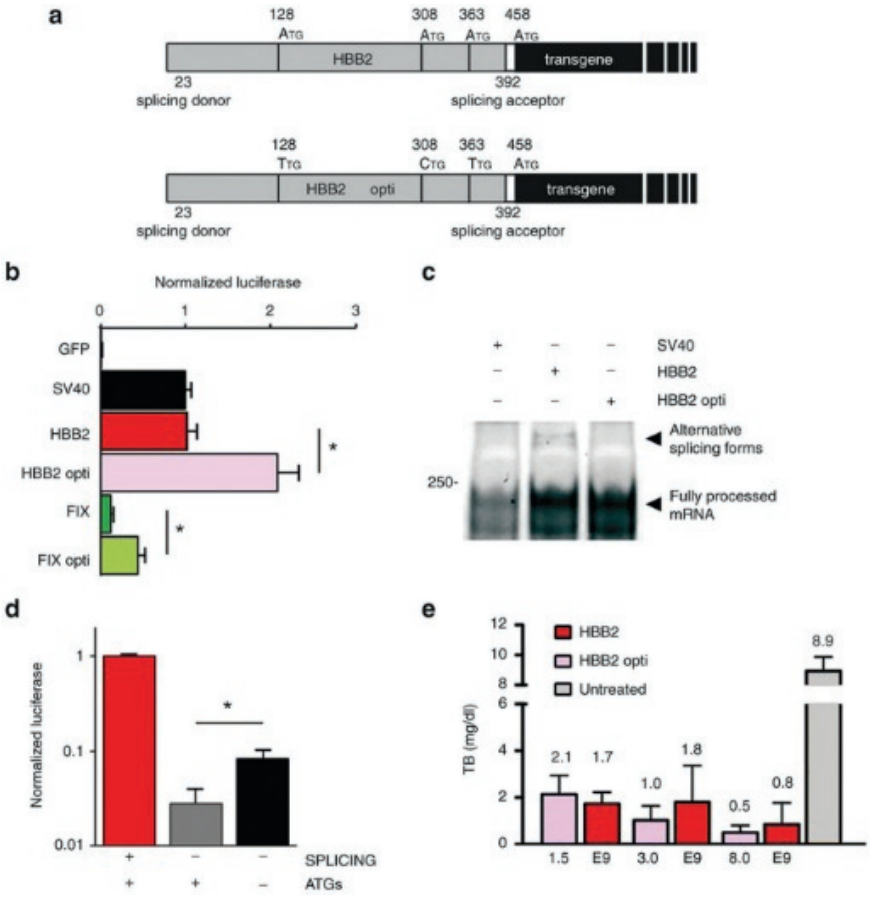

Figure 2. (**a**) Schematic diagram illustrating the position of ATGs, splicing donor (SD) and acceptor (SA) in the sequence of the HBB2 intron. The position is calculated from the start of the HBB2 intron (441 base pair). The optimized version of the intron (HBB2 opti) does not contain ATGs. (**b**) Huh-7 cells were transfected with plasmids expressing GFP or luciferase fused with introns SV40, HBB2, HBB2 opti, FIX and FIX opti described in Materials and Methods under the transcriptional control of a liver specific promoter. The graph shows the mean ± SD of luciferase levels measured 48 hours after transfection and normalized to the levels observed with construct carrying the SV40 intron. Data derived from three independents experiments, statistical analysis was performed by non-parametric t-test (* = $p<0.05$). (**c**) Primer elongation assay performed on the mRNA obtained from Huh-7 cells transfected with a plasmid expressing firefly luciferase fused with the SV40 or the HBB2 intron. The size of the band of a molecular weight standard running in parallel with the sample is indicated on the left. (**d**)

Huh-7 cells were transfected with plasmids expressing luciferase under the transcriptional control of a liver specific promoter and fused with the HBB2 intron (in red), the HBB2 sequence mutagenized to remove all the predicted splicing sites (in gray) or the same sequence mutagenized to remove all the predicted splicing sites and all the cryptic ATGs (in black). The graph shows the mean ± SD of luciferase levels measured in three different samples 48 hours after transfection and normalized on the levels observed in first sample. Data derived from three independents experiments, statistical analysis has been performed by non-parametric t-test (* = $p<0.05$). (**e**) Total plasma bilirubin levels (TB) in mutant mice treated with the indicated doses of AAV8 vectors (expressed in vg/mouse) containing the UGT1A1 transgene under the control of the hAAT promoter and carrying either the HBB2 (in red) or the optimized HBB2 intron (in pink). TB levels were measured 1 month post-injection. For each dose/vector n=4. Untreated animals (in gray) were used as negative control (n=4).

Together these results indicate that the presence of cryptic ATGs in introns can negatively influence transgene expression levels. This negative effect correlates, at least for the HBB2 synthetic intron, with the presence of unprocessed and partially processed forms of mRNA. This leads to the formation of mRNAs containing cryptic ATGs that are inefficiently transcribed and possibly leading to the production of aberrant proteins.

Next, we produced AAV8 vectors carrying the UGT1A1 transgene under the control of the hAAT promoter and carrying either the HBB2 or the optimized, ATG-free HBB2 intron. AAV8 vectors were injected intraperitoneally (i.p.) at escalating doses to Ugt1a1$^{-/-}$ mice at postnatal day 11 (P11). Ugt1a1$^{-/-}$ mice present a phenotype that closely resembles the human condition, therefore unless treated they need to be exposed to phototherapy to survive.[24,36] Phototherapy was discontinued two days after gene transfer and the animals were housed in normal light conditions. One-month post injection plasma bilirubin levels were determined. We reported a partial correction of serum TB levels (~2.0 mg/dl or 34 µmol/l) at the lowest vector dose (1.5E9 vector genomes (vg)/mouse, corresponding to 3.0E11 vg/kg). This level of TB is considered well below the limit of toxicity.25,36 We observed a dose-dependent effect on TB levels, reaching the levels of wild type littermates24 at the highest dose, corresponding to 1.6E12 vg/kg (8.0E9 vg/mous). In the midand high-dose treatment groups, lower levels of TB (although not statistically significant) were measured in animals treated with the construct carrying the optimized HBB2 intron (Figure 2e).

The degree of codon-optimization does not accurately predict UGT1A1 transgene expression levels in vivo

Based on codon optimization results (Figure 1) we developed three AAV8 vectors expressing the UGT1A1 transgene under the control of the hAAT promoter, AAV8-hAAT-wtUGT1A1, AAV8-hAAT-v1UGT1A1, and AAV8-hAAT-v2UGT1A1. All constructs carried the optimized HBB2 intron. Vectors were produced, titered side

by side and injected via the tail vein into 6 to 8-weeks old Gunn rats[22] (*n* = 10/group) and TB wasmeasured over time for about 7 weeks after vector delivery. Gunn rats have a milder phenotype compared with Ugt1a1$^{-/-}$ mice, as they do not need phototherapy to survive despite the high serum levels of TB (8.2 ± 1.9 mg/dl or 140 ± 32 µmol/l, mean ± SD, in our colony). At a dose of 5E10 vg/kg, partial correction of TB levels was observed with all 3 constructs tested, however, the v2 construct, the one displaying the highest CAI and GC content (Figure 1a, b, respectively), resulted in the lowest and most transient levels of correction of serum TB (Figure 3a, left), indicating lower potency. At a higher vector dose, 5E11 vg/kg, full correction of TB levels was observed in all animals treated (Figure 3a, right), comparable to those of wild-type animals (0.2 ± 0.3 mg/dl, or 3.3 ± 4.6 µmol/l, in our colony). No difference in expression levels was observed in animals treated with the wt or v1 UGT1A1 constructs, regardless of the dose administered (Figure 3a). No statistical differences in serum TB levels were observed between male and female animals from the same treatment cohorts (two-way analysis of variance, sex per treatment, $P > 0.05$ for all treatments).

Vector genome copy number (VGCN) and mRNA levels were also measured in all treated animals, 50 days after vector administration. While VGCN/cell were similar in animals treated with the three vectors (Figure 3b), animals treated with the v2UGT1A1 vector construct showed slightly lower levels of mRNA in liver compared with wt and v1 UGT1A1 treated animals, particularly at the low vector dose (Figure 3c). Differences in mRNA levels were not statistically significant. Being the UGT1A1 transgene based on the human sequence, we also measured antitransgene antibodies. As already reported,[22] several animals were scored positive for anti-UGT1A1 antibodies in all treatment groups, however these antibodies did not affect transgene expression levels or degree of correction of TB (Supplementary Figure S4).

Finally, as a measure of enzyme activity, we collected bile from all treated animals and measured presence of mono- and di- glucuronidated bilirubin (MCB, and DCB, respectively). Untreated Gunn rats have no detectable conjugated bilirubin in bile.22 Levels of conjugated bilirubin in bile correlated with levels of TB in serum (R2 = 0.44; *P*-value = 2.33E-8). Content of conjugated bilirubin (expressed as % of MCB and DCB over TB) was the highest in animals treated with the highest dose of wt and v1 UGT1A1 vectors, 5E11 vg/kg, while bile from animals treated with the highest dose of v2 UGT1A1 had CB levels equal to animals treated with a log lower dose of wt and v1 UGT1A1 vectors (Figure 3d). This highlighted significant differences in phenotype correction among experimental groups. The

lowest levels of CB in bile and highest levels of TB in serum were found in animals treated with 5E10 vg/kg of v2UGT1A1 vector (Figure 3d).

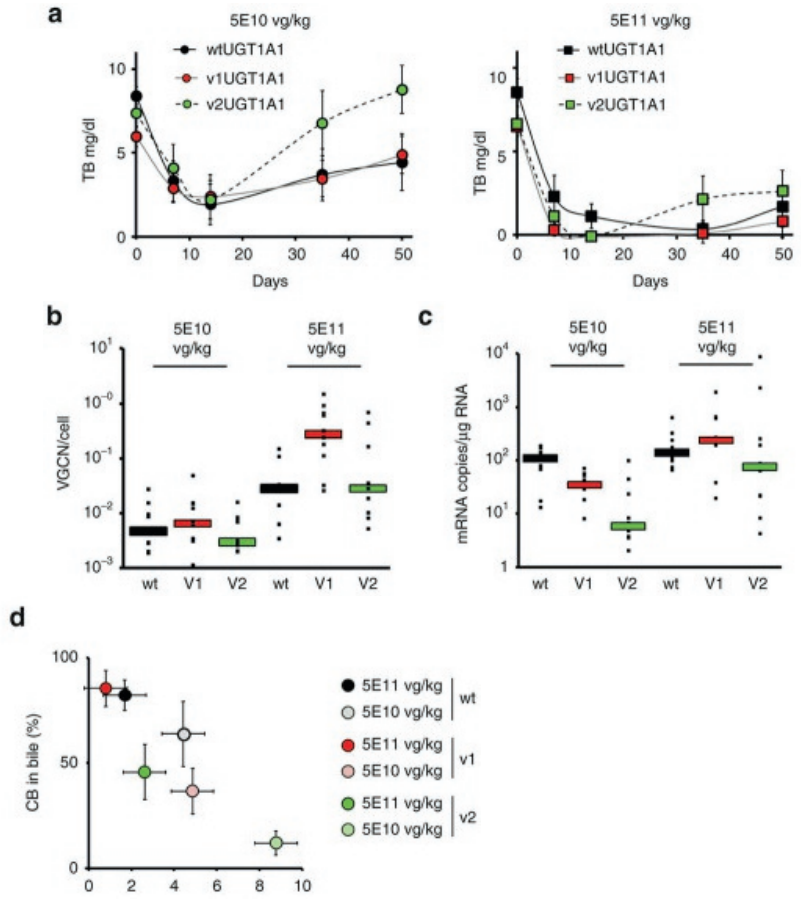

Figure 3. Comparison of the therapeutic efficacy of AAV8 vectors carrying the wt, v1, or v2 UGT1A1 transgenes in Gunn rats. (**a**) Animals (n=5 females and 5 males) were injected with AAV8 vectors expressing the wtUGT1A1 (black), v1UGT1A1 (red), and v2 UGT1A1 (green) transgene at the dose of 5E10 (left) and 5E11 vg/kg (right). Animals were bled weekly and sacrificed two months after the injection of the vectors. Results are shown as mean ± SD. (**b**) Vector genome copy number (VGCN) per cell measured by qPCR. Values were normalized to the number of copies of titin measured in each sample. In the figure are showed the single values measured (small black dots) and the median value for each treatment (line with the same color code as in **a**). (**c**) UGT1A1 mRNA levels normalized to the levels of rat albumin mRNA. (**d**) Bilirubin conjugates measured by HPLC in the bile of AAV treated Gunn rats. The graph shows the mean ± SD of the levels of total conjugated bilirubin observed in bile (CB in bile) as a function of the total bilirubin (TB) measured in serum. Darker colors represented the values observed for the high dose whereas lighter colors represented the low dose. Statistical analysis has been performed by ANOVA (* = $p<0.05$).

These results indicate that the outcome of codon-optimization is highly dependent on the transgene, and that testing transgene expression in multiple models *in vitro* and *in vivo* is key to identify the ideal candidate sequence.

Optimized AAV vectors expressing the UGT1A1 transgene correct the disease phenotype in a severe mouse model of CN syndrome

To assess the ability of the newly developed AAV vector construct to correct the phenotype of CN syndrome in a clinically-relevant scenario, Ugt1a1$^{-/-}$ mice were treated at P11 with 1.5E9, 3.0E9, or 8.0E9 vg/mouse of the vectors carrying the wt and v1 transgenes. The v2 transgene was not tested because less efficacious in rats. Complete correction of serum TB was observed in animals treated with the wt and v1 UGT1A1 transgenes (Figure 4a), to levels undistinguishable from the wild-type littermates.24 Conversely, despite phototherapy, untreated Ugt1a1$^{-/-}$ animals shown elevated TB levels in serum. One-month postinjection livers were harvested and VGCN/cell was determined. We showed good correlation of VGCN/cell with TB levels, with the exception of the animals treated with the highest dose of vector, in which lower VGCN/cell in liver (not significant) was measured in v1UGT1A1 treated animals (Figure 4b), despite the completely correction of TB in serum (Figure 4a). This result is likely due to the variability of the assay. Likewise, levels of UGT1A1 protein determined by western blot in livers at sacrifice correlated well with serum TB levels and VGCN/cell results (Figure 4c).

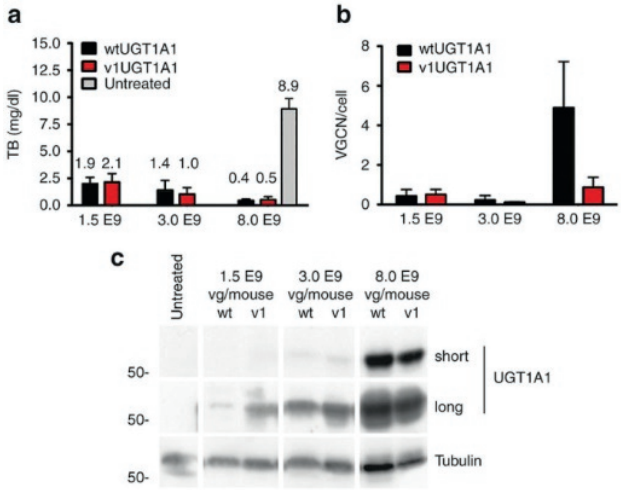

Figure 4. In vivo comparison of WT and v1 vectors efficiency in a mouse model of hyperbilirubinemia. (**a**) Total plasma bilirubin levels (TB, mg/dL) in mutant mice treated with escalating doses of optimized AAV8 vectors containing UGT1A1 cDNA. TB levels were measured 1 month post-injection. For each dose/vector, n≥3. Untreated animals were used as negative control (n=4). (**b**) Viral genome determination (VGCN/cell) performed 1 month post-injection. (**c**) Western blot analysis of total liver extract obtained from UGT1A1 KO mice, untreated or treated with AAV8-UGT1A1 wt or v1 (wt and v1, respectively). Antitubulin was used as loading control.

Differently from rats, none of the animals treated developed antihuman UGT1A1 antibodies (Supplementary Figure S5), indicating that the observation in rats was likely to be species-specific.

These results indicate that AAV8-hAAT-UGT1A1 vector mediated liver gene transfer with an optimized expression cassette can correct CN syndrome in a model closely mimicking severely affected individuals.

Long-term correction of CN syndrome with an optimized AAV8-UGT1A1 vector

To assess the ability of the optimized AAV8-hAAT-v1UGT1A1 vector to safely and effectively correct CN syndrome long-term, 6–8 week-old Gunn rats were treated with 5E12 vg/kg of vector delivered via the tail vein. In this experiment, five male and five female animals were treated. Animals were followed for ~400 days after vector delivery. As expected vector genome biodistribution showed the highest levels of VGCN/cell in liver (Supplementary Figure S6a).

After vector administration, TB levels quickly decreased to undetectable levels and remained undetectable for the duration of the observation period (>1 year) in seven out of 10 animals. This well correlated with VGCN/cell and human UGT1A1 protein levels in liver (Supplementary Figure S6b, c). In three animals,

all males, a gradual loss of correction was observed. None of the female rats lost therapeutic efficacy (Figure 5a). At sacrifice, no gross lesions were evident following necropsy performed by a certified pathologist (not shown), however it was noted that male rats had heavier livers compared with females. To further investigate this point, we collected and weighted livers in untreated male and female Gunn rats from birth to 26 weeks of age. This analysis revealed that livers of male rats grew in weight after birth for a longer period of time and reached larger size than the livers isolated from female rats (Figure 5b). In particular, the liver of male rats appeared still in the growth phase at the time of vector delivery, 6–8 weeks, while in female animals of similar age the liver already reached the final size.

These results indicate that long-term correction of CN syndrome can be achieved in Gunn rats following a single administration of an optimized AAV vector expressing the UGT1A1 transgene. They also correlate the stability of gene transfer with liver growth, suggesting that gene transfer in young children may require later vector readministration.

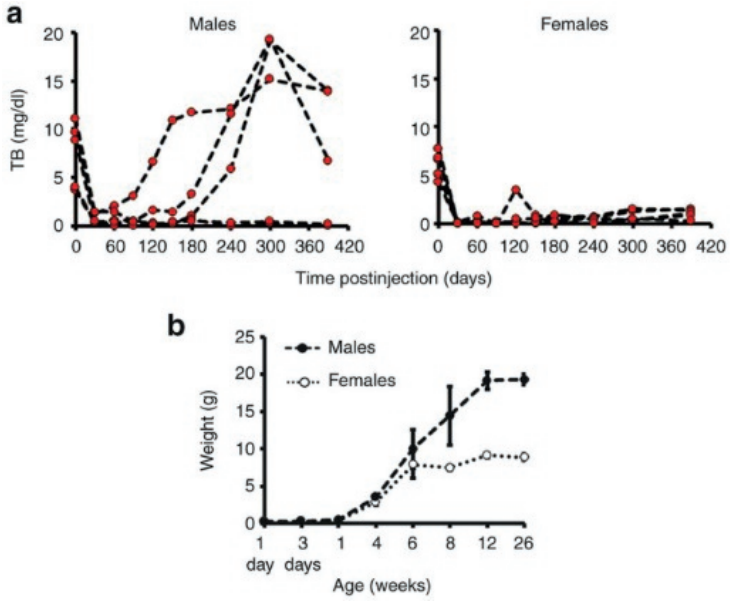

Figure 5. AAV8-UGT1A1 mediates long-term correction of hyperbilirubinemia in a rat model of CN syndrome. 6-8 weeks old male and female Gunn rats (n=5 per group) were intravenously injected with 5E12 vg/kg of AAV8-v1UGT1A1 vector. (**a**) Total bilirubin (TB) levels were measured for 13 months after the injection in serum in male rats (left) and females (right). (**b**) Monitoring of liver growth in Gunn rats. Livers were collected at day 1 (1d), day 3 (3d) and between 1 and 26 weeks after birth. In the graph are reported the mean ± SD of the weight of livers obtained from male and female expressed in grams (g). n=3 per group.

Discussion

CN syndrome is a rare disease for which the current treatment options are suboptimal and no permanent cure is available except for liver transplantation.[2,10] Advanced therapies based on allogeneic hepatocytes or hepatocyte progenitors have been attempted in CN patients, however achieving only limited and transient benefit,[28,37,38] likely due to the poor levels of engraftment of transplanted cells. This was also shown in a recent cell transplantation trial.[39]

Using a translationally optimized AAV-UGT1A1 vector, here we showed that it is possible to obtain long-lasting correction of serum TB in two animal models of the disease, and notably to rescue the lethal phenotype of CN syndrome in Ugt1a1$^{-/-}$ mice. Additionally, following a single intravenous infusion of our AAV-UGT1A1 vector, we observed long term (>1 year) correction of TB levels in serum, and the appearance of the MCB and DCB forms of conjugated bilirubin in bile, evidence of restoration of enzyme activity and active bilirubin excretion.[22]

We previously published proof-of-concept of correction of CN syndrome with AAV vector mediated gene therapy in Gunn rats[22] and Ugt1a1−/− mice.[24,25] Similarly, correction of the CN phenotype was also obtained with helper-dependent adenoviral[40] and lentiviral[41] vectors. In order to translate these results to humans in an AAV vectorbased gene therapy clinical trial, we performed an optimization of the human UGT1A1 expression cassette to achieve complete removal of UCB from serum and enhance the safety and the efficacy of the approach. With this strategy, we were able to obtain full correction of serum TB levels in both mouse and rat models of CN syndrome at vector doses lower than those previously described.[22,25]

We first codon-optimized the UGT1A1 transgene cDNA, a maneuver that has been broadly employed in the field of gene transfer to enhance transgene expression.[42,43] In parallel, alternate open reading frames were also removed from the coding sequence of the transgene to avoid unwanted immune responses triggered by aberrant antigens.[33] Results in mice and rats failed to evidence higher levels of TB correction in serum from animals treated with codonoptimized versus wt UGT1A1 transgenes. This is in contrast with results *in vitro* in human cell lines, which showed superior level of expression deriving from codon-optimized UGT1A1 cDNA. This result is plausible, as the transgene coding sequences were optimized for expression in human cells. Additionally, it should also be pointed out that the UGT1A1 transgene is a membrane protein, and its activity can only be followed indirectly via bilirubin measures, which makes the *in vivo* readout of expression levels less quantitative when compared with other secreted transgenes.[31]

One unexpected finding was that, in the case of the UGT1A1 transgene, the cDNA with the best CAI (*i.e.*, predicted to be ideally optimized) was also the worse performing *in vivo*. Conversely, the UGT1A1 cDNA version with CAI and GC content closer to the native sequence drove the highest levels of transgene expression *in vitro* in human cell lines, and at least equal levels of expression compared with the wild-type UGT1A1 sequence in Ugt1a1$^{-/-}$ mice and Gunn rats. This underscores our poor understanding of some aspect of transgene optimization, for example the role of codon usage in protein folding,[44] which may play an important role in the function of the membrane protein UGT1A1. It also suggests that when approaching the optimization of a transgene expression cassette, multiple codon-optimization algorithms should be empirically tested.

Next, we examined the role of synthetic intron sequences frequently included in AAV vector expression cassettes to enhance mRNA processing and stability.[45] Bioinformatics analysis of the HBB2 intron, and of other introns commonly used in gene transfer (Supplementary Table S1), revealed the presence of ATGs that could originate aberrant proteins if incorrectly spliced. Our data indicate that, at least *in vitro*, splicing of synthetic introns does not always occur with 100% efficiency, and that removal of all possible ATGs is essential to enhance transgene expression. This has obviously an impact on the safety of gene transfer, as cryptic ATGs could originate potentially immunogenic proteins.46 This is a finding not pertaining exclusively to gene transfer for CN syndrome, thus requiring careful evaluation.

Obtaining therapeutic levels of transgene expression at the lowest vector dose possible is an important goal for AAV vector mediated gene transfer. This is particularly true for what it concerns avoidance of potentially detrimental cytotoxic immune responses directed against transduced hepatocytes.[17,18,47,48] With the construct we developed, therapeutic levels of transgene expression were measured at doses of AAV8-UGT1A1 vector of 5E11 vg/kg, possibly below the threshold of activation of CD8+ T- cells, as suggested by results from the AAV hemophilia B trials,[16,18,47] although ultimately the efficacy of this approach can only be tested in humans. Importantly, long-term rescue of the hyperbilirubinemic phenotype in Gunn rats, and correction of the disease in Ugt1a1$^{-/-}$ mice, the animal model closest to the human condition,[24] is highly encouraging. Additional optimization steps could involve the use of novel AAV capsids that target specifically and efficiently human hepatocytes[49,50] or evolutionary intermediates of ancestor AAVs.[51]

Like for other pediatric diseases,[26] an ideal scenario for CN syndrome

would be to achieve stable, long-lasting correction of the disease in young children. As AAV vectors do not efficiently integrate in the host genome,[52] it is expected that the transduction of actively replicating liver cells with AAV would lead to at least partial vector dilution over time, with reduction in transgene expression levels. We recently described this by injecting neonate Ugt1a1$^{-/-}$ mice with AAV vectors 4 days after birth.[25] This led to partial vector dilution over time, resulting in lower levels of transgene expression 17 months after gene transfer, which still remained within the therapeutic range.[25] While it is a known fact that proliferation of the neonate liver over time leads to dilution of the effect of gene transfer,[53] for diseases like CN syndrome for which the amount of transgene expression needed to rescue the diseased phenotype is relatively low, a single administration of an AAV vector at an appropriate dose may be sufficient to achieve lifelong correction of the disease. This would argue in favour of performing gene transfer in young pediatric subjects. Results presented here in Gunn rats support the idea that gene transfer in a rapidly growing liver leads to a variable therapeutic outcome. How these preclinical results will correlate with the stability of AAV mediated liver gene transfer in children is currently unknown. Based on the growth curve of human liver[54] it is expected that gene transfer in younger children will be less stable than in adults.[17] Several possible strategies have been proposed to address the issue of loss of efficacy following AAV vector delivery, which include strategies to allow for vector readministration,[55,56] *in vivo* gene editing,[57,58] or promoterless integrative AAV vectors.[59]

In summary, our work provides a framework for AAV vector optimization for safe and efficient liver gene transfer for CN syndrome in humans. Our results indicated that empirical testing of codon optimization algorithms for the UGT1A1 transgene, together with careful optimization of the noncoding sequences of the transgene expression cassette, are fundamental steps for the translation of preclinical results to humans. For pediatric diseases like CN syndrome, the age at which gene transfer is performed can have a profound influence on the outcome of gene transfer in terms of transgene persistence.

REFERENCES

1. Crigler, J.F., Jr. & Najjar, V.A. Congenital familial nonhemolytic jaundice with kernicterus. *Pediatrics* 10, 169-180 (1952).
2. van der Veere, C.N. et al. Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. *Hepatology* 24, 311-315 (1996).
3. Chowdhury, J., Wolkoff, A., Chowdhury, N. & Arias, I. Hereditary jaundice and disorders of bilirubin metabolism. *The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill* 2, 3063-3101 (2001).
4. Erlinger, S., Arias, I.M. & Dhumeaux, D. Inherited disorders of bilirubin transport and conjugation: new insights into molecular mechanisms and consequences. *Gastroenterology* 146, 1625-1638 (2014).
5. Bosma, P.J. Inherited disorders of bilirubin metabolism. *Journal of hepatology* 38, 107-117 (2003).
6. Kadakol, A. et al. Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype. *Human mutation* 16, 297-306 (2000).
7. Skierka, J.M., Kotzer, K.E., Lagerstedt, S.A., O'Kane, D.J. & Baudhuin, L.M. UGT1A1 genetic analysis as a diagnostic aid for individuals with unconjugated hyperbilirubinemia. *The Journal of pediatrics* 162, 1146-1152, 1152 e1141-1142 (2013).
8. Labrune, P.H., Myara, A., Francoual, J., Trivin, F. & Odievre, M. Cerebellar symptoms as the presenting manifestations of bilirubin encephalopathy in children with Crigler-Najjar type I disease. *Pediatrics* 89, 768-770 (1992).
9. Ostrow, J.D. Photocatabolism of labeled bilirubin in the congenitally jaundiced (Gunn) rat. *J Clin Invest* 50, 707-718 (1971).
10. Strauss, K.A. et al. Management of hyperbilirubinemia and prevention of kernicterus in 20 patients with Crigler-Najjar disease. *European journal of pediatrics* 165, 306-319 (2006).
11. Sellier, A.L. et al. Successful plasmapheresis for acute and severe unconjugated hyperbilirubinemia in a child with crigler najjar type I syndrome. *JIMD reports* 2, 33-36 (2012).
12. Kaneko, K. et al. Bilirubin adsorption therapy and subsequent liver transplantation cured severe bilirubin encephalopathy in a long-term survival patient with Crigler-Najjar disease type I. *Internal medicine* 39, 961-965 (2000).
13. Adam, R. et al. Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *Journal of hepatology* 57, 675-688 (2012).
14. Immordino, G. et al. Predictability and survival in liver replantransplantation: monocentric experience. *Transplantation proceedings* 46, 2290-2292 (2014).
15. Pedersen, M. & Seetharam, A. Infections after orthotopic liver transplantation. *Journal of clinical and experimental hepatology* 4, 347-360 (2014).
16. Manno, C.S. et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 12, 342-347 (2006).
17. Nathwani, A.C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 371, 1994-2004 (2014).
18. Nathwani, A.C. et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B.

N Engl J Med 365, 2357-2365 (2011).
19. Muzyczka, N. & Berns, K.I. *Parvoviridae*: The viruses and their replication., Edn. 4th. (Lippincott, Williams and Wilkins, Philadelphia; 2001).
20. Buchlis, G. et al. Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood* 119, 3038-3041 (2012).
21. Mingozzi, F. & High, K.A. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* 12, 341-355 (2011).
22. Seppen, J. et al. Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats. *Mol Ther* 13, 1085-1092 (2006).
23. Pastore, N. et al. Sustained reduction of hyperbilirubinemia in Gunn rats after adenoassociated virus-mediated gene transfer of bilirubin UDP-glucuronosyltransferase isozyme 1A1 to skeletal muscle. *Hum Gene Ther* 23, 1082-1089 (2012).
24. Bortolussi, G. et al. Rescue of bilirubin-induced neonatal lethality in a mouse model of Crigler-Najjar syndrome type I by AAV9-mediated gene transfer. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* 26, 1052-1063 (2012).
25. Bortolussi, G. et al. Life-long correction of hyperbilirubinemia with a neonatal liverspecific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* 25, 844-855 (2014).
26. Junge, N., Mingozzi, F., Ott, M. & Baumann, U. Adeno-associated virus vector-based gene therapy for monogenetic metabolic diseases of the liver. *Journal of pediatric gastroenterology and nutrition* 60, 433-440 (2015).
27. Fagiuoli, S., Daina, E., D'Antiga, L., Colledan, M. & Remuzzi, G. Monogenic diseases that can be cured by liver transplantation. *Journal of hepatology* 59, 595-612 (2013).
28. Fox, I.J. et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 338, 1422-1426 (1998).
29. Sneitz, N. et al. Crigler-Najjar syndrome in The Netherlands: identification of four novel UGT1A1 alleles, genotype-phenotype correlation, and functional analysis of 10 missense mutants. *Human mutation* 31, 52-59 (2010).
30. Iyanagi, T. Molecular basis of multiple UDP-glucuronosyltransferase isoenzyme deficiencies in the hyperbilirubinemic rat (Gunn rat). *The Journal of biological chemistry* 266, 24048-24052 (1991).
31. Nathwani, A.C. et al. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 107, 2653-2661 (2006).
32. Sharp, P.M. & Li, W.H. The codon Adaptation Index--a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic acids research* 15, 1281-1295 (1987).
33. Li, C. et al. Cellular immune response to cryptic epitopes during therapeutic gene transfer. *Proc Natl Acad Sci U S A* 106, 10770-10774 (2009).
34. Snyder, R.O. et al. Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum Gene Ther* 8, 1891-1900 (1997).
35. Miao, C.H. et al. Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro. *Mol Ther* 1, 522-532 (2000).
36. Bortolussi, G. et al. Age-dependent pattern of cerebellar susceptibility to bilirubin neurotoxicity

in vivo in mice. *Disease models & mechanisms* 7, 1057-1068 (2014).
37. Ambrosino, G. et al. Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1. *Cell transplantation* 14, 151-157 (2005).
38. Horslen, S.P. et al. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. *Pediatrics* 111, 1262-1267 (2003).
39. Dobbelaere, D. et al. Phase I/II multicenter trial of liver derived mesenchymal stem cells (HepastemR) for the treatment of urea cycle disorder and Crigler Najjar syndrome. interim analysis at 6 months post infusion. *Journal of inherited metabolic disease* 37, O-027 (2014).
40. Dimmock, D., Brunetti-Pierri, N., Palmer, D.J., Beaudet, A.L. & Ng, P. Correction of hyperbilirubinemia in gunn rats using clinically relevant low doses of helperdependent adenoviral vectors. *Hum Gene Ther* 22, 483-488 (2011).
41. Schmitt, F. et al. Lentiviral vectors that express UGT1A1 in liver and contain miR-142 target sequences normalize hyperbilirubinemia in Gunn rats. *Gastroenterology* 139, 999-1007, 1007 e1001-1002 (2010).
42. Binny, C. et al. AAV-mediated gene transfer in the perinatal period results in expression of FVII at levels that protect against fatal spontaneous hemorrhage. *Blood* 119, 957-966 (2012).
43. Wu, Z. et al. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther* 16, 280-289 (2008).
44. Zhou, M., Wang, T., Fu, J., Xiao, G. & Liu, Y. Nonoptimal codon usage influences protein structure in intrinsically disordered regions. *Molecular microbiology* 97, 974-987 (2015).
45. Powell, S.K., Rivera-Soto, R. & Gray, S.J. Viral expression cassette elements to enhance transgene target specificity and expression in gene therapy. *Discovery medicine* 19, 49-57 (2015).
46. Bullock, T.N. & Eisenlohr, L.C. Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. *The Journal of experimental medicine* 184, 1319-1329 (1996).
47. Mingozzi, F. et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* 13, 419-422 (2007).
48. Mingozzi, F. & High, K.A. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* (2013).
49. Lisowski L, Dane AP, chu K *et al* (2014) Selection and evaluation of clnicallyn relevant AAV variants in a xenograft liver model. *Nature* 506: 382-286
50. Vercauteren K, Hoffman BE, Zolotukhin U *et al.* (2016) Superior *in vivo* transduction of human hepatocytes using engineerd AAV3 capsid. *Mol Ther* 24: 1042-1049
51. Zinn E, Pacouret S, Khaychuck V, Turunen HT *et al.* (2015) In silico reconstruction of the viral evolutionary lineage yields a potent gene therapy vector. *Cell Rep*
52. Ehrhardt, A., Xu, H. & Kay, M.A. Episomal persistence of recombinant adenoviral vector genomes during the cell cycle in vivo. *J Virol* 77, 7689-7695 (2003).
53. Wang, L., Wang, H., Bell, P., McMenamin, D. & Wilson, J.M. Hepatic gene transfer in neonatal mice by adeno-associated virus serotype 8 vector. *Hum Gene Ther* 23, 533-539 (2012).
54. Coppoletta, J.M. & Wolbach, S.B. Body Length and Organ Weights of Infants and Children: A Study of the Body Length and Normal Weights of the More Important Vital Organs of the Body between Birth and Twelve Years of Age. *The American journal of pathology* 9, 55-70 (1933).
55. Nathwani, A.C. et al. Safe and efficient transduction of the liver after peripheral vein infusion

of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* 109, 1414-1421 (2007).

56. Amine Meliani, F.B., Giuseppe Ronzitti, Fanny, Collaud, C.L., Takashi Kei Kishimoto, Federico & Mingozzi in ASGCT 19th Annual Meeting, Vol. Poster 77 2016).
57. Li, H. et al. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 475, 217-221 (2011).
58. Anguela, X.M. et al. Robust ZFN-mediated genome editing in adult hemophilic mice. *Blood* 122, 3283-3287 (2013).
59. Barzel, A. et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature* 517, 360-364 (2015).
60. Thummel, C., Tjian, R. & Grodzicker, T. Construction of adenovirus expression vectors by site-directed in vivo recombination. *Journal of molecular and applied genetics* 1, 435-446 (1982).
61. Ayuso, E. et al. High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. *Gene Ther* 17, 503-510 (2010).
62. Matsushita, T. et al. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* 5, 938-945 (1998).
63. Ying BW, Fourmy D and Yoshizawa S *et al.* (2007) Substitution of the use of radioactivity by fluorescence for biochemical studies of RNA. *RNA* 13: 2042-2050
64. Cornelius, C.E. & Arias, I.M. Animal model of human disease. Crigler-Najjar Syndrome. Animal model: hereditary nonhemolytic unconjugated hyperbilirubinemia in Gunn rats. *The American journal of pathology* 69, 369-372 (1972).
65. Montenegro-Miranda, P.S. et al. Adeno-associated viral vector serotype 5 poorly transduces liver in rat models. *PloS one* 8, e82597 (2013).

SUPPLEMENTAL MATERIAL

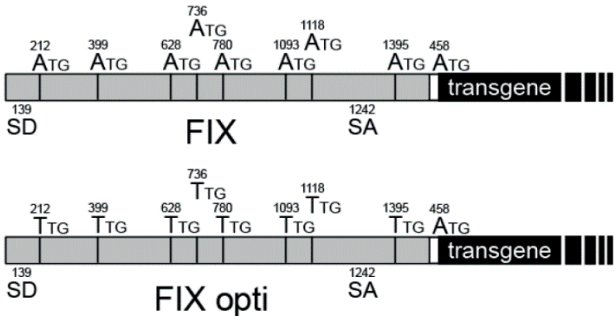

Figure S1. Diagram displaying the alternative open reading frames (aORFs) predicted in the human coagulation factor IX (FIX) intron 1 and their modification in the FIX optimized intron (FIX opti). The position of each aORF calculated from the start of the intron sequence is indicated.

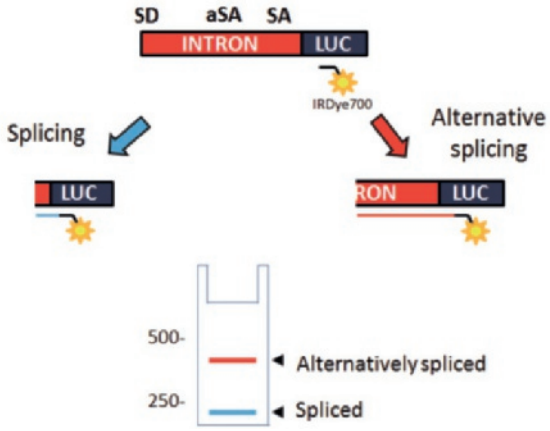

Figure S2. Schematic representation of the primer elongation assay. The intron contains a splicing donor (SD), a splicing acceptor (SA) and an alternative splicing acceptor (aSA). The primer elongation assay has been performed using an oligo conjugated with IRDye700. The elongation of a mature messenger RNA obtained by the splicing of SD and SA leads to a low molecular weight product (in blue). Conversely the alternative splicing of SD and aSA produces a longer elongation product (in red). The presence of the two elongation products after separation in a denaturing gel indicates that the intron is alternatively spliced.

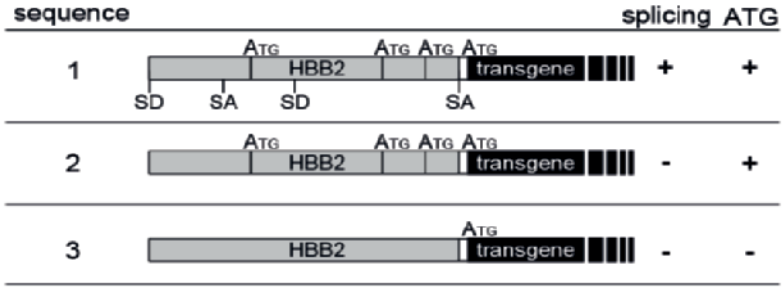

Figure S3. Schematic representation of the splicing-incompetent HBB2 introns. The original HBB2 intron sequence (sequence 1) contains 3 ATGs originating 3 aORFs, two acceptors (SA) and two donors (SD) splicing sites. The splicingincompetent HBB2 introns (sequence 2 and 3) have been obtained by mutagenizing the four splicing sites to reduce their predicted score below the threshold of 0.7 (http://www.fruitfly.org/seq_tools/splice.html). The removal of ATGs in the splicing incompetent HBB2 intron originated sequence 3.

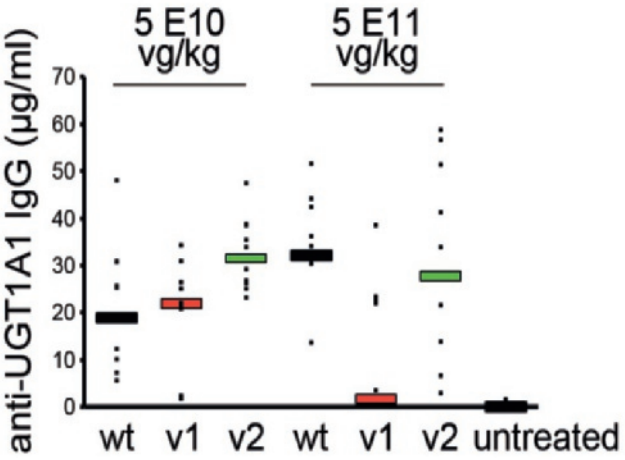

Figure S4. Measurement of anti-UGT1A1 antibodies in treated Gunn rats. Animals (5 females and 5 males) were injected with AAV8 vectors expressing the wtUGT1A1 (black), v1UGT1A1 (red), and v2 UGT1A1 (green) transgene at a dose of 5E10 and 5E11 vg/kg. In the graph are depicted the levels of anti-UGT1A1 IgG measured in serum two months after vector delivery. Total IgG were measured by ELISA as described in Materials and Methods. In the figure are showed the individual values measured and the median value for each treatment (horizontal lines). Statistical analysis has been performed by ANOVA (* = $p<0.05$).

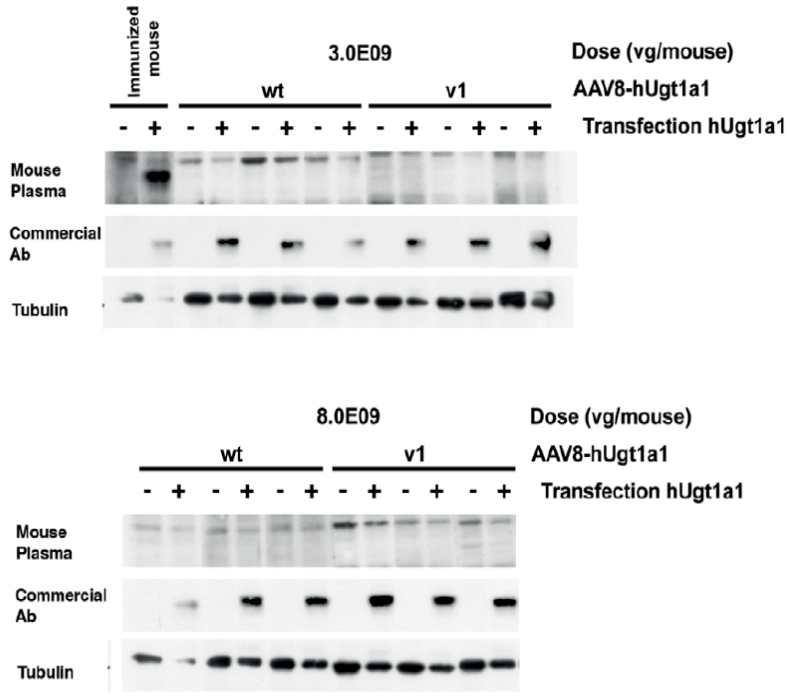

Figure S5. To detect circulating antibodies against the transgene (human UGT1A1) total cell protein extracts derived from untransfected (-) and transfected (+, UGT1A1) cells were run in a SDS PAGE gel and blotted onto nitrocellulose membrane. Membranes were probed with plasma derived from immunized mice (positive control) or mice treated with indicated doses of AAV8-UGT1A1 wt or v1. Next, membranes were stripped and probed with a commercial antibody against UGT1A1 (Santa Cruz, sc-25847). Anti-Tubulin was used as loading control.

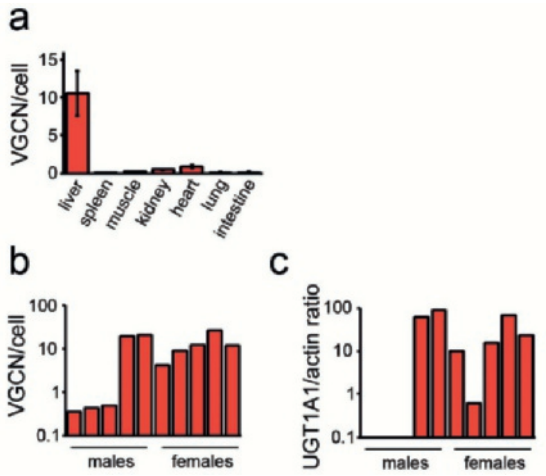

Figure S6. Biodistribution of AAV8-UGT1A1 vector genomes and UGT1A1 protein expression in Gunn rats. 6-8 weeks old male and female Gunn rats (n=5 per sex) were intravenously injected with 5E12 vg/kg of an AAV8 vector expressing v1UGT1A1 and sacrificed 13 months after the injections. (**a**) Vector biodistribution was measured by qPCR in different organs as described in the graph. Vector genome copy number (VGCN) values were normalized to the number of copies of titin measured in each sample. Results are shown as mean ± SEM. (**b**) VGCN per cell measured in single livers from rats treated as described above. (**c**) Western Blot analysis of UGT1A expression levels in the livers of Gunn rats treated as described above. In the graph are showed the UGT1A1/actin ratio obtained by densitometry on western blot performed on microsomal extracts using antibody specific for UGT1A1 and actin.

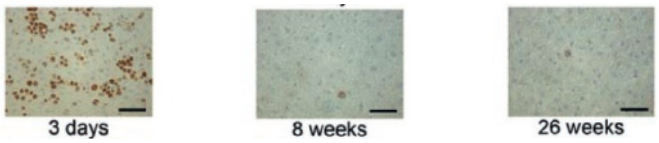

Figure S7. Evaluation of hepatocytes proliferation in the liver of Gunn rats at day 3 or 8, and 26 weeks; liver sections were stained with an antibody specific for Ki67. Hepatocytes were counterstained with hematoxylin-eosin. Scale bar = 50 μm

Intron[a]	aORF (>bp)[b]	Donor (score>0.9)[c]	Acceptor (score>0.9)[c]
HBB2	3	2	2
hFIXint1	8	2	1
CBA-rHBB	3	1	1
CBA	2	1	1
hGH	1	3	1
hFIX synth	1	1	1
hALB synt	0	2	1

Table S1: alternative open reading frame (aORF) and splicing donor and acceptor analysis of different introns commonly used in gene therapy expression cassettes. HBB2: intron 2 of the human hemoglobin beta gene hFIX int1: intron 1 of the human coagulation factor IX gene CBA-rHHB: synthetic intron derived from the fusion of the intron 1 of the chicken beta actin gene and intron 2 of the rabbit hemoglobin beta CBA: intron 1 of the chicken beta actin gene hGH: intron 1 of the human growth hormone gene hFIX synth: synthetic intron derived from different portions of the human coagulation factor IX gene and present in the pLIVE vector (Mirus Bio, Madison, WI) hALB synth: synthetic intron derived from different portions of the human albumin gene and present in the pLIVE vector (Mirus Bio, Madison, WI). [a]alternative open reading frames (aORF) originating reading frames of at least base pairs. [c]donor and acceptor splicing sites predicted using an online tool (http://www.fruitfly.org/seq_tools/splice.html) with a minimum score of 0.9.

3

POLYINOSINIC ACID BLOCKS ADENO-ASSOCIATED VIRUS MACROPHAGE ENDOCYTOSIS *IN VITRO* AND ENHANCES ADENO-ASSOCIATED VIRUS LIVER-DIRECTED GENE THERAPY *IN VIVO*

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Abstract

Adeno-associated virus serotype 8 (AAV8) has been demonstrated to be effective for liver-directed gene therapy in humans. Although hepatocytes are the main target cell for AAV8, there is a loss of the viral vector because of uptake by macrophages and Kupffer cells. Reducing this loss would increase the efficacy of viral gene therapy and allow a dose reduction. The receptor mediating this uptake has not been identified; a potential candidate seems the macrophage scavenger receptor A (SR-A) that is involved in the endocytosis of, for instance, adenovirus. In this study we show that SR-A can mediate scAAV8 endocytosis and that blocking it with polyinosinic acid (poly[i]) reduces endocytosis significantly in vitro. Subsequently, we demonstrate that blocking this receptor improves scAAV-mediated liver-directed gene therapy in a model for inherited hyperbilirubinemia, the uridine diphospho-glucuronyl transferase 1A1–deficient Gunn rat. In male rats, preadministration of poly[i] increases the efficacy of a low dose ($1 \cdot 10^{11}$ gc/kg) but not of a higher dose ($3 \cdot 10^{11}$ gc/kg) scAAV8-LP1-UT1A1. Administration of poly[i] just before the vector significantly increases the correction of serum bilirubin in female rats. In these, the effect of poly[i] is seen by both doses but is more pronounced in the females receiving the low vector, where it also results in a significant increase of bilirubin glucuronides in bile. In conclusion, this study shows that SR-A mediates the endocytosis of AAV8 in vitro and in vivo and that blocking this receptor can improve the efficacy of AAV-mediated liver-directed gene therapy.

Introduction

Adeno-associated virus (AAV) is a small, nonenveloped virus that depends on a helper virus such as adeno or herpes virus for replication. In the absence of a helper virus, this non-pathogenic virus is replication defective but can persist in nondividing tissues[1]. This sustained persistence renders AAV vectors good candidates for liver-directed gene therapy. Several AAV serotypes have been identified, of which AAV serotype 8 (AAV8) and AAV9 appeared to have a more pronounced tropism for the liver[2]. The efficacy of AAV8 for liver-directed gene therapy has been demonstrated in several animal models[3]. A recent clinical trial has shown that AAV8 is an effective and safe vector for liver-directed gene therapy in the human setting, thereby underscoring the potential of this vector for correcting inherited liver disorders[4].

Although hepatocytes are the main target cell especially for AAV8, other cells such as macrophages and Kupffer cells are able to take up AAV particles[5]. Overcoming the loss of vector to these nontarget cells may increase its efficacy and thus allow additional dose reduction. It is unknown which receptor is involved in the uptake of AAV uptake by Kupffer cells and other macrophages. Potential candidates are the scavenger receptors expressed on macrophages, Kupffer cells, and certain endothelial cells. This family is involved not only in the endocytosis of apoptotic cells and certain macromolecules such as lipoproteins, but also in that of blood-borne pathogens, including viruses[6, 7]. Previously, we showed that the macrophage scavenger receptor A (SR-A/CD204) present on Kupffer cells can mediate endocytosis and transduction of macrophages and Chinese hamster ovary (CHO) cells by adenoviral vectors in vitro[8]. Blocking of SR-A with polyinosinic acid (poly[i]) in mice in vivo inhibited the clearance of adenovirus by Kupffer cells and thereby increased adenovirus-mediated transgene expression in hepatocytes[8]. For AAV it has been shown that in human serum the capsid associates with iC3b and that this mediates uptake of this vector by macrophages[5, 9]. Since iC3b is recognized by SR-A1, upon association with this complement factor AAV might indeed be endocytosed via this receptor. Blocking it may therefore increase the efficacy of AAV-mediated liver-directed gene therapy.

Our goal is to develop gene therapy for Crigler-Najjar syndrome type 1. This inherited liver disorder is caused by a single gene defect resulting in deficiency of uridine diphospho-glucuronyl transferase 1A1 (UGT1A1), which leads, when left untreated, to a fatal unconjugated hyberbilirubinemia[10, 11]. By catalysing the glucuronidation of the hydrophobic unconjugated bilirubin (UCB) in

the liver, UGT1A1 is of vital importance in the bilirubin homeostasis. The current standard treatment for Crigler-Najjar type I consists of phototherapy during early childhood. When later in life serum bilirubin levels start to increase, a liver transplantation will be required[12]. The burden of extensive phototherapy, the lack of sufficient liver donors, and the need for lifelong immune suppression makes the development of new effective treatments for Crigler-Najjar syndrome, such as liver directed gene therapy, desirable. Using single-stranded and double-stranded AAV, we have demonstrated that AAV mediated gene therapy is effective in correcting serum bilirubin in the Gunn rat, the appropriate animal model for Crigler-Najjar syndrome type 1 [13, 14]. The aim of this study was to investigate whether SR-A can mediate the endocytosis of AAV by macrophages and if so whether blocking it can increase the efficacy of AAV-mediated gene therapy for Crigler-Najjar syndrome type 1.

First, the effect of SR-A expression on the endocytosis of scAAV8 was determined in CHO cells. This revealed that SR-A expression resulted in a significant increase of AAV8 endocytosis. Blocking scavenger receptors with the general ligand poly[i] reduces endocytosis in these cells, in the human monocytic leukemia cell line THP-1, and in primary human interleukin 4 differentiated macrophages. In vivo, administration of poly[i] just before the injection of scAAV8- LP1-UGT1A1 improved the correction of hyperbilirubinemia in the Gunn rat. These results demonstrate that the human SR-A can mediate endocytosis of AAV by macrophages and suggest that specifically blocking this receptor improves AAV-mediated liver-directed gene therapy via the systemic circulation.

Materials and Methods

Cells

The CHO cell line was cultured in F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 2mM Lglutamine, penicillin (100 IU/ml), and streptomycin (100 IU/ ml) at 37_C in a humidified 5% CO2 atmosphere. The human acute monocytic leukemia cell line THP-1 was cultured in RPMI medium (Gibco) supplemented with 10% FBS, 2mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 IU/ ml) at 37_C in a humidified 5% CO2 atmosphere. THP-1 cells were differentiated and polarized as described by Daigneault et al. (2010). Briefly, THP-1 cells were seeded in a 12-well plate at a density of $1.0 \cdot 10^6$ cells per well and incubated with 200 nM phorbol-12-myristate-13-acetate (PMA) for 3 days, washed with HBSS, and subsequently cultured for 5 days in RPMI containing 10%FBS, 2mML-glutamine, penicillin (100 IU/ml), and streptomycin (100 IU/ml). Human peripheral blood mononuclear cells were isolated and differentiated to type 2a macrophages as described Monocytes were polarized and validated for specific phenotypic markers as described[15].

Fluorescence-activated cell sorting analysis

CHO, CHO SR-A, differentiated THP1, and differentiated primary human macrophages were seeded in a 12-well plate at a density of $1.0 \cdot 10^6$ cells per well and cultured overnight. The next day, cells were washed and incubated with Alexa-647-labeled scAAV8-LP1-UGT1A1 – poly[i] 25 µg/ml preincubation for 5 min at 4°C or 37°C in medium supplemented with 10% human serum. After incubation for 4 hr, cells were washed, isolated, fixed in 2% formaldehyde, and resuspended in fluorescence-activated cell sorting buffer. Flow cytometry analysis was performed within 30 min after harvesting of the cells.

Animal experiments

All animal experiments were performed in accordance with the Animal Ethics Committee guidelines of the Academic Medical Center of Amsterdam. Gunn rats from our own colony with a weight between 160 and 260 g for males and between 140 and 170 g for females were used. The rats were fed ad libitum and randomly assigned to different treatment groups. Poly[i] 4 mg/kg body weight was injected in the tail vein 5 min before the injection of $1 \cdot 10^{11}$ or $3 \cdot 10^{11}$ of scAAV8-LP1-UGT1A1. Intravenous tail vein injection, blood sampling, and bile collection were performed as described by[16].

Bilirubin quantification

Total bilirubin in serum was determined by the routine clinical chemistry department using a standard colorimetric assay. UCB and bilirubin conjugates in bile were analysed and quantified by high-performance liquid chromatography as described with the modification that a pursuit column (Agilent) was used[13, 17].

Construction of production of AAV vectors

The recombinant AAV vector was constructed by replacing the factor IX cDNA with the UGT1A1 cDNA, using the EcoR1 and Bsb1 sites of plasmid scAAV-LP1-hFIXco (kindly provided by Dr. A. Nathwani, University College of London, London, United Kingdom). The plasmid was sequenced to confirm proper insertion of UGT1A1. Recombinant AAV was produced with AAV2 Rep and pseudotyped with capsid from AAV8 using the adenovirus-free method. Briefly, AAV8scLP1UGTA1 vectors were produced after triple-transient transfection into adherent HEK293 cells. AAV8 particles were purified using AVB chromatography affinity gel and were finally formulated using diafiltration into D-PBS. Titration of AAV vectors was performed by quantitative PCR. AAV vectors were labeled with an Alexa Fluor 647 following instructions as provided by the manufacturer (Molecular Probes) followed by extensive washing to remove unbound label.

Results

Human SR-A mediates AAV endocytosis that can be blocked by poly[i] in vitro

To evaluate whether the SR-A can mediate endocytosis of scAAV8 vector, stable expression of the human receptor was established using a lentiviral vector as reported previously[8] in CHO cells that do not have endogenous expression[18]. SR-A expression resulted in a 3-fold induction of scAAV8 endocytosis, 74% versus 24% in the parental cell line after a 4 hr incubation at 37^{o}C. To confirm uptake and to exclude a specific binding of the vector, a cold incubation was performed with these cells for 4 hr at 4^{o}C. The lack of a detectable signal in those cells showed that binding to these cells is minimal, indicating that the signal at 37^{o}C results from internalized vector (Fig. 1). The macromolecule complex poly[i] is shown to be an SR-A ligand[19]. Preincubation of the CHO SR-A cell line with poly[i] (25 μg/ml for 5 min) resulted in a significant 58% reduction of scAAV8 endocytosis (Fig. 2B). In the parental control CHO cell line, no effect of poly[i] on the uptake of scAAV8 was seen (Fig. 2A). Since these data indicate that SR-A mediates uptake of scAAV8,

it was investigated subsequently if it could play a role in the endocytosis of this vector by human macrophages. First, the uptake was determined in the human monocytic cell line THP-1, which upon PMA differentiation resembles the type 2 macrophage (MF2) and exhibits significant expression of SR-A/CD204[20]. Upon incubation for 4 hr at 37°C, 26% of the differentiated THP-1 cells were positive for scAAV8 endocytosis. This percentage was dramatically reduced to 2% after preincubation of these cells with poly[i], indicating the important role of SR-A in this process (Fig. 2B). In the cells incubated with the vector at 4°C, the signals were very low, indicating that the fluorescence represents internalized vector.

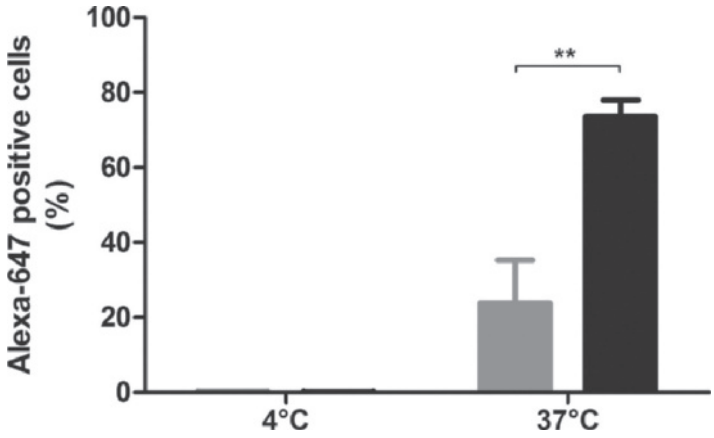


Figure 1. Scavenger receptor A1 can mediate AAV vector endocytosis. CHO and CHO-expressing SR-A1 were incubated for 4 hr with Alexa-647-labeled scAAV8 (300 gc/cell) in Dulbecco's modified Eagle's medium plus 10% HS at 4°C or at 37°C and were subsequently analyzed for scAAV8 endocytosis using flow cytometry. The percentage of CHO (gray bar n = 3) and of CHO-expressing SR-A (black bar n = 3) containing Alexa-647-labeled scAAV8 is presented – SD; **p < 0.01. AAV, adeno-associated virus; CHO, Chinese hamster ovary; HS, human serum; SR-A1, scavenger receptor A1; SD, standard deviation.

Next we investigated the relevance of this receptor in endocytosis in primary human macrophages. Human peripheral blood mononuclear cells can be polarized into a wide range of different macrophages (Mϕ)[21, 22]. Monocytes were polarized into Mϕ1, Mϕ2a, and Mϕ2b as described[15]. Extensive profiling revealed that monocytes that were differentiated into Mϕ2a with interleukin 4 (20 ng/ml) have higher expression of SR-A in comparison to Mϕ1 differentiated macrophages (100 ng/ml LPS plus 20 ng/ml IFN-γ) (Supplementary Fig. S1)[23]. Mϕ2a differentiated macrophages were exceptionally capable of endocytosis of scAAV8, and ~88% of all macrophages were positive for Alexa-647-labeled scAAV8 after a

4 hr incubation at 37°C, while no signal was detectable at 4°C. Preincubation with poly[i] before scAAV8 incubation reduced endocytosis significantly by more than 80% (Fig. 3).

Altogether, these *in vitro* data demonstrate that enhancing the expression of the SR-A leads to increased uptake of AAV by CHO cells, differentiated THP1 cells, and primary human Mϕ2a. Blocking this receptor by the potent ligand poly[i] leads to a large reduction of AAV uptake in all these cell types. This suggests that SR-A present on macrophages and Kupffer cells can mediate endocytosis of scAAV8 and therefore seems relevant in the loss of this vector to these cells upon systemic injection.

Preadministration of poly[i] enhances serum bilirubincorrection and glucuronidated bilirubin secretion in Gunn rats treated with self-complementary AAV vector

To assess whether poly[i] affects scAAV-mediated liver directed gene therapy, its effect on the correction of serum bilirubin level was studied in the Gunn rat[14]. Efficacy of scAAV8-LP1-UGT1A1 as a single agent or in combination with poly[i] was studied by determining the correction of serum bilirubin level and the secretion of bilirubin glucuronides into bile 16 weeks after viral injection. Male and female Gunn rats were injected via the tail vein with a low dose ($1 \cdot 10^{11}$ gc/kg) or high dose ($3 \cdot 10^{11}$ gc/kg) of scAAV8-LP1-UGT1A1. Blocking of SR-A was established by injecting poly[i] (4 mg/kg) 5 min before vector administration. In male Gunn rats, tail vein injection of only scAAV8-LP1-UGT1A1 resulted in a significant reduction of serum bilirubin level by approximately 55%. Although the effect seemed somewhat more pronounced with the threefold Higher dose, the difference between both treatment groups did not reach significance. Preadministration of poly[i] resulted in an increased correction of serum bilirubin in the male rats treated with the low viral vector dose ($p < 0.05$). In the rats treated with the higher vector dose, the effect of poly[i] was minimal and not significant (Fig. 4A). Correction of serum bilirubin level in female rats upon tail vein injection of scAAV8-LP1-UGT1A1 in comparison to male rats was small. With the low vector dose, the effect only reached significance 1 week after injection, while the higher dose did provide a significant correction ($p < 0.01$). Preadministration of poly[i] improved the efficacy of this vector in female rats and especially in the group treated with the low dose. In this group the reduction of serum bilirubin was significantly increased by poly[i] preadministration ($p < 0.05$).

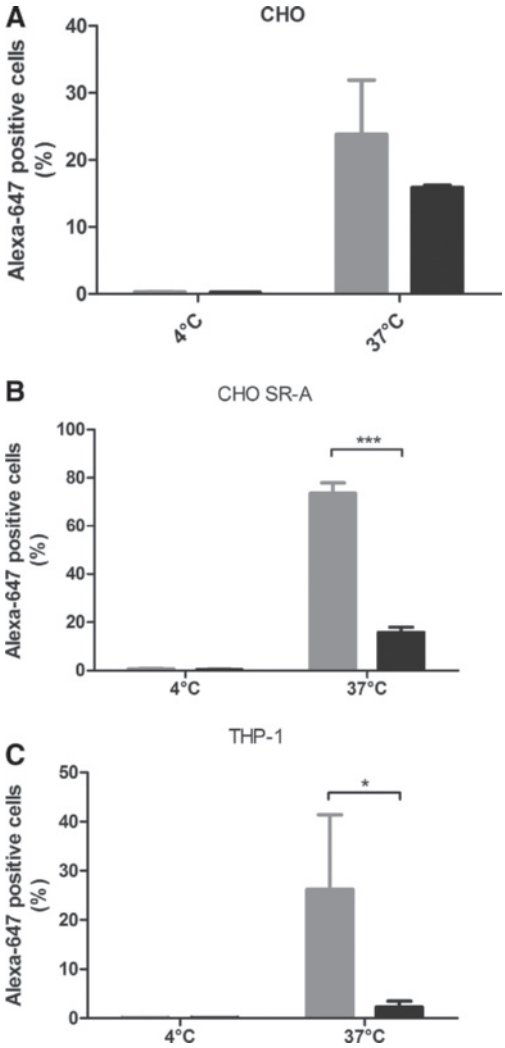


Figure 2. The scavenger receptor ligand poly[i] reduces endocytosis of the scAAV8. (A) CHO and (B) CHO-expressing SRA1 cells were incubated for 4 hr with Alexa-647-labeled scAAV8 (300 gc/cell) inulbecco's modified Eagle's medium plus 10% HS in the presence (black bars) or absence (gray bar) of poly[i] (25 µg/ml) added 5min before the vector. After 4 hr incubation at 4°C or 37°C, cells were subsequently analyzed for scAAV8 endocytosis using flow cytometry. (C) Phorbol-12-myristate-13-acetate differentiated THP-1 cells were incubated for 4 hr with Alexa-647-labeled scAAV8 (300 gc/cell) in RPMI plus 10% HS in the presence (black bars) or absence (gray bar) of poly[i] (25 lg/ml) added 5min before the vector. After 4 hr incubation at 4°C or 37°C, cells were subsequently analyzed for scAAV8 endocytosis using flow cytometry. Data are presented as mean –SD; *$p < 0.05$, ***$p < 0.001$. Poly[i], polyinosinic acid.

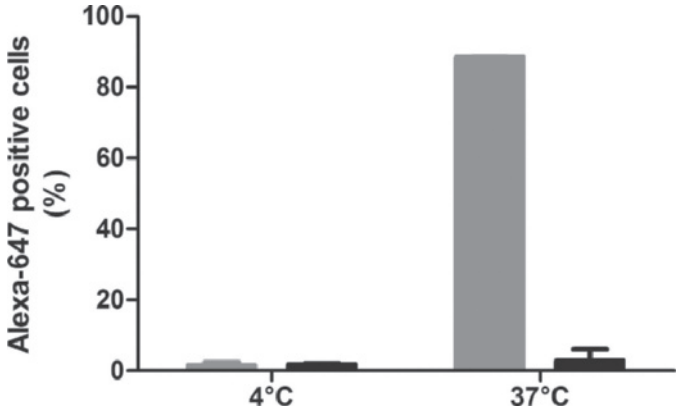


Figure 3. Blocking of the scavenger receptor reduces scAAV8 uptake in differentiated primary human macrophages. Human monocytes were isolated from blood and differentiated into type 2a macrophages (MΦ2a) with interleukin 4. After 4 days, cells were incubated with Alexa-647-labeled scAAV8 (300 gc/cell) in RPMI plus 10% HS in the presence (black bars) or absence (gray bar) of poly[i] (25 µg/ml) added 5 min before the vector. After 4 hr incubation at 4°C or 37°C, cells were subsequently analyzed for scAAV8 endocytosis using flow cytometry. Data are presented as average (n = 2)

Although UGT1A1 is the major role determinant, other processes such as bilirubin production from heme, its consumption by oxidative processes, and its re-uptake from the intestine do also affect serum bilirubin levels. The presence of bilirubin glucuronides in bile seems another possibly more sensitive parameter for the UGT1A1 activity re-established in the liver by scAAV8-LP1-UGT1A1-mediated gene therapy. At the time of sacrifice, 16 weeks after injection of the vector, bile was obtained and analyzed for the presence of bilirubin and bilirubin glucuronides. In both male and female rats, the amount of conjugated bilirubin secreted in bile was increased in the rats pretreated with poly[i] (Table 1). However, this only reached significance ($p < 0.05$) in female Gunn rats treated with a low vector dose. Also, the amount of bilirubin diglucuronide in bile was significantly increased in the female rats pretreated with poly[i]. In this group 19% of total bilirubin in bile was bilirubin diglucuronide, while in the female rats receiving the low dose of the scAAV8-LP1- UGT1A1 but no poly[i], this was only 5% (Table 1).

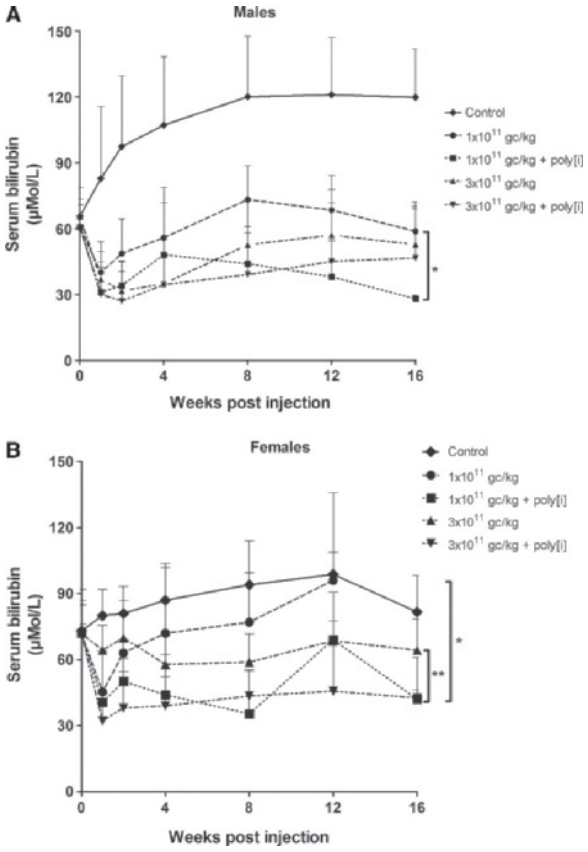


Figure 4. Poly[i] increases efficiency of scAAV8 viral gene therapy in rats. Gunn rats were injected in the tail vein with low- or high-dose scAAV8-LP1- UGT1A1 ($1 \cdot 10^{11}$ resp. $3 \cdot 10^{11}$ gc/kg) with or without preadministration of poly[i]. (A) Total serum bilirubin levels were measured in untreated (♦, n = 15), in low-dose-injected (•, n = 4), in highdose- injected (▲, n = 4), in low dose injected with poly[i] (■, n = 3), and in high-dose-injected male rats with poly[i] (▼, n = 6). Data represent the mean and standard deviation per time point. *Statistically significant difference of $p < 0.05$ of the poly[i] treatment in the low-dose group as assessed by a mixed linear model analysis of variance. (B) Total serum bilirubin levels in untreated female rats (♦, n = 13), in low-dose-injected (•, n = 3), in highdose-injected (▲, n = 4), in low dose injected with poly[i] (■, n = 3), and in high-dose-injected female rats with poly[i] (▼, n = 5). Data represent the mean and standard deviation per time point. *Statistically significant difference of $p < 0.05$; **statistically significant difference of $p < 0.01$ of the poly[i] treatment in the low- and high-dose group as assessed by a mixed linear model analysis of variance.

Discussion

Crigler-Najjar syndrome is a rare but severe congenital disease that, when left untreated, leads to the development of kernicterus and can eventually be lethal. At this moment liver transplantation is the only curative option, but because of the lack of available donors and the burden of life-long immunosuppression development, a novel therapy is warranted. Liver-directed gene therapy with an AAV vector has shown to be a feasible and effective option for the treatment of monogenetic liver diseases such as Crigler-Najjar[24]. A recent trial demonstrated that scAAV8- mediated liver-directed gene therapy is safe and effective, providing sustained correction of factor IX deficiency in haemophilia B, an x-linked bleeding disorder[4]. The healthy liver has an enormous capacity for the glucuronidation of bilirubin. Restoring only 5% of the normal UGT1A1 activity in Crigler-Najjar patients will reduce serum bilirubin levels effectively and will minimize the risk of the development of kernicterus[25]. In six haemophilia B patients, a dose of $2 \cdot 10^{12}$ gc/kg scAAV8-LP1-hFIX restored 8–12% of the normal expression levels of FIX.

Although this dose was well tolerated and did not cause any acute toxicity, after 4–6 weeks elevated aminotransferase levels in the high-dose cohort indicated that this vector dose did induce an immune response in one patient. A short treatment with prednisone rapidly reduced these levels and prevented loss of efficacy[4]. Although there was not a clear correlation between the vector dose and immune responses in this small group of patients, the dose required for correction was high enough to induce a CTL response at least in some of them. This indicates that there is room for optimizing vector delivery to the liver to lower the therapeutic vector dose.

It has been shown that cells of the innate immune system, such as macrophages and Kupffer cells, are capable of taking up virus particles, thereby providing protection toward viral infections[26]. When aiming to treat inherited liver disorders with viral vectors, this reduces the amount of vector that reaches the hepatocyte and thus impairs the efficacy. Indeed, depletion of Kupffer cells increased hepatocyte transduction with adenovirus[27, 28]. We demonstrated previously that uptake of adenoviral vectors in these cell types could be reduced by the scavenger receptor ligand poly[i], showing that the scavenger receptors are involved in vector clearance[8]. Preventing the loss of viral vector to non-target cells such as Kupffer cells by pretreatment with poly[i] indeed increased adenoviral transduction of hepatocytes, thereby enhancing vector efficacy. Although for AAV Kupffer cell depletion did reduce the innate immune response toward AAV[29, 30],

the potential beneficial effect on vector efficacy has not yet reported. The fact that Kupffer cells are directly involved in the induction of an innate immune response toward AAV indicates that there will be loss of the viral vector to these cells. The uptake of AAV by macrophages has been shown to depend on binding to the complement factor iC3b. Since this factor is recognized by SR-A, endocytosis of AAV may be mediated via this receptor[9].

Data presented in this study indeed demonstrate that like adenovirus, endocytosis of AAV8 can also be mediated by SR-A. Furthermore, these data show that AAV uptake in human macrophages *in vitro* can be effectively reduced by the SR-A ligand poly[i]. Previously, we showed that poly[i] by reducing the sequestering of adenoviral vectors by Kupffer cells enhances transgene expression in hepatocyte in mice and rats almost 10-fold[8]. In mice a single dose of poly[i] appeared safe and did not induce hepatocellular damage. Now our data show that intravenous administration of poly[i] just before vector injection also significantly increased the efficacy of scAAV8-LP1-UGT1A1 in the Gunn rat, although to a lesser extent as previously observed with adenoviral vectors. Although the increased efficacy was seen in male and female rats, poly[i] established the most pronounced effect in female rats treated with a low vector dose. As seen in female rats, also in males, poly[i] had the largest effect on the group injected with a low viral dose. One could envision that injection of a vector dose high enough to saturate the Kupffer cell system will allow the vector to disseminate and to transduce other cell types, especially the hepatocytes. Blocking of the scavenger receptor will therefore have a smaller effect on liver transduction when injecting a high saturating viral vector dose. In addition to vector dose, the effect of poly[i] also seems to depend on sex. The most pronounced effect of SR-A blocking is seen in female Gunn rats. This suggests a larger uptake of AAV by Kupffer cells and other macrophages, resulting in a higher threshold requiring a higher vector dose to saturate this system in females. A more active uptake by these innate immune cells may explain the somewhat reduced efficacy of double-stranded scAAV8 as observed in female mice[31, 32]. Our data in rats now seem to indicate that in part this difference may result from an increased loss of vector to the macrophages. It is known that sex does influence the activity of the immune system in processes such as inflammation and cancer development[33, 34]. In addition, it has been shown that in males and ovariectomized and aged females, there is a reduced immune function[35]. Furthermore, in vivo estrogen administration does induce Kupffer cell phagocytosis activity in rats[36, 37]. This hormonal effect is reflected in a greater than 1-log increase in AAV-mediated transgene expression in ovariectomized females

compared with normal females, which then returned to normal levels upon addition with 17-b-estradiol[38]. All these data do suggest that in female rats the macrophages clearance of AAV may indeed be higher in comparison to male rats. Such an increased phagocytosis may explain why the effect of SR-A blocking by poly[i] may be more effective in female rats. Since most inherited liver disorders are inherited autosomally, this sex effect on the efficacy is very relevant for the clinical use scAAV liver-directed gene therapy in both sexes.

Table 1. Bile Composition Showing Increased Bilirubin Conjugation After Polyinosinic Acid readministration in Female Gunn Rats

Animal sex	Dose (gc/kg)	Poly[i]	Bile composition		
			% BDG	% BMG	% UCB
Male (n=2)	$1x10^{11}$	-	14.5±1.7	51.0±2.5	34.5±4.2
Male (n=2)	$1x10^{11}$	+	18.1±1.9	55.5±4.9	26.4±3.0
Male (n=6)	$3x10^{11}$	-	15.4±2.5	57.5±7.4	27.2±9.2
Male (n=2)	$3x10^{11}$	+	19.1±7.2	67.2±6.4	13.8±13.6
Female (n=2)	$1x10^{11}$	-	5.2±0.5	21.0±6.4	73.8±6.9
Female (n=2)	$1x10^{11}$	+	19.0±4.9*	42.3±0.01**	38.8±4.9
Female (n=6)	$3x10^{11}$	-	6.5±3.4	29.2±9.8	64.3±13.0
Female (n=3)	$3x10^{11}$	+	8.8±4.3	42.9±9.5#	48.3±11.5

The percentage of unconjugated (%UCB), monoglucuronidated (%BMG), and diglucuronidated (%BDG) bilirubin in bile was determined by high-performance liquid chromatography. All studies were performed at 16 weeks after vector administration. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared between female low-dose ($1 \cdot 10^{11}$) vector and female low dose plus poly[i] preadministration. $^{\#}p < 0.01$ compared between female high-dose ($3 \cdot 10^{11}$) vector and female high dose plus poly[i] preadministration. Poly[i], polyinosinic acid.

In conclusion, our data demonstrate that macrophage scavenger receptor can mediate the endocytosis of AAV serotype 8. In addition, we showed that in vivo administration of poly[i] before the viral vector improved outcome of liver-directed gene therapy in the Gunn rat, especially in females, suggesting that the lower efficacy in this sex seems to be caused by an increased uptake by the macrophages. Therefore, these results seem of relevance for scAAV8-mediated liver-directed gene therapy when aiming to treat autosomally inherited liver disorders.

Author Disclosure Statement

No competing financial interests exist.

References

1. Srivastava, A., Lusby, E.W., and Berns, K.I. (1983). *Nucleotide sequence and organization of the adeno-associated virus 2 genome.* J. Virol. 45, 555–564.
2. Gao, G., Vandenberghe, L.H., and Wilson, J.M. (2005*). New recombinant serotypes of AAV vectors. Curr. Gene Ther.* 5, 285–297.
3. Jacobs, F., and Wang, L. (2011). *Adeno-associated viral vectors for correction of inborn errors of metabolism: progressing towards clinical application.* Curr. Pharm. Des. 17, 2500–2515.
4. Nathwani, A.C., Tuddenham, E.G., Rangarajan, S., et al. (2011). *Adenovirus-associated virus vector mediated gene transfer in hemophilia B*. N. Engl. J. Med. 365, 2357–2365.
5. Zaiss, A.K., and Muruve, D.A. (2008). *Immunity to adenoassociated virus vectors in animals and humans: a continued challenge*. Gene Ther. 15, 808–816.
6. Peiser, L., Mukhopadhyay, S., Gordon, S. (2002). *Scavenger receptors in innate immunity.* Curr. Opin. Immunol. 14, 123–128.
7. Kzhyshkowska, J., Neyen, C., Gordon, S. (2012). *Role of macrophage scavenger receptors in atherosclerosis.* Immunobiology 217, 492–502.
8. Haisma, H.J., Kamps, J.A., Kamps, G.K., et al. (2008*). Polyinosinic acid enhances delivery of adenovirus vectors in vivo by preventing sequestration in liver macrophages*. J. Gen. Virol. 89, 1097-1105.
9. Goh, J.W., Tan, Y.S., Dodds, A.W., et al. (2010). *The class A macrophage scavenger receptor type I (SR-AI) recognizes complement iC3b and mediates NF-kappaB activation*. Protein Cell 1, 174-187.
10. Axelrod, J., Schmid, R., and Hammaker, L. (1957). *A biochemical lesion in congenital, nonobstructive, non-haemolytic jaundice*. Nature 180, 1426–1427.
11. Bosma, P.J., Chowdhury, N.R., Goldhoorn, B.G., et al. (1992). *Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I*. Hepatology 15, 941–947.
12. Pett, S., and Mowat, A.P. (1987). *Crigler-Najjar syndrome types I and II. Clinical experience—King's College Hospital 1972– 1978. Phenobarbitone, phototherapy and liver transplantation.* Mol. Aspects Med. 9, 473–482.
13. Seppen, J., Bakker, C., de, J.B., et al. (2006). *Adeno-associated virus vector serotypes mediate sustained correction of b ilirubin UDP glucuronosyltransferase deficiency in rats.* Mol. Ther. 13, 1085–1092.
14. Montenegro-Miranda, P.S., ten, B.L., Kunne, C., et al. (2011). *Mycophenolate mofetil impairs transduction of single-stranded adeno-associated viral vectors.* Hum. Gene Ther. 22, 605-612.
15. Ambarus, C.A., Krausz, S., van, E.M., et al. (2012). *Systematic validation of specific phenotypic markers for in vitro polarized human macrophages.* J. Immunol. Methods 375, 196–206.
16. Montenegro-Miranda, P.S., Sneitz, N., de Waart, D.R., et al. (2012). *Ezetimibe: a biomarker for efficacy of liver directed UGT1A1 gene therapy for inherited hyperbilirubinemia.* Biochim. Biophys. Acta 1822, 1223–1229.
17. Seppen, J., Tada, K., Hellwig, S., et al. (1996). *Bilirubin glucuronidation by intact Gunn rat fibroblasts expressing bilirubin UDP-glucuronosyltransferase.* Biochem. J. 314 (Pt 2), 477–483.
18. Penman, M., Lux, A., Freedman, N.J., et al. (1991). *The type I and type II bovine scavenger*

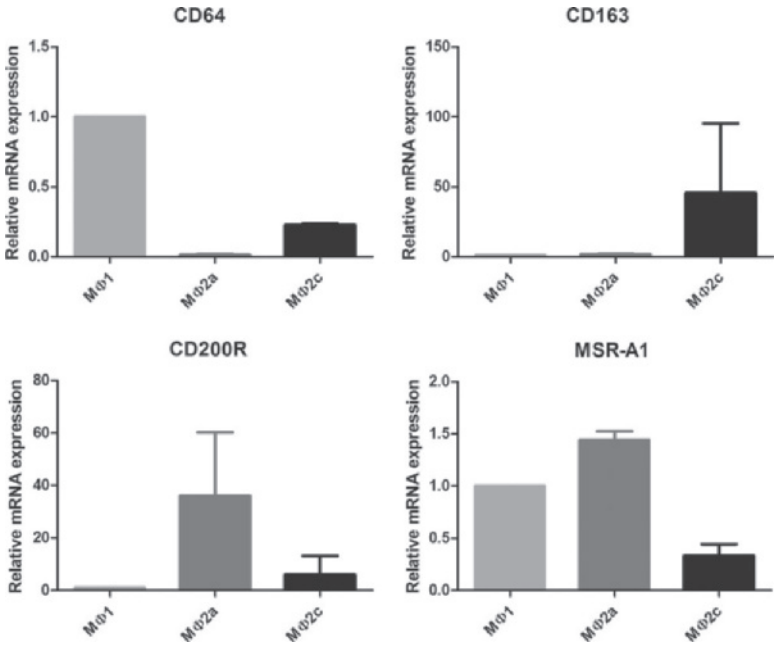
receptors expressed in Chinese hamster ovary cells are trimeric proteins with collagenous triple helical domains comprising noncovalently associated monomers and Cys83-disulfide-linked dimers. J. Biol. Chem. 266, 23985–23993.

19. Platt, N., and Gordon, S. (2001). *Is the class A macrophage scavenger receptor (SR-A) multifunctional? —The mouse's tale.* J. Clin. Invest. 108, 649–654.
20. Tjiu, J.W., Chen, J.S., Shun, C.T., et al. (2009*). Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction.* J. Invest. Dermatol. 129, 1016–1025.
21. Sica, A., Schioppa, T., Mantovani, A., and Allavena, P. (2006). *Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy.* Eur. J. Cancer 42, 717–727.
22. Daigneault, M., Preston, J.A., Marriott, H.M., et al. (2010). *The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages.* PLoS One 5, e8668.
23. Martinez, F.O., Gordon, S., Locati, M., and Mantovani, A. (2006). *Transcriptional profiling of the human monocyte-tomacrophage differentiation and polarization: new molecules and patterns of gene expression.* J. Immunol. 177, 7303–7311.
24. Miranda, P.S., and Bosma, P.J. (2009). *Towards liver-directed gene therapy for Crigler-Najjar syndrome*. Curr. Gene Ther. 9, 72–82.
25. Fox, I.J., Chowdhury, J.R., Kaufman, S.S., et al. (1998). *Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation.* N. Engl. J. Med. 338, 1422–1426.
26. Smith, J.S., Xu, Z., and Byrnes, A.P. (2008). *A quantitative assay for measuring clearance of adenovirus vectors by Kupffer cells.* J. Virol. Methods 147, 54–60.
27. Kuzmin, A.I., Finegold, M.J., and Eisensmith, R.C. (1997). *Macrophage depletion increases the safety efficacy and persistence of adenovirus-mediated gene transfer in vivo.* Gene Ther. 4, 309-316.
28. Shayakhmetov, D.M., Li, Z.Y., Ni, S., and Lieber, A. (2004*). Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fibermodified vectors.* J. Virol. 78, 5368–5381.
29. Zaiss, A.K., Liu, Q., Bowen, G.P., et al. (2002). *Differential activation of innate immune responses by adenovirus and adenoassociated virus vectors.* J. Virol. 76, 4580–4590.
30. Martino, A.T., Suzuki, M., Markusic, D.M., et al. (2011). *The genome of self-complementary adeno associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver.* Blood 117, 6459–6468.
31. Paneda, A., Vanrell, L., Mauleon, I., et al. (2009). *Effect of adenoassociated virus serotype and genomic structure on liver transduction and biodistribution in mice of both genders.* Hum. Gene Ther. 20, 908–917.
32. Dane, A.P., Wowro, S.J., Cunningham, S.C., and Alexander, I.E. (2012). *Comparison of gene transfer to the murine liver following intraperitoneal and intraportal delivery of hepatotropic AAV pseudo-serotypes.* Gene Ther. 20, 460–464.
33. Csaba, G., and Pallinger, E. (2009). *Gender dependence in the hormone content of the immune cells*. Acta. Physiol. Hung. 96, 45–50.
34. Melgert, B.N., Oriss, T.B., Qi, Z., et al. (2010). *Macrophages: regulators of sex differences in*

asthma? Am. J. Respir. Cell Mol. Biol. 42, 595–603.

35. Angele, M.K., Schwacha, M.G., Ayala, A., and Chaudry, I.H. (2000). *Effect of gender and sex hormones on immune responses following shock.* Shock 14, 81–90.
36. Yang, J.M., Kim, S.S., Kim, J.I., et al. (1999). *The metabolic effects of estriol in female rat liver.* J. Korean Med. Sci. 14, 277–285.
37. Hsieh, C.H., Nickel, E.A., Chen, J., et al. (2009). *Mechanism of the salutary effects of estrogen on kupffer cell phagocytic capacity following trauma-hemorrhage: pivotal role of Akt activation.* J. Immunol. 182, 4406–4414.
38. Lonning, S.M., Ford, C.A., Taylor, K.M. (2002). *Influence of gender-specific hormones on aden-associated virus vector gene transfer and expression [abstract].* Mol. Ther. 5, S2.

Supplementary Figure



Supplementary Figure 1. Expression of macrophage scavenger receptor A is induced in type 2a human macrophages. Primary human monocytes were polarized into type 1 (Mϕ1), 2a (Mϕ2a), and 2c (Mϕ2c) macrophages. Specific markers were checked for successful polarization: CD64 for Mϕ1, CD200R for MF2a, and CD163 for Mϕ2c. Quantitative polymerase chain reaction for macrophage scavenger receptor A showed highest expression in Mϕ2a.

4

BILIVERDIN REDUCTASE INHIBITORS DID NOT IMPROVE SEVERE UNCONJUGATED HYPERBILIRUBINEMIA *IN VIVO*

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Abstract

We aimed to identify potent biliverdin reductase (BVRA) inhibitors as a novel concept for the treatment of severe unconjugated hyperbilirubinemia. 1280 FDA-approved compounds were screened *in vitro* for their ability to inhibit human and rat BVRA activity and 26 compounds were identified as BVRA inhibitors. Montelukast and Disulfiram were selected as potentially clinically applicable drugs and tested to reduce serum unconjugated bilirubin (UCB) levels in the Ugt1a1-deficient rat, a model for chronic unconjugated hyperbilirubinemia. Oral administration of Disulfiram was toxic in the Ugt1a1-deficient rat (weight loss, transaminase elevation). Oral Montelukast administration led to low serum concentrations and did not alter serum UCB levels. Intraperitoneal injections of Montelukast resulted in concentrations up to 110 µmol/L in serum and 400 µmol/L in the liver. Still, serum UCB levels remained unaltered. This first study on biliverdin reductase inhibition as a novel concept for treatment of unconjugated hyperbilirubinemia identified putative *in vitro* BVRA inhibitors. Montelukast, the clinically most suitable inhibitor, did not result in reduction of serum UCB in the Ugt1a1-deficient rat. The proposed treatment strategy will not result in amelioration of severe unconjugated hyperbilirubinemia in humans without the identification or development of more potent BVRA inhibitors.

Introduction

Severe unconjugated hyperbilirubinemia is a critical condition which can lead to irreversible brain damage (kernicterus) already in early childhood and eventually can be lethal when left untreated [1]. Accumulation of unconjugated bilirubin (UCB) results from an imbalance between UCB production and elimination and can be caused by several pathological conditions such as extensive hemolysis or severe impaired bilirubin glucuronidation known as Crigler-Najjar syndrome [2,3].

Bilirubin is the final product of heme catabolism. Old erythrocytes, which are degraded in splenocytes and liver sinusoidal cells, and cytochrome P_{450} [4], are the main source of heme production. Heme is catabolized into equimolar amounts of carbon monoxide (CO), free iron (Fe^{2+}) and biliverdin by the enzyme heme oxygenase (HO) [5]. Iron is recycled for the production of heme, CO functions as a local signaling molecule and biliverdin is further reduced by the cytosolic enzyme biliverdin reductase (BVRA) into UCB (figure 1). The lipophilic and toxic UCB can be detoxified in the hepatocyte via conjugation with one or two glucuronide groups by the enzyme UDP-glucuronosyl transferase 1A1 (UGT1A1)[6]. Glucuronidation will render bilirubin hydrophilic, an essential modification for its excretion into bile over the apical membrane via the multidrug resistance-associated protein 2 (*ABCC2*/MRP2) transporter[7]. Under physiological conditions serum total bilirubin concentration is less than 17 µmol/L (1mg/dL).

Newborns are likely to develop increased unconjugated bilirubinemia with total bilirubin levels above 17 µmol/L (1 mg/dL). However, only a small group will develop severe unconjugated hyperbilirubinemia, with levels above 428 µmol/L (25 mg/dL), requiring treatment. Severe neonatal jaundice is in general caused by a combination of increased bilirubin production, due to extensive breakdown of fetal hemoglobin and a shorter life span of erythrocytes, and a reduced clearance of bilirubin due to low UGT1A1 expression in the newborn liver [8]. Also other pathological conditions such as isoimmune-mediated hemolysis (e.g. Rh(D) incompatibility), erythrocyte enzymatic defects (e.g. hereditary spherocytosis and heterozygous beta-thalassemia) or inherited erythrocyte membrane defects (e.g. glucose-6-phosphate dehydrogenase deficiency) can cause severe neonatal hyperbilirubinemia [9]. In neonatal jaundice, phototherapy is the first choice of treatment. When phototherapy is not effective or infants show signs of bilirubin-induced neurologic dysfunction, treatment via exchange transfusion can be

considered [10].

Crigler-Najjar syndrome (CNS) is an autosomal recessive disorder characterized by severe unconjugated hyperbilirubinemia caused by a deficiency of bilirubin glucuronidation, due to impaired or complete lack of UGT1A1 function [11]. Phototherapy is the first line treatment for CNS [12,13]. It is based on the formation of hydrophilic UCB photo-isomers in the skin upon exposure to light that can readily be excreted via bile or urine. Drawbacks of phototherapy are the long exposure time (up to 14 hours daily), and reduced efficacy over time. Liver transplantation, both orthotopic and auxiliary, is currently the only curative therapy for CNS [12,13]. Due to the risk for complications and mortality, the need of lifelong immunosuppression and the long waiting list, the use of liver transplantation in CNS has its limitations.

In both disease entities described above, all treatment strategies (phototherapy, exchange transfusion and liver transplantation) are directed to eliminate the toxic UCB from the systemic circulation [14]. Rather than inducing bilirubin elimination, inhibition of bilirubin production is another potential strategy for decreasing serum UCB levels. This principle was first proven to be effective by the use of synthetic metalloporphyrins that inhibit the breakdown of heme into biliverdin by competitive binding to HO [15]. Small clinical trials showed a beneficial effect of metalloporphyrins in jaundiced neonates by lowering the maximum serum bilirubin concentration, lower frequency of severe hyperbilirubinemia, decrease the need for phototherapy, and shorter duration of hospitalization [16]. Unfortunately no reports were made on the amount and severity of neurotoxicity, death and long term neurological outcome of these patients. Although these clinical trials looked very promising, the use of metalloporphyrins has never been registered due to its severe side effects, such as photo-sensitivity of the skin, diminished CYP_{450} activity and iron deficiency anemia [17]. Metalloporphyrins also induce HO expression [17], and will therefore not be suitable for long term use, as required in CNS.

Inhibition of the downstream step in the generation of bilirubin, the conversion of biliverdin to bilirubin, has not yet been investigated as a potential treatment for severe unconjugated hyperbilirubinemia. This step is catalyzed by biliverdin reductase (BVRA). Inhibition of this enzyme will reduce bilirubin production without affecting heme catabolism, resulting in biliverdin as the end product. Biliverdin is a hydrophilic, non-toxic compound which can be excreted in both bile and urine without any further conjugation steps [18]. Conversion of biliverdin to bilirubin is not

seen in all species. Most bird and fish species completely lack biliverdin reductase and excrete exclusively biliverdin via their faeces [19,20]. Although bilirubin has been recognized as an anti-oxidant [21,22] and shows to have anti-inflammatory properties [23,24], the necessity for the conversion of biliverdin to bilirubin still forms an enigma. The identification of two adult patients with complete lack of BVRA activity due to a nonsense mutation in the BVRA gene, revealed that it is compatible with life and does not result in disease [25]. This indicates that finding an effective, non-toxic BVRA inhibitor could be an option to treat severe unconjugated hyperbilirubinemia without major adverse effects.

Aiming to treat severe unconjugated hyperbilirubinemia by decreasing bilirubin production, we developed a screen for potential BVRA inhibitors. A semi-high-throughput assay was set up to determine BVRA activity after which 1280 FDA/EMA approved compounds were screened for their ability to inhibit BVRA. Two inhibitors that could potentially be applied in a clinical setting were selected for subsequent testing *in vivo* in the Ugt1a1 deficient Gunn rat, an animal model for inherited severe unconjugated hyperbilirubinemia.

Materials and methods

Cloning of rat and human biliverdin reductase A

Reverse transcribed RNA isolated from rat and human liver was amplified with Phusion high-fidelity Polymerase (New England Biolabs Inc, Ipswich, MA, USA) using the primers listed in supplementary table 1, and upon incubation with Taq Polymerase amplicons were cloned into a TA-cloning vector (Sigma-Aldrich, Steinheim, Germany) and sequenced completely using BigDye Terminator v1.1 (Life technologies, Carlsbad, USA). Both BVRA cDNAs were inserted into a lentiviral vector behind a constitutive CMV promoter (pLV.CMV.bc.Puro) and lentivirus was produced in HEK293T cells using transient transfection [26].

Production of rBVRA and hBVRA containing cell lysates

HEK293T cells were seeded in a T162cm² flask and upon reaching 30-40% confluence lentivirus containing the hBVRA or rBVRA transgene was added in the presence of 10 µg/ml DEAE Dextran. Four hours thereafter, the medium was refreshed with complete DMEM. 48 hours after transduction the cells were washed and harvested in ice-cold PBS. Cells were then pelleted at 1500 rpm at

4°C for 10 minutes and taken up in a hypertonic buffer (10mM HEPES, 40mM KCl, 2mM $MgCl_2$, 10% gycerol). After 15 minutes on ice, 25 stokes with a dounce homogenizer were used to break the cells. To remove cell membranes and debris, the homogenate was centrifuged at 80.000 RCF (xg) for 1 hour at 4°C and the supernatant was harvested, aliquoted and stored at -80°C.

Biliverdin Reductase assay and semi-high-throughput drug screen
The biliverdin reductase activity assay was conducted in a 96-wells format in a total volume of 200µL at 37°C. HEK293T cytosol (5µg total protein) was pre-incubated at 37°C with 10 µmol/L biliverdin (Sigma-Aldrich, Steinheim, Germany), BSA (400µg/ml) and 50mM TrisHCL pH8.7. After 5 minutes the reaction was started by adding NADPH to a final concentration of 100 µmol/L. The conversion of biliverdin to bilirubin was determined by measuring absorbance at 453nm (bilirubin) and 670nm (biliverdin) every

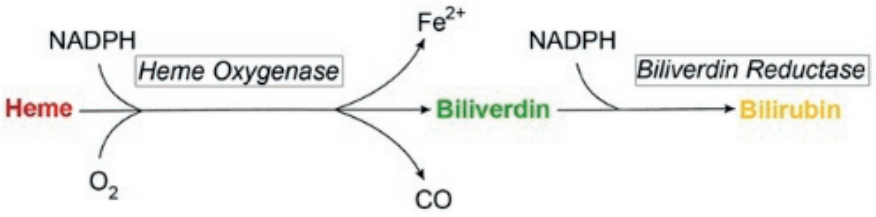


Figure 1: Schematic representation of the conversion of Heme into bilirubin. Heme is catabolised into biliverdin by the enzyme Heme Oxygenase, releasing carbon monoxide (CO) and free iron (Fe^{2+}). Biliverdin is further reduced by Biliverdin Reductase into unconjugated bilirubin. Both enzymes depend on nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agent.

2 minutes for 60 minutes until the plateau phase was reached using a Synergy HT multi-detection reader (BioTek Instruments Inc, Winooski, VT, USA). BVRA activity was determined by calculating the slope over the linear part of the reaction, expressed as the ratio 453/670nm per minute (Figure 2a-b).

A library containing 1280 FDA and EMA approved small molecules was purchased from Prestwick Chemical (Illkirch-Graffenstaden, France). The effect of 10 µM of each compound, dissolved in DMSO (Sigma-Aldrich, Steinheim, Germany) on biliverdin reductase activity was tested in duplo in two independent assays. The quality and reproducibility of this semi-high-throughput drug screen was assured with a Z-factor of > 0.5 at all performed assays.

Animal experiments

All animal experiments were performed in accordance with the guidelines and with approval of the Animal Ethics Committee of the Academic Medical Center of Amsterdam. Male Gunn rats, strain RHA j/j, from our breeding colony, were fed ad libitum and were randomly assigned to different treatment groups. Blood samples were taken by tail or saphenous vein puncture and collected in lithium heparin tubes after which plasma was separated by centrifugation. Bile samples were collected by bile duct cannulation after distal ligation of the bile duct at the time of sacrifice. Serum and bile samples were protected from light during sampling and analysis and stored at -80°C.

Oral drug administration

Ascending doses of Disulfiram (90, 180, or 360 mg/kg/day), Montelukast (10, 30, or 90 mg/kg/day) in 1% DMSO or vehicle control were administered to male Gunn rats (12-16 weeks old) over a period of 3 weeks via oral gavage.

Intraperitoneal drug administration

Male Gunn rats weighing 30-50 grams (4 weeks of age) were administered 300mg/kg/day Montelukast in Hanks' Balanced Salt solution (HBSS) buffered with 15mM HEPES to pH 8.0 or vehicle control by bi-daily injection (150mg/kg per injection) in the peritoneal cavity using a 23 gauge needle for 2,5 days. In total each rat received 5 i.p. injections.

Bilirubin and biliverdin measurements

Serum total and direct bilirubin were determined with standardized diazo assay by the Department of Clinical Chemistry of the hospital. For High-performance liquid chromatography (HPLC) analysis of both bilirubin fractions and biliverdin in serum, urine and bile the protocol was adapted from the method described by Spivak and Carey [27]. In brief, urine and plasma samples were de-proteinized with 2 volumes of methanol. Following centrifugation for 2 minutes at 14000 rpm at 4°C, 100µl of the supernatant was applied to a Pursuit C18, 5 µm, 10 cm HPLC column (Varian, Middelburg, The Netherlands). Starting eluent consisted of 40% MeOH/60% ammonium acetate (1%, pH4.5), followed by a linear gradient to 100% MeOH in 15 minutes. Detection of biliverdin and bilirubin was performed at 390 and 450 nm, respectively. Quantification of biliverdin was done by using a calibration curve of biliverdin (Sigma-Aldrich, Steinheim, Germany). Quantification of bilirubin and conjugates was executed by using a calibration curve of unconjugated bilirubin (Sigma-Aldrich, Steinheim, Germany).

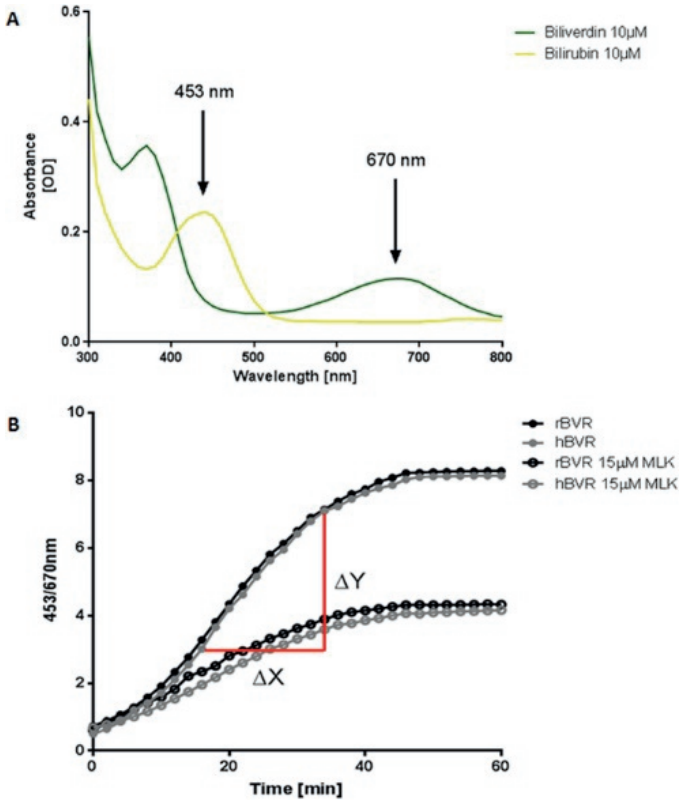


Figure 2: (A) The biliverdin reductase activity assay makes use of the different wavelengths at which bilirubin (453nm) and biliverdin (670nm) have their peak absorbance [OD]. The absorbance spectrum was measured at a concentration of 10µmol/L in a total volume of 200µL at 37°C by a Synergy HT multi-detection reader.
(B) Biliverdin reductase activity is derived from the speed at which biliverdin is converted to bilirubin by calculating the slope over the linear part of the enzymatic reaction. Cytosolic rat or human biliverdin reductase (rBVRA or hBVRA) was pre-incubated at 37°C with 10 µmol/L biliverdin, bovine serum albumin (BSA 400µg/ml) and 50mM TrisHCL pH8.7. The reaction was started by adding NADPH to a final concentration of 100 µmol/L in a total volume of 200µL. The conversion of biliverdin to bilirubin was determined by measuring absorbance every 2 minutes for 60 minutes and expressed as the ratio 453/670 nm. When 15µM Montelukast (MLK) is added to the reaction, the reduced slope (ΔY/ΔX) indicates reduced BVRA activity.

Montelukast measurements

Serum Montelukast concentration was measured using the above described HPLC protocol with a starting eluent consisting of 50% acetonitrile/ 50% 20 mmol/L ammonium acetate buffer, pH 5.5, followed by a linear gradient to 100% acetonitrile. Peaks were detected at 345nm of the UV spectrum. Quantification of Montelukast was executed by using a calibration curve of Montelukast.

Tissue analysis

Tissue (liver, spleen and kidney) was snap-frozen in liquid nitrogen and stored at -80°C. Homogenates were made in 100 mmol/L Tris-HCL pH 7,8 buffer and bilirubin, biliverdin and Montelukast concentrations were measured by HPLC.

Statistical analysis

To determine significance of BVRA inhibition *in vitro* between different concentrations a one way ANOVA with Bonferroni's correction was used. A student's t test was used to calculate differences in serum bilirubin concentrations between the treated and vehicle group

Results

Biliverdin reductase inhibitor in vitro drug library screen

After establishing a stable and highly reproducible BVRA-activity assay (figure 2a) a drug library containing 1280 FDA and EMA approved small molecules (Prestwick Chemical) was tested for their ability to inhibit hBVRA activity *in vitro*. 26 compounds were found to inhibit hBVRA activity between 34 to 99% at the used concentration of 10 µmol/L (figure 3a). Of those potential candidates, those that can be administered orally and can be given long-term without potential toxic effect in the clinical setting were selected. Based on these criteria, two potential candidates for further dose response testing remained (figure 3b).

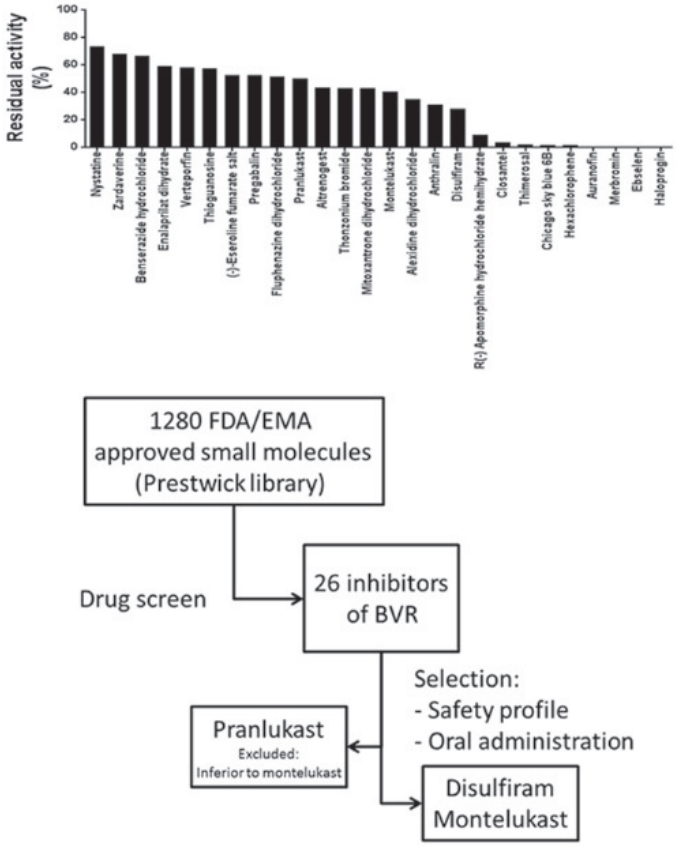


Figure 3: (A) 26 compounds were identified to inhibit human Biliverdin Reductase at a concentration of 10 µmol/l during a semi-high-throughput screen of a drug library containing 1280 FDA and EMA approved small molecules. **(B) Two compounds were selected for *in vivo* studies** after consideration of safety profile for long-term dosing and possibility of oral administration**.**

The leukotriene receptor antagonist Montelukast (Singulair), which is used as a complementary therapy for asthma, reduced BVRA activity by 60% during the drug library screen. A second leukotriene receptor antagonist, Pranlukast (Onon), also showed capacity to reduce BVRA activity by 50% during the drug library screen. Because Montelukast showed more potency, Pranlukast was left out for further studies (figure 3a).

The second compound selected was the irreversible acetaldehyde dehydrogenase inhibitor Disulfiram (Antabus) which is used to support the treatment of chronic alcoholism. Disulfiram reduced BVRA activity by 75% in the semi high-through put screen (figure 3a).

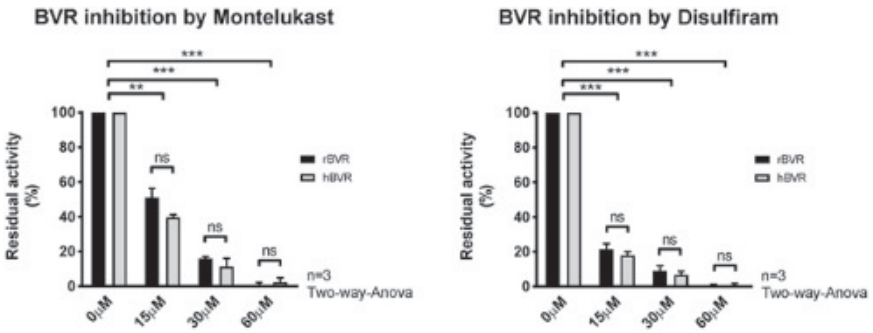


Figure 4: (A) Comparison between rat and human Biliverdin Reductase (rBVRA and hBVRA) activity in the presence of 0, 15, 30 or 60 µM Montelukast or (B) 0, 15, 30 or 60 µM Disulfiram. The conversion of biliverdin to bilirubin was determined by measuring absorbance over a period of 60 minutes at wavelengths 453nm (bilirubin) and 670nm (biliverdin). Biliverdin reductase activity was determined by calculating the slope over the linear part of the reaction and is expressed as residual activity relative to the vehicle control 1% DMSO. Data represent the mean ± SD of the data obtained from three independent experiments (n=3). Two-way analysis of variance (ANOVA) was used for statistical analysis, ** p<0.01, ***p<0.001.

Montelukast and Disulfiram BVRA activity inhibition in vitro

Montelukast and Disulfiram were tested for their ability to inhibit both human and rat BVRA in four different concentrations *in vitro*. Indeed both compounds showed a dose-response effect on BVRA activity. As observed during the drug library screen, both compounds reduced BVRA activity with more than 50% with concentrations as low as 15 µmol/L. At a concentration of 30µmol/L Montlelukast inhibited hBVRA and rBVRA by 75%. Disulfiram was even more effective and showed almost complete BVRA inhibition at concentrations above 30 µmol/L (figure 4a-b). Because of these positive findings both Disulfiram and Montelukast were tested *in vivo* for their ability to reduce serum bilirubin in the chronic hyperbilirubinemic Gunn rats.

Disulfiram is hepatotoxic in the Gunn rat

Adult male Gunn rats were administered Disulfiram via oral gavage in a weekly increasing dose starting from 90mg/kg/day increasing 2 fold every week. During the first week the rats started to lose weight in comparison to the vehicle control group. This weight loss progressed during the second week when the dose of Disulfiram was increased to 180 mg/kg/day as per protocol (supplementary figure 1a). Besides losing weight the animals also showed a rise in both serum bilirubin and liver enzyme (ALT) levels (supplementary figure 1b-c), indicating a hepatotoxic effect of Disulfiram in Gunn rats. The experiment was terminated after the second

week, due to the progressive weight loss of the animals. Because of the (hepato-) toxic profile no further *in vivo* experiments involving Disulfiram were conducted.

No effective BVRA inhibition upon oral administration of Montelukast

Adult male Gunn rats received Montelukast via oral gavage at a starting dose of 10 mg/kg/day and increasing 3-fold every week. In contrast to Disulfiram, Montelukast was well tolerated by the Gunn rat. However, even the highest dose of Montelukast (90 mg/kg/day) did not alter serum bilirubin levels (supplementary figure 2a). To investigate if serum Montelukast concentrations were in the range for potential rBVRA inhibition trough levels were determined using HPLC. This revealed serum Montelukast trough levels were only between 1 and 8 µmol/l, clearly below the concentration needed to inhibit rBVRA (supplementary figure 2b).

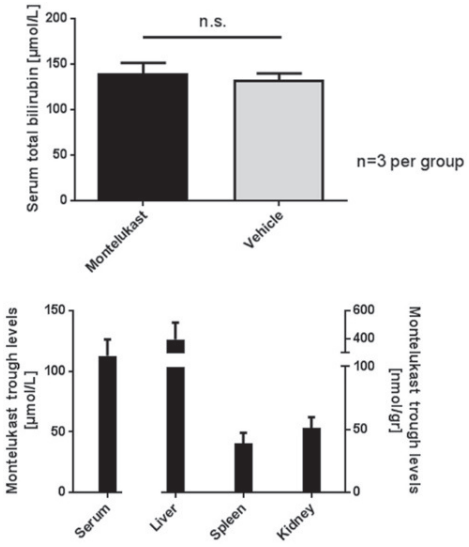


Figure 5: (A) Total serum bilirubin is not decreased in 4 weeks old male Gunn rats after bi-daily intraperitoneal injection of 150mg/kg Montelukast for 2,5 days (5 injections), compared to animals receiving vehicle control. Data represent the mean ± SD of three animals per group. **(B) Concentration of Montelukast in serum,liver, spleen and kidney of 4 weeks old male Gunn rats after bi-daily intraperitoneal injection of 150mg/kg Montelukast for 2,5 days (5 injections).** Animals were sacrificed 5 hours after last Montelukast administration. Tissue homogenates were made in 100 mmol/L Tris-HCL pH 7,8 buffer and concentration of Montelukast was measured by HPLC. Data represent the mean ± SD of three animals per group.

High serum Montelukast concentrations do not result in reduced serum bilirubin in young Gunn rats

To achieve higher *in vivo* concentrations, young male Gunn rats were injected intraperitoneally with Montelukast. For three days they received bi-daily injections (150mg/kg). This indeed resulted in high serum Montelukast concentrations up to 112.0±27.0 µmol/L. The Montelukast concentration was variable in different tissues ranging from 38.7±14.9 nmol/gram in the spleen to 51.1±15.0 nmol/gram in the kidney and accumulating in the liver to 396.0±168 nmol/gram (figure 5b). Although these concentrations would strongly inhibit BVRA activity *in vitro*, no effect was observed on the serum bilirubin concentrations in comparison to the vehicle treated group (figure 5a). Furthermore, no biliverdin was detectable in serum, urine, liver and spleen (data no shown), suggesting the inhibition of rBVRA, if any, was insufficient.

Discussion

In the present study, we have demonstrated that biliverdin reductase (BVRA) activity can be successfully inhibited *in vitro* by at least 26 FDA/EMA approved compounds, including the irreversible acetaldehyde dehydrogenase inhibitor Disulfiram and the leukotriene receptor antagonist Montelukast. However, *in vivo* administration of Montelukast did not result in a decrease of serum bilirubin, while Disulfiram appeared to be hepatotoxic to the Ugt1A1 deficient Gunn rat, the animal model for hereditary unconjugated hyperbilirubinemia. Both compounds therefore appeared not suitable to treat severe unconjugated hyperbilirubinemia in this animal model.

Both neonatal jaundice and Crigler-Najjar syndrome, marked by highly elevated serum levels of unconjugated bilirubin, can cause severe central nervous damage with life-long disabilities and can even be lethal when left untreated. Current established treatments are based on the elimination of UCB, rather than on reducing the production of UCB. Based on two subjects described in literature lacking biliverdin reductase activity that did not express a disease phenotype [25], we postulated that inhibition of this enzyme is an interesting new target for the treatment of severe unconjugated hyperbilirubinemia. Two out of the 26 FDA approved compounds that showed to inhibit BRVA had a favorable safety profile for potential clinical application in the treatment of CNS.

Disulfiram has been used for more than 60 years in treatment of alcohol dependence. By inhibiting the enzyme acetaldehyde dehydrogenase an accumulation of acetaldehyde will occur after alcohol intake, resulting in a disulfiram-ethanol reaction with symptoms including: flushing of the skin, accelerated heart rate, shortness of breath, nausea, vomiting, throbbing headache, postural syncope, and circulatory collapse [28]. Disulfiram is also known to inhibit cytochrome P_{450} 2E1 and by forming complexes with metals it can function as a protease inhibitor [29]. In this study we found for the first time that Disulfiram also acts as a biliverdin reductase inhibitor. *In vitro* Disulfiram showed to be a very effective BVRA inhibitor with a concentration of only 10 µmol/L resulting in a 75% activity reduction. However, *in vivo* oral administration of Disulfiram resulted in severe weight loss and possible hepatotoxicity in male Gunn rats. Several clinical studies have shown that the use of Disulfiram can lead to liver tests abnormalities in 25% of the patients of whom 10-16% suffered from severe hepatotoxicity resulting in liver transplantation or death [30]. Although the underling mechanism is not fully understood yet, it is thought that different processes such as hypersensitivity and direct toxic effects of Disulfiram metabolites may play a role [30,31]. A large proportion of Disulfiram is eliminated via bile after glucuronidation of its metabolite diethyldithiocarbamate [32]. Absence of this detoxification mechanism could in part explain the hepatotoxicity of disulfiram in our Ugt1a1 deficient animal model, rendering this drug unsuitable for the use in patients with CNS. Further evaluation of the pharmacokinetics of disulfiram in this animal model is challenging since methods to accurately measure disulfiram and its metabolites in serum lack [33,34]

Montelukast has also shown to be a potent *in vitro* inhibitor of both human and rat BVRA. Montelukast is a cysteinyl leukotriene receptor type 1 antagonist which is clinically used as add-on therapy to inhaled corticosteroids for asthma patients [35]. By blocking the leukotriene pathway it improves symptoms of asthma and reduces inflammation [36]. Montelukast is generally well tolerated in both pediatric and adult patients, with few adverse reactions reported [35]. This lipophilic compound with high protein binding affinity[37] could possibly displace UCB from albumin by competitive binding, although literature to support this assumption lacks. No effect was seen on the bilirubin metabolism in the Gunn rat after both oral and intraperitoneal administration. Montelukast is a very effective cysteinyl leukotriene receptor type 1 antagonist with only low nanomolar concentrations needed to achieve clinical effects [38]. Although a much higher oral dose was used

(90 mg/kg/day vs 0.125 mg/kg/day), serum concentrations reached a maximum of only 4 µmol/L in the Gunn rat. As observed in the *in vitro* studies, a concentration above 60 µmol/L is most likely necessary to completely inhibit BVRA and to result in a clinical effect on the bilirubin metabolism. We did not observe any alteration in the serum bilirubin levels in Gunn rats treated with oral Montelukast, which indeed could be caused by inadequate serum concentrations of the drug. To obtain proof of concept, younger male Gunn rats were bi-daily injected intraperitoneally for three days with Montelukast. Although this resulted in a significant increase in serum Montelukast concentrations up to 112 µmol/L and as high as 396 nmol/gram in the liver, the required concentration for complete BVRA inhibition was not reached in all tissues. Serum bilirubin levels remained unaltered and biliverdin did not appear in serum or urine during treatment. Whether complete BVRA inhibition in al tissues would lead to a decrease of serum bilirubin levels remains uncertain, but with the proposed inhibitors it is unlikely that complete BVRA inhibition *in vivo* can be achieved.

The other compounds identified as potential BVRA activity inhibitors during the semi-high-throughput screen are either not orally administrable (e.g. Anthralin and Haloprogin), used as stabilizers/preservatives (e.g. Thimerosalm) or have inferior safety profiles (e.g. Auranofin and Apomorphine) and were therefore not further investigated both *in vitro* and *in vivo.*

The reticuloendothelial system is the site of erythrocyte degradation and heme catabolism. Kupffer cells and other macrophages express large amount of HO-1 and are responsible for 50-75% of all bilirubin formation [39,40]. Biliverdin reductase is expressed widely throughout the body, including macrophages. Measurement of Montelukast after intraperitoneal administration showed high concentrations in serum and liver, but possibly insufficient concentrations in spleen and kidney for complete BVRA inhibition. Unaltered serum bilirubin levels could indicate that only a small amount of residual BVRA activity could be sufficient to support bilirubin production, resembling the high efficiency of UGT1A1 [41]. Biliverdin reductase is a cytosolic protein [42], therefore it is important that Montelukast is taken up by the macrophages to have any effect on the bilirubin metabolism. The exact mechanism, however, by which Montelukast is taken up by cells remains to be determined [43].

In conclusion, we report for the first time that multiple compounds have the ability to inhibit BVRA activity *in vitro*. Because of the potential clinical use in patients

with severe unconjugated hyperbilirubinemia, Disulfiram and Montelukast were further investigated in the appropriate animal model. However, both compounds did not lower serum bilirubin levels *in vivo*, and Disulfiram even proved to be toxic in rats lacking Ugt1a1 activity. Unless a potent BVRA inhibitor with high bioavailability in all tissues is identified, the proposed treatment strategy will not result in amelioration of severe unconjugated hyperbilirubinemia in humans.

References

1. Shapiro, S. M., Bhutani, V. K. & Johnson, L. Hyperbilirubinemia and kernicterus. *Clin. Perinatol.* 33, 387-410, doi:10.1016/j.clp.2006.03.010 (2006).
2. Erlinger, S., Arias, I. M. & Dhumeaux, D. Inherited disorders of bilirubin transport and conjugation: new insights into molecular mechanisms and consequences. *Gastroenterology* 146, 1625-1638, doi:10.1053/j.gastro.2014.03.047 (2014).
3. Maisels, M. J. & Kring, E. The contribution of hemolysis to early jaundice in normal newborns. *Pediatrics* 118, 276-279, doi:10.1542/peds.2005-3042 (2006).
4. Bissell, D. M., Hammaker, L. & Schmid, R. Hemoglobin and erythrocyte catabolism in rat liver: the separate roles of parenchymal and sinusoidal cells. *Blood* 40, 812-822 (1972).
5. Kikuchi, G., Yoshida, T. & Noguchi, M. Heme oxygenase and heme degradation. *Biochem. Biophys. Res. Commun.* 338, 558-567, doi:10.1016/j.bbrc.2005.08.020 (2005).
6. Bosma, P. J. *et al.* Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J. Biol. Chem.* 269, 17960-17964 (1994).
7. Paulusma, C. C. *et al.* Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* 271, 1126-1128 (1996).
8. Kawade, N. & Onishi, S. The prenatal and postnatal development of UDP-glucuronyltransferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. *Biochem. J.* 196, 257-260 (1981).
9. Christensen, R. D. & Yaish, H. M. Hemolytic Disorders Causing Severe Neonatal Hyperbilirubinemia. *Clin. Perinatol.* 42, 515-527, doi:10.1016/j.clp.2015.04.007 (2015).
10. American Academy of Pediatrics Subcommittee on, H. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics* 114, 297-316 (2004).
11. Sinaasappel, M. & Jansen, P. L. The differential diagnosis of Crigler-Najjar disease, types 1 and 2, by bile pigment analysis. *Gastroenterology* 100, 783-789 (1991).
12. Strauss, K. A. *et al.* Management of hyperbilirubinemia and prevention of kernicterus in 20 patients with Crigler-Najjar disease. *Eur. J. Pediatr.* 165, 306-319, doi:10.1007/s00431-005-0055-2 (2006).
13. van der Veere, C. N. *et al.* Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. *Hepatology* 24, 311-315, doi:10.1002/hep.510240205 (1996).
14. Funato, M., Teraoka, S., Tamai, H. & Shimida, S. Follow-up study of auditory brainstem responses in hyperbilirubinemic newborns treated with exchange transfusion. *Acta Paediatr. Jpn.* 38, 17-21 (1996).
15. Kappas, A. & Drummond, G. S. Synthetic metalloporphyrins: a class of compounds of pharmacological interest. *Bioessays* 3, 256-259, doi:10.1002/bies.950030606 (1985).
16. Suresh, G. K., Martin, C. L. & Soll, R. F. Metalloporphyrins for treatment of unconjugated hyperbilirubinemia in neonates. *Cochrane Database Syst Rev*, CD004207, doi:10.1002/14651858.CD004207 (2003).
17. Schulz, S., Wong, R. J., Vreman, H. J. & Stevenson, D. K. Metalloporphyrins - an update. *Front. Pharmacol.* 3, 68, doi:10.3389/fphar.2012.00068 (2012).
18. Grossbard, M. L., Boyer, J. L. & Gordon, E. R. The excretion pattern of biliverdin and bilirubin in

bile of the small skate (Raja erinacea). *J. Comp. Physiol. B* 157, 61-66 (1987).

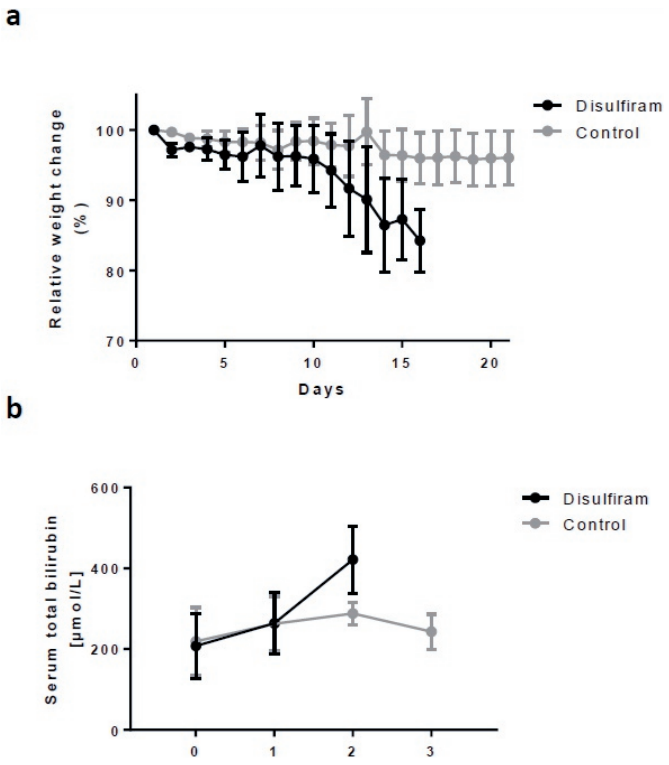
19. Mateo, R., Castells, G., Green, A. J., Godoy, C. & Cristofol, C. Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography-ultraviolet detection analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 810, 305-311, doi:10.1016/j.jchromb.2004.08.019 (2004).
20. Ding, Z. K. & Xu, Y. Q. Purification and characterization of biliverdin IXalpha from Atlantic salmon (Salmo salar) bile. *Biochemistry (Mosc.)* 67, 927-932 (2002).
21. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N. & Ames, B. N. Bilirubin is an antioxidant of possible physiological importance. *Science* 235, 1043-1046 (1987).
22. Stocker, R., McDonagh, A. F., Glazer, A. N. & Ames, B. N. Antioxidant activities of bile pigments: biliverdin and bilirubin. *Methods Enzymol.* 186, 301-309 (1990).
23. Lanone, S. *et al.* Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. *FASEB J.* 19, 1890-1892, doi:10.1096/fj.04-2368fje (2005).
24. Wang, W. W., Smith, D. L. & Zucker, S. D. Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. *Hepatology* 40, 424-433, doi:10.1002/hep.20334 (2004).
25. Nytofte, N. S. *et al.* A homozygous nonsense mutation (c.214C->A) in the biliverdin reductase alpha gene (BLVRA) results in accumulation of biliverdin during episodes of cholestasis. *J. Med. Genet.* 48, 219-225, doi:10.1136/jmg.2009.074567 (2011).
26. Soneoka, Y. *et al.* A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res.* 23, 628-633 (1995).
27. Spivak, W. & Carey, M. C. Reverse-phase h.p.l.c. separation, quantification and preparation of bilirubin and its conjugates from native bile. Quantitative analysis of the intact tetrapyrroles based on h.p.l.c. of their ethyl anthranilate azo derivatives. *Biochem. J.* 225, 787-805 (1985).
28. Mutschler, J., Grosshans, M., Soyka, M. & Rosner, S. Current Findings and Mechanisms of Action of Disulfiram in the Treatment of Alcohol Dependence. *Pharmacopsychiatry* 49, 137-141, doi:10.1055/s-0042-103592 (2016).
29. Cvek, B. & Dvorak, Z. Targeting of nuclear factor-kappaB and proteasome by dithiocarbamate complexes with metals. *Curr. Pharm. Des.* 13, 3155-3167 (2007).
30. Bjornsson, E., Nordlinder, H. & Olsson, R. Clinical characteristics and prognostic markers in disulfiram-induced liver injury. *J. Hepatol.* 44, 791-797, doi:10.1016/j.jhep.2005.12.016 (2006).
31. Berlin, R. G. Disulfiram hepatotoxicity: a consideration of its mechanism and clinical spectrum. *Alcohol Alcohol.* 24, 241-246 (1989).
32. Masuda, Y., Yasoshima, M. & Shibata, K. Effects of carbon disulfide, diethyldithiocarbamate, and disulfiram on drug metabolism in the perfused rat liver. *Res. Commun. Chem. Pathol. Pharmacol.* 61, 65-82 (1988).
33. Zhang, L. *et al.* A novel UPLC-ESI-MS/MS method for the quantitation of disulfiram, its role in stabilized plasma and its application. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 937, 54-59, doi:10.1016/j.jchromb.2013.08.009 (2013).
34. Cobby, J., Mayersohn, M. & Selliah, S. The rapid reduction of disulfiram in blood and plasma. *J. Pharmacol. Exp. Ther.* 202, 724-731 (1977).
35. Cingi, C., Muluk, N. B., Ipci, K. & Sahin, E. Antileukotrienes in upper airway inflammatory diseases. *Curr Allergy Asthma Rep* 15, 64, doi:10.1007/s11882-015-0564-7 (2015).

36. Barbosa, J. S., Almeida Paz, F. A. & Braga, S. S. Montelukast medicines of today and tomorrow: from molecular pharmaceutics to technological formulations. *Drug Deliv*, 1-9, doi:10.3109/10717544.2016.1170247 (2016).
37. Okumu, A., DiMaso, M. & Lobenberg, R. Dynamic dissolution testing to establish in vitro/in vivo correlations for montelukast sodium, a poorly soluble drug. *Pharm. Res.* 25, 2778-2785, doi:10.1007/s11095-008-9642-z (2008).
38. Zhao, J. J. *et al.* Pharmacokinetics and bioavailability of montelukast sodium (MK-0476) in healthy young and elderly volunteers. *Biopharm. Drug Dispos.* 18, 769-777 (1997).
39. Naito, M., Hasegawa, G., Ebe, Y. & Yamamoto, T. Differentiation and function of Kupffer cells. *Med. Electron Microsc.* 37, 16-28, doi:10.1007/s00795-003-0228-x (2004).
40. Hirano, K. *et al.* Role of heme oxygenase-1 and Kupffer cells in the production of bilirubin in the rat liver. *Arch. Histol. Cytol.* 64, 169-178 (2001).
41. Sneitz, N. *et al.* Crigler-Najjar syndrome in The Netherlands: identification of four novel UGT1A1 alleles, genotype-phenotype correlation, and functional analysis of 10 missense mutants. *Hum. Mutat.* 31, 52-59, doi:10.1002/humu.21133 (2010).
42. Kutty, R. K. & Maines, M. D. Purification and characterization of biliverdin reductase from rat liver. *J. Biol. Chem.* 256, 3956-3962 (1981).
43. Brannstrom, M. *et al.* Montelukast Disposition: No Indication of Transporter-Mediated Uptake in OATP2B1 and OATP1B1 Expressing HEK293 Cells. *Pharmaceutics* 7, 554-564, doi:10.3390/pharmaceutics7040554 (2015).

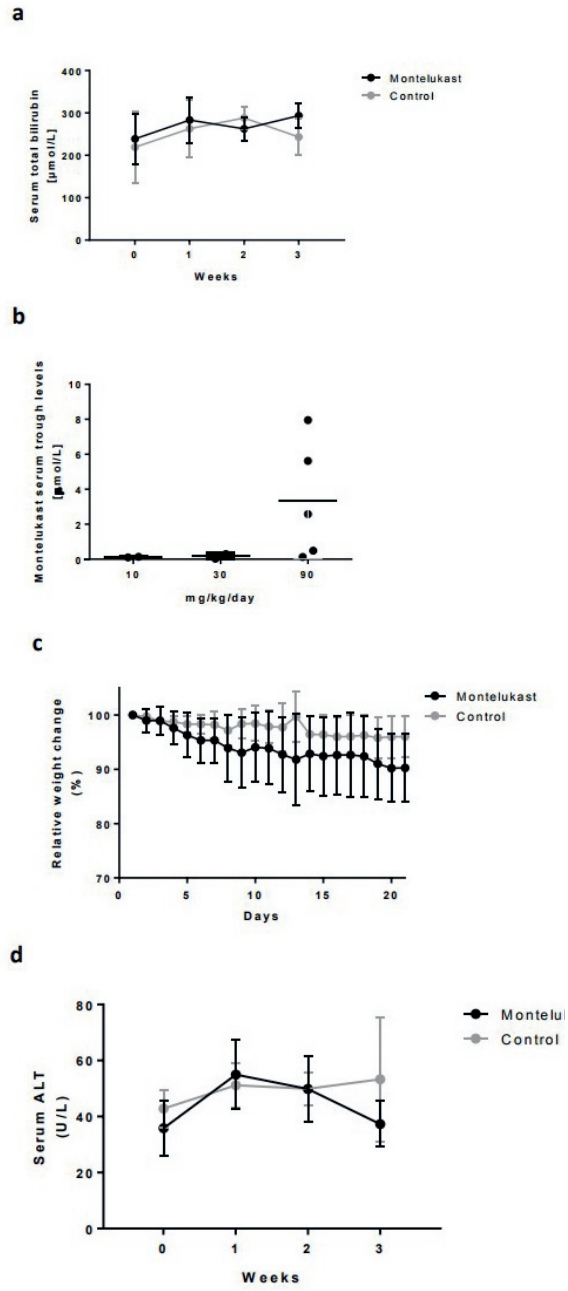
Supplementary figures

Gene (exon)	Primer sequence		Length bp
Rat BLVRA	F	TCTGTCTGTCTTCGGACACT	977
	R	TGGTTGATAGGACCAAACCCTG	
Human BLVRA	F	CGTCAGTGACCGAAGGAA	959
	R	ATGCTGGTGCCATCTTGGA	

Supplementary tabel 1: Primer sequences used for the cloning of rat and human biliverdin reductase A (rBVRA, hBVRA)



Supplementary figure 1: Hepatotoxic effect of Disulfiram in adult Gunn rats after 2 weeks of oral administration (90-180mg/kg/day) resulted in (A) weight loss compared to the control group (B) increase in serum total bilirubin (µmol/L) and (C) elevation of alanine aminotransferase (ALT), leading to termination of the experiment. Data represent the mean ± SD of five animals per group.



Supplementary figure 2: Oral administration of Montelukast (up to 90 mg/kg/day) does (A) not result in reduction of serum total bilirubin, due to (B) insufficient serum concentrations to achieve complete BVRA inhibition (>60 µmol/L). (C) no significant weight loss compared to control or (D) elevation of alanine aminotransferase (ALT) were seen in these animals. Data represent the mean ± SD of five animals per group.

5

DISRUPTION OF HNF1α BINDING SITE CAUSES INHERITED SEVERE UNCONJUGATED HYPERBILIRUBINEMIA

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Abstract

Crigler-Najjar syndrome presents as severe unconjugated hyperbilirubinemia and is characteristically caused by a mutation in the UGT1A1 gene, encoding the enzyme responsible for bilirubin glucuronidation. Here we present a patient with Crigler-Najjar syndrome with a completely normal UGT1A1 coding region. Instead, a homozygous 3 nucleotide insertion in the UGT1A1 promoter was identified that interrupts the HNF1α binding site. This mutation results in almost complete abolishment of UGT1A1 promoter activity and prevents the induction of UGT1A1 expression by the liver nuclear receptors CAR and PXR, explaining the lack of a phenobarbital response in this patient. Although animal studies have revealed the importance of HNF1α for normal liver function, this case provides the first clinical proof that mutations in its binding site indeed result in severe liver pathology stressing the importance of promoter sequence analysis.

Introduction

A 25 year old female patient from Turkey presented with serum unconjugated bilirubin (UCB) levels around 300 µmol/L and conjugated bilirubin around 15 µmol/L, in line with Crigler-Najjar syndrome (CNS) type II[1]. In contrast to patients with CNS type II, this patient had not responded to administration of phenobarbital (50 mg/kg) in the past, an established treatment for CNS type II patients. Therefore, the patient had been treated with phototherapy (4 h/day), which reduced serum bilirubin levels to about 200 µmol/L.

The severe unconjugated hyperbilirubinemia characteristic for CNS is caused by deficiency of hepatic UGT1A1 activity[2]. In contrast to type I, patients with CNS type II do have some residual UGT1A1 activity that can be induced by phenobarbital administration via activation of the constitutive androstane receptor (CAR; NR1I3) resulting in a >30% reduction of serum bilirubin levels. This transcriptional activation of the UGT1A1 promoter by CAR depends on its binding to the specific binding site in the gtPBREM region located between -3499/-3210 and the HNF1α binding site between -75 and -95 present in the UGT1A1 promoter. To clarify the cause of UGT1A1 deficiency and the non-responsiveness to phenobarbital a detailed genetic analysis was performed.

Methods

UGT1A1 coding and promoter sequence analysis

Genomic DNA was isolated from the patient and her parents. The entire coding region, the splice sites, the proximal and the distal gtPBREM promoter regions of the UGT1A1 gene were amplified using Taq-polymerase (Promega, Madison, USA). For primer sequence see Supplementary Table 1. Amplicons were isolated from 1% agarose (Seakem LE agarose, Rockland, USA) electrophoresis gel by Zymoclean Gel DNA recovery kit (Zymo Research, Irvine, USA) and sequenced using BigDye Terminator v1.1 (Life technologies, Carlsbad, USA).

Cell culture

The human hepatoma cell line HepG2, overexpressing CAR (HepG2^{CAR}) was generated by transduction with a lentiviral vector encoding the CAR cDNA behind the constitutive CMV promoter, HepG2 overexpressing pregnane X receptor (PXR) (HepG2^{PXR}) behind a PGK promoter was generated previously[3]. HepG2, HepG2^{CAR},

HepG2^{PXR}, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza, Cologne, Germany) supplemented with 10% foetal calf serum (FCS), 4 mmol/L L-glutamine and a mixture of antibiotics (5 mg/ml penicillin, 5 mg/ml streptomycin.

Functional analysis of UGT1A1 promoter activity by luciferase reporter assay
UGT1A1 proximal promoter fragments with the wild-type (wt) allele (6 TA repeats) or the UGT1A1⁄28 allele with 7 TA repeats with and without the CAT insertion were amplified by PCR from genomic DNA using Phusion high-fidelity Polymerase (New England Biolabs Inc) and cloned into a pGL4.22 [luc2CP/PURO] vector (Promega). The gtPBREM region was amplified from genomic DNA and inserted upstream of the various proximal constructs. All constructs were sequenced to confirm absence of mutations (for primer sequence see Supplementary Table 1).

Transient transfection was used to determine transcriptional activity of the different promoter constructs. HepG2, HepG2^{CAR}, HepG2^{PXR}, and HEK293T cells were plated at a confluence between 40 to 60%, and 24 h later transfected with 1 µg of a luciferase reporter plasmid and 25 ng of a renilla plasmid (to allow normalization of transfection efficiency), using polyethyleneImine (PEI) in DMEM without FCS. Four hours later the transfection medium was replaced with DMEM supplemented with 10% FCS. Promoter activity was measured 48 h after transfection using the Dual-Luciferase Reporter Assay system (Promega). To show the effect of HNF1α, co-transfection with an HNF1α expressing plasmid (a kind gift of Prof. Dr P. Mackenzie) was performed.

Induction of endogenous UGT1A1 and UGT1A1 promoter constructs by phenobarbital and rifampicin
HepG2 and HepG2 transfected with the UGT1A1 promoter constructs were cultured overnight in serum-free medium containing 0.2% BSA. The next day the cells were washed and incubated at 37^{0}C in 5% CO2 with DMEM/0.2% BSA containing rifampicin (10 µm/L) or phenobarbital (2 mmol/L) or 0.1% dimethyl sulfoxide (DMSO) as a solvent control, 24 h thereafter cells were harvested. RNA extraction, cDNA synthesis and quantitative PCR were performed as described previously[8]. Induction of the promoter activity was measured using the Dual-Luciferase Reporter Assay system (Promega). Rifampicin, phenobarbital and DMSO were purchased from Sigma Aldrich (Steinheim, Germany).

Results

Genetic analysis of the UGT1A1 gene and promoter

No mutations were found in the coding region and the splice donor and acceptor sites of exons 1 to 5, indicating that the gene encodes the normal UGT1A1 protein. In the UGT1A1 promoter region of the patients several sequence abnormalities were identified (Fig. 1B). The patient appeared to be homozygous for the UGT1A1⁄28 allele, an extra TA in the TATAA box, corresponding to the genotype present in Caucasians with Gilbert syndrome[4]. In addition, the patient was found to be homozygous for a common single nucleotide polymorphism (SNP) -3279T>G in the gtPBREM site. The combination of these two sequence abnormalities has been reported to decrease the transcription of the UGT1A1 gene by 50%[5]. This reduction however is not severe enough to cause the high serum UCB concentration seen in this patient.

In addition to these two common sequence abnormalities the patient appeared homozygous for a 3 nucleotide (nt) (CAT) insertion between position -83 to -85 of the proximal promoter region (Fig. 1B). This insertion interrupts the HNF1α binding site that is located between -79 to -95 in the UGT1A1-promoter[6]. Both parents appeared heterozygous carriers of the UGT1A1⁄28 allele and the 3nt CAT insertion, indicating both mutations are present in the same allele. To distinguish the role of all three UGT1A1 promoter sequence abnormalities in the phenobarbital-unresponsive severe hyperbilirubinemia seen in this patient, functional studies were performed.

Functional promoter studies

To determine the implications of each of these sequence abnormalities and their combined effect, four UGT1A1 proximal promoter constructs were generated and inserted upstream of a luciferase reporter gene. Using this reporter the transcriptional activity of the wt promoter (TA_6-TAA) was compared to the UGT1A1⁄28 allele (TA_7-TAA) with the wt HNF1α site and with the HNF1α site having the 3nt CAT insertion (CAT-TA_6) and (CAT-TA_7).

The basal transcriptional activity of the proximal UGT1A1 promoter with the TA insertion, TA_7-TAA, was reduced by 30% in comparison to that of the wt, TA_6-TAA, promoter (Fig. 1C). This reduction is in agreement with previous reports on the UGT1A1⁄28 allele[4]. Irrespective of the presence of this additional TA, the effect of the 3nt insertion in the HNF1α binding site was much more deleterious since it reduced basal promoter activity by >95% (Fig. 1C). Previous studies have

reported the important role of HNF1α in the transcriptional activity of the UGT1A1 promoter. To confirm that this insertion affects the activating role of HNF1α on the UGT1A1 promoter we co-transfected the various promoter constructs with an HNF1α expression construct in HEK293T cells. The HEK293T cell line lacks endogenous expression of this transcription factor. Co-transfection of a small amount of pCMV-HNF1α induced the luciferase expression of the wt construct up to 7-fold. In contrast, the construct with the 3nt insertion appeared insensitive to HNF1α overexpression (Fig. 1D). Addition of empty vector was used to ensure that constant amounts of DNA were transfected. Altogether these data indicate that the 3nt insertion interferes with HNF1α mediated activation of the UGT1A1 promoter explaining the severe hyperbilirubinemia seen in this patient. Next we wanted to know if this sequence abnormality could also be responsible for the lack of a response to phenobarbital treatment.

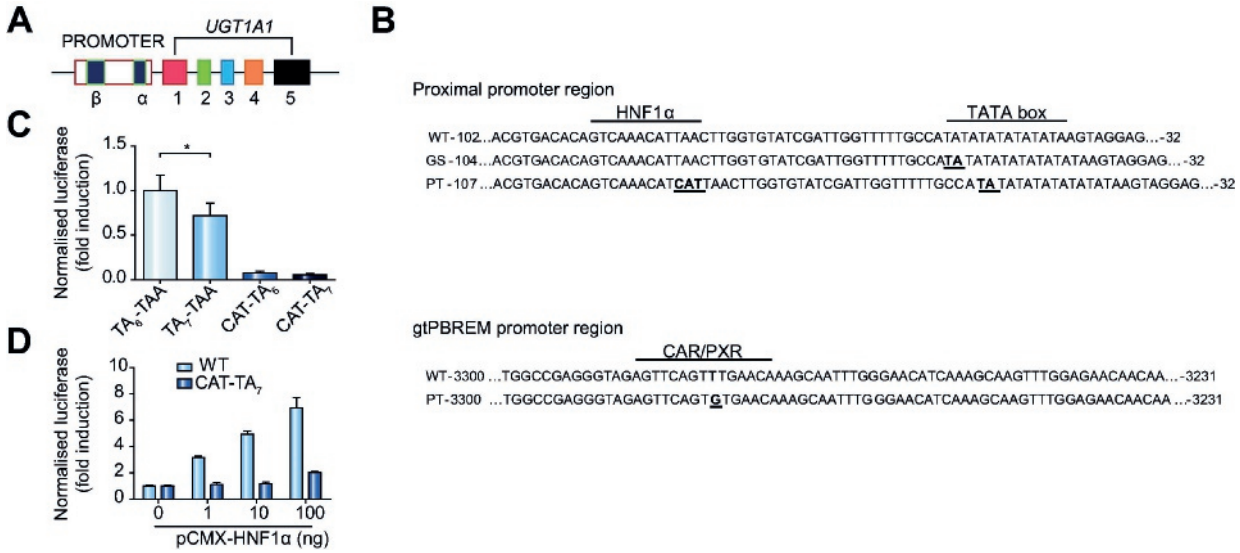

Figure 1. UGT1A1 gene structure and sequence and the effects of SNP on UGT1A1 activity. **(A)** Human UGT1A1 gene structure. α proximal promoter region (-181/-1) containing the HNF1α binding site and the TATA box. β distal gtPBREM module (-3499/-3210). **(B)** UGT1A1 gene promoter sequence. Sequences of the proximal promoter of a control (wt) of a Gilbert syndrome (GS) and of the patient (PT) indicating the HNF1α-binding site and the TATA box, and the distal gtBREM promoter indicating the -3279T>G SNP are shown. **(C)** Activity of the UGT1A1 proximal promoter in HepG2 cells. HepG2 cells were transfected with luciferase reporter plasmids containing UGT1A1 gene proximal promoters with the wt (TA_6–TAA), the Gilbert (TA_7–TAA), the CAT insertion in the HNF1α binding site (CAT-TA_6) and the patients sequence (CAT insertion and TA_7TAA (CAT-TA_7)) and a plasmid expressing renilla luciferase. Data represent the mean ± SD of the firefly-renilla luciferase ratio (RLX/RLU) determined 48 h after transfection, obtained in four independent experiments (n = 4) *p <0.05, One-way analysis of variance (ANOVA) was used for statistical analysis. **(D)** HNF1α does not activate mutated UGT1A1 gene proximal promoter. HEK293T were transfected with luciferase reporter plasmids containing the wt (light blue bar) or the mutated CAT-TA_7 (dark blue bar) UGT1A1-proximal promoter, increasing amounts of CMV-HNF1α or an empty control plasmid and a renilla luciferase plasmid. Data represent the mean ± SD of the firefly-renilla luciferase ratio (RLX/RLU) determined 48 h after transfection, obtained in two independent experiments (n = 2).

Induction of UGT1A1 transcription

The xenobiotic phenobarbital is used to reduce serum bilirubin in patients suffering from CNS type II by inducing residual UGT1A1 expression. Phenobarbital induces nuclear translocation of CAR in primary hepatocytes and in intact livers resulting in its binding to the 290-bp distal gtPBREM and activation of the UGT1A1 promoter. To study if the lack of a phenobarbital effect in this patient is due to the 3nt insertion we generated constructs containing the patients gtPBREM -3279G region upstream of the different proximal promoter constructs of the UGT1A1 gene. Functional analysis of these vectors in the human hepatoma cell line HepG2 showed that a combination of gtPBREM -3279G and TA_7-TAA reduces transcription activity with 50% compared to the wt TA_6-TAA promoter (Fig. 2A). Presence of the 3nt CAT insertion in the proximal promoter also abolished the transcriptional activity of this longer promoter. The residual promoter activity was less than 5%, demonstrating that the presence of the gtPBREM region did not overcome the deleterious effect of this 3nt insertion.

To investigate if this insertion affects the induction of transcriptional activity via CAR, phenobarbital was added to HepG2 cells. No UGT1A1 induction was seen after phenobarbital incubation suggesting that CAR expression could be limiting in these cells (Fig. 2B). Therefore, cells overexpressing CAR, $HepG2^{CAR}$ cells, were generated. Although this resulted in a significant 7-fold increase in expression of the endogenous UGT1A1 gene compared to the control HepG2 cells, phenobarbital did not further boost the expression of this gene (Fig. 2B). This suggests that upon overexpression of CAR, nuclear translocation resulting in UGT1A1 promoter activation occurs irrespective of the presence of a specific ligand, as shown previously[7]. To study if the presence of CAR did induce UGT1A1 promoter activity, HepG2 and $HepG2^{CAR}$ were transfected with luciferase vectors with the patient's gtPBREM upstream of the four different proximal promoters. The promoter with the wt proximal promoter was induced 1.5-fold by CAR overexpression while the promoter containing the 3nt CAT insertion was not induced (Fig. 2C).

Besides phenobarbital, also rifampicin can be used to induce UGT1A1 expression by activating the PXR (NR1I2) that also binds to the gtPBREM region[8]. To investigate if this compound could be therapeutic in this patient by inducing transcriptional activity of the mutated promoter, we studied its effect on all proximal promoter constructs in combination with the gtPBREM region. As seen with phenobarbital, this compound also did not induce the expression of the endogenous UGT1A1, suggesting limiting expression of PXR in HepG2 cells. Cells overexpressing this

nuclear receptor were generated. In contrast to CAR, overexpression of PXR did not result in spontaneous transcription of the endogenous UGT1A1 gene (Fig. 2B). Subsequent treatment with phenobarbital and rifampicin resulted in a 17- and 20-fold increased expression of the endogenous UGT1A1 gene. However, the promoter constructs with the 3nt insertion were not induced significantly by PXR overexpression nor by the addition of rifampicin, indicating the mutated promoter present in this patient seems unresponsive to this drug (Fig. 2D).

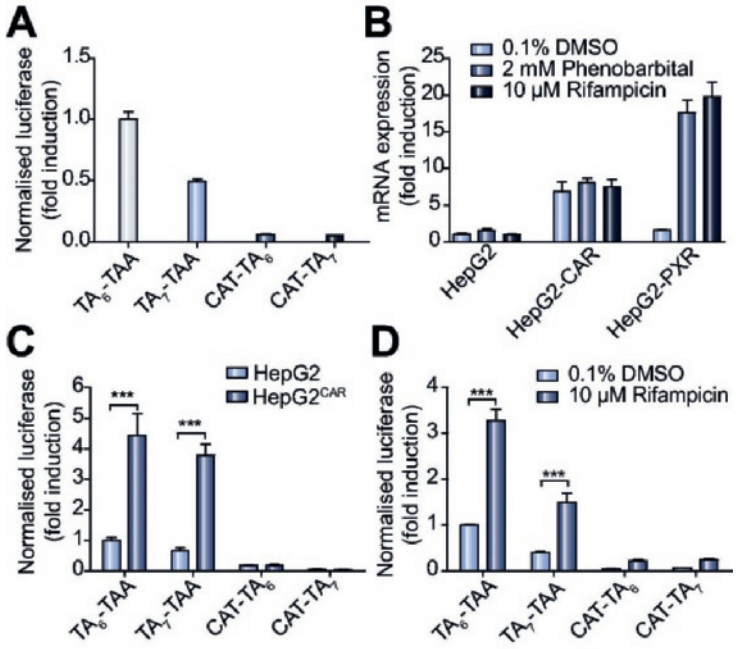

Figure 2. (A) HepG2 were transfected with luciferase plasmids containing the distal gtPREM promoter region ligated to UGT1A1 gene proximal promoters with the wt (TA_6–TAA), the Gilbert (TA_7–TAA), the CAT insertion in the HNF1α binding site (CAT-TA_6) or the patients sequence (CAT insertion and TA_7-TAA (CAT-TA_7)) and a plasmid expressing renilla luciferase. Data represent the mean ± SD of the fireflyrenilla luciferase ratio determined 48h after transfection (n = 4). **(B)** HepG2, $HepG2^{CAR}$, and $HepG2^{PXR}$ cells were incubated for 24h with phenobarbital (2 mM) (dark blue bar), rifampicine (10 µM) (navy blue bar) or vehicle (DMSO 0.1%) (light blue bar). UGT1A1 mRNA levels were determined by RT-qPCR. Data represent the mean ± SD of the data (n= 3). **(C)** HepG2 (light blue bar) and $HepG2^{CAR}$ (dark blue bar) were transfected with luciferase plasmids containing the distal gtPREM region ligated to UGT1A1 gene proximal promoters with the wt, Gilbert, the CAT insertion or the patients sequence. Data represent the mean ± SD of the firefly-renilla luciferase ratio determined 48h after transfection (n = 3). **(D)** $HepG2^{PXR}$ cells were transfected with luciferase vector containing the gtPREM promoter region ligated to the UGT1A1 gene proximal promoter with the wt, the Gilbert, CAT insertion and the patients sequence and a plasmid expressing renilla luciferase. Cells were incubated for 24h with 10 µmol/L rifampicin (dark blue bars) or vehicle control (light blue bar). Data represent the mean ± SD of the firefly-renilla luciferase ratio (RLX/RLU) determined 48h after transfection (n= 3). ***p <0.001, Two-way ANOVA was used for statistical analysis.

Discussion

Here we present a patient with unconjugated serum bilirubin around 300 µmol/L without treatment, a level reported for patients with CNS type II. In contrast to the latter, this patient did not respond to phenobarbital. Genome analysis revealed that the UGT1A1 coding sequence did not contain any mutations. Instead three sequence abnormalities were found in the promoter region of this gene, two common polymorphisms in the gtPBREM and the TATA box, and a 3nt insertion in the HFN1α binding site present in the proximal promoter. Functional promoter studies showed that this 3nt insertion caused a strong reduction of basal promoter activity and made the promoter non-responsive to CAR activation and to a potential alternative treatment via PXR activation with rifampicin, explaining the unique presentation of the disorder seen in this patient.

The combination of two polymorphisms present in the promoter of the UGT1A1 gene in this patient, the -3279T>G in the gtPBREM region and the extra TA-repeat in the TATA box, did result in a 50% reduction of the promoter activity, comparable to the effect reported in previous studies[4, 5]. Although significant, this 2-fold reduction could not explain the high serum levels of UCB (>300 µmol/L; with daily phototherapy >200 µmol/L) seen in this patient. The third sequence abnormality, a 3nt CAT insertion at position -85/-83 present in the proximal promoter, reduced basal promoter activity by more than 95% ($p < 0.0001$) (Fig. 1C) in the human hepatoblastoma cell line HepG2. This 20-fold reduction was independent of the presence of one of the other sequence abnormalities and did correlate with the impairment of UGT1A1 activity explaining the severe UCB accumulation seen in this patient. Both parents were heterozygous carriers for this mutation. Although no serum bilirubin data of the parents were available, most likely both will have Gilbert syndrome as reported previously for this allele in a cohort of Gilbert patients in India[9].

The 3nt insertion is present in the binding site for HNF1α, a transcription factor reported to play an essential role in the activity of the promoter of the UGT1A1 gene[10]. In addition to the basal activity this site is also needed for the induction of this promoter by activated CAR. Although CAR binds to the gtPBREM region -3499 to -3210, for transcriptional activation binding of HNF1α is required[6, 7]. By co-expressing HNF1α we confirmed that the promoter with the 3nt insertion indeed does not respond to HNF1α (Fig. 1D). Additionally, we also showed that this mutation explains the lack of a response to phenobarbital in this patient and predicts activation via PXR will also be ineffective, leaving

phototherapy and liver transplantation as the only therapeutic options.

In vitro studies have shown that HNF1α is required for the proper expression of a large number of liver and pancreas specific genes. It was shown that HNF1α plays an essential role in the regulation of amino acid, glucose, bile acid, cholesterol and drug metabolism[11, 12]. Studies with conditional and total HNF1α/TCF1 knock-out mice showed that several metabolic processes are affected. These mice developed hypercholesterolemia, increased serum bile acids, hyperbilirubinemia as well as non-insulin dependent diabetes mellitus[12, 13]. In humans no patients with complete absence of HNF1α have been reported most likely because of lethality. Partial inactivation of HNF1α has been found to be associated with the occurrence of maturity-onset diabetes of the young[14]. Our data showed that a 3nt insertion in the HNF1α binding site abolished transcriptional activation of the UGT1A1 gene to a large extent, resulting in the severe liver disorder seen in this patient. As such this case provides the first clinical evidence of the relevance of HNF1α for normal liver function and especially for biotransformation capacity.

In conclusion, we report a case intermediate between CNS type I and type II, with serum UCB characteristic for CNS type II but non-responsive to phenobarbital. This non-responsiveness was caused by a mutation in the HNF1α binding site in the proximal promoter of the UGT1A1 gene. The severe presentation of this patient confirms the pivotal role of HNF1α for UGT1A1 expression as reported in several in vitro studies. Recently several patients presenting with unconjugated hyperbilirubinemia characteristic for CNS were reported in which no causative mutation in the UGT1A1 coding region could be identified[15]. Given the central role of UGT1A1 in bilirubin metabolism and the presence of the UGT1A1⁄28, linked to this 3nt insertion, in these patients, further sequence analysis of the UGT1A1 promoter should be considered.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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References

1. Arias IM, Gartner LM, Cohen M, Ezzer JB, Levi AJ. (1969) *Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologic and genetic evidence for heterogeneity.* Am J Med;47:395–409
2. Bosma PJ. (2003) *Inherited disorders of bilirubin metabolism.* J Hepatol;38:107–117
3. Nibourg GA, Huisman MT, van der Hoeven TV, van Gulik TM, Chamuleau RA, Hoekstra R. (2010) *Stable overexpression of pregnane X receptor in HepG2 cells increases its potential for bioartificial liver application.* Liver Transpl;16:1075–1085
4. Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, et al. (1995) *The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome.* N Engl J Med;333:1171–1175
5. Li Y, Buckley D, Wang S, Klaassen CD, Zhong XB. (2009) *Genetic polymorphisms in the TATA box and upstream phenobarbital-responsive enhancer module of the UGT1A1 promoter have combined effects on UDP-glucuronosyltransferase 1A1 transcription mediated by constitutive androstane receptor, pregnane X receptor, or glucocorticoid receptor in human liver. Drug Metab Dispos*;37:1978–1986
6. Belanger AS, Tojcic J, Harvey M, Guillemette C. (2010) *Regulation of UGT1A1 and HNF1 transcription factor gene expression by DNA methylation in colon cancer cells.* BMC Mol Biol;11:9
7. Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M. (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. Mol Cell Biol;19:6318–6322
8. van Dijk R, Kremer AE, Smit W, van den Elzen B, van Gulik T, Gouma D, et al. (2014) *Characterization and treatment of persistent hepatocellular secretory failure.* Liver Int. 2015 35(4):1478-88.
9. Farheen S, Sengupta S, Santra A, Pal S, Dhali GK, Chakravorty M, et al. (2006) *Gilbert's syndrome: high frequency of the (TA)7 TAA allele in India and its interaction with a novel CAT insertion in promoter of the gene for bilirubin UDP-glucuronosyltransferase 1 gene*. World J Gastroenterol;12: 2269–2275
10. Sugatani J. (2013*) Function, genetic polymorphism, and transcriptional regulation of human UDP-glucuronosyltransferase (UGT) 1A1.* Drug Metab Pharmacokinet;28:83–92.
11. Liu SY, Gonzalez FJ. (1995) *Role of the liver-enriched transcription factor HNF-1 alpha in expression of the CYP2E1 gene.* DNA Cell Biol;14:285–293
12. Shih DQ, Bussen M, Sehayek E, Ananthanarayanan M, Shneider BL, Suchy FJ, et al. (2001) *Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism.* Nat Genet;27:375–382
13. Pontoglio M, Barra J, Hadchouel M, Doyen A, Kress C, Bach JP, et al. (1996) *Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome.* Cell;84:575–585
14. Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H, Vaxillaire M, et al. (1996) *Mutations in the hepatocyte nuclear factor-1alpha gene in maturity-onset diabetes of the young (MODY3).* Nature;384:455–458
15. Skierka JM, Kotzer KE, Lagerstedt SA, O'Kane DJ, Baudhuin LM. (2013) *UGT1A1 genetic analysis as a diagnostic aid for individuals with unconjugated hyperbilirubinemia.* J Pediatr;162:1146–1152, 1152.

Supplementary table 1. Primer list for UGT1A1 promoter sequence and cloning.

Primer	Forward primer	Reverse primer
UGT1A1 proximal promoter (sequence)	GTCTGGCTCACCTCATGGC	CGTCAGGTGCTAGGACAACTA
UGT1A1 gtPBREM region (sequence)	TAACCTGAAACCCGGACTTGG	CATGCTATCACTCAGGTGCC
UGT1A1 proximal promoter (cloning into pGL4.22)	GCTAGCGAGTCTGGCTCACCTCACCTCATGG	AAGCTTGCCTTTGCTCCTGCCAGA
UGT1A1 gtPBREM region (cloning into pGL4.22)	GGTACCATTAAGACTTGCAGGCC	GCTAGCGCTCTGGACATGTTTCT
pGL4.22 (Sequence)	CTAGCAAAATAGGCTGTCCC	-

PART II

6

AN INTRODUCTION INTO THE ENTEROHEPATIC CIRCULATION, CHOLESTASIS AND CHOLESTASIS-ASSOCIATED PRURITUS

van Dijk R, Bosma PJ and Beuers U

To be submitted

The enterohepatic circulation

Bile acids are one of the main components and driving force of bile formation. Approximately 200-600mg are synthesised each day by the liver. Bile acid synthesis starts with the hydroxylation of cholesterol by the cytochrome P450 cholesterol 7 alpha-hydroxylase (CYP7A1), or alternatively by CYP27A1[1, 2]. In humans, the two primary bile acids synthesised are cholic acid (CA) and chenodeoxycholic acid (CDCA)[3]. Both CA and CDCA are hydrophobic and can exert cytotoxic effects on the hepatocyte and cholangiocyte[4, 5]. To render primary bile acids more water-soluble, CA and CDCA are conjugated with a taurine or glycine group by the hepatic microsomal enzymes bile acid: CoA synthase (BACS) and bile acid: amino acid transferase (BAT)[6] (Figure 1). The conjugated bile acids are transported over the hepatic canalicular membrane via the ATP-dependent bile salt export pump (BSEP/*ABCB11*)[7, 8]. Other transporters involved in bile formation are MDR3/*ABCB4,* a phospholipid floppase which mediates phosphatidylcholine (PC) secretion into bile[9, 10], and the heterodimer ABCG5/ABCG8 responsible for mainly canalicular cholesterol secretion[11, 12]. Bile acids are about 1000-fold enriched in bile in comparison to blood and form mixed micelles with PC and cholesterol in the bile duct lumen. Mixed micelles protect cholangiocytes from the toxic-detergent effects of bile acid monomers. In humans, bile containing mixed micelles is stored in the gallbladder. A (fatty) meal stimulates the release of the peptide hormone cholecystokinin by the duodenal entero-endocrine cells which results in the contraction of the gallbladder, the relaxation of the sphincter of Oddi and thereby the secretion of bile into the duodenum[13]. Once in the small intestine bile acid aid in the emulsification of lipids and facilitate the absorption of lipid nutrients and lipid soluble vitamins. In the terminal ileum up to 95% of all secreted bile acids are reabsorbed by the ileocytes mainly via active transport by the apical sodium dependent bile acid transporter (ASBT/*SLC10A2*)[14, 15]. A small amount of bile acids that reach the colon are metabolised into secondary bile acids via deconjugation, 7-dehydroxylation, esterification, desulfatation or oxidation by the gut microbiota[16, 17]. For example, the secondary bile acid lithocholic acid is formed by dehydroxylation of CDCA[18], and the 7α-dehydroxylation of cholic acid results in the formation of deoxycholic acid[19].

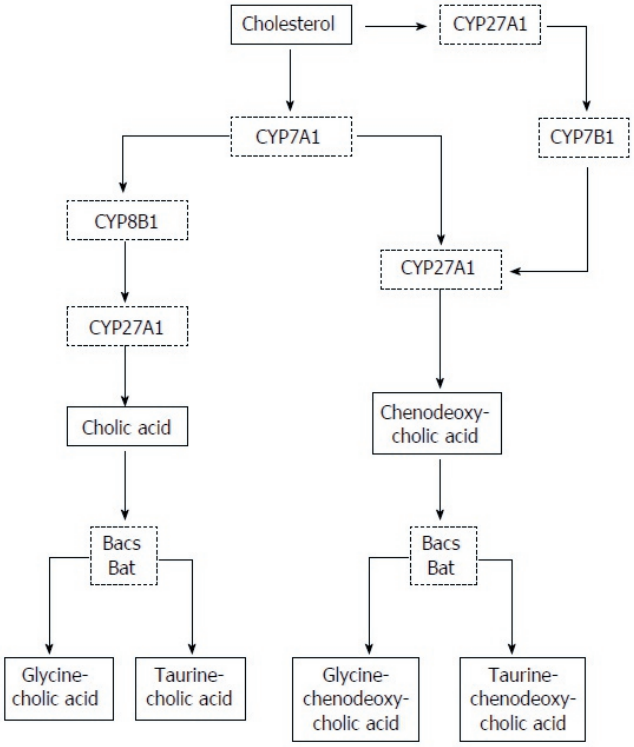

Figure 1. Schema of bile acid synthesis and conjugation. CYP7B1: 7α-hydroxylase; CYP7A1: Enzyme cholesterol-7α-hydroxylase; CYP8B1: Sterol 12 α-hydroxylase; CYP27A1: Mitochondrial sterol 27 hydroxylase.

Once in the ileocyte the bile acids are secreted by the basolateral OSTα-OSTβ transporter into the mesenteric and portal venous blood[20, 21], from which they are efficiently cleared by the liver. Hepatocellular re-uptake of bile acids occurs predominantly via the Na^+-taurocholate co-transporting polypeptide (NTCP/ *SLC10A1*) which facilitates the transport of glycine and taurine-conjugated bile acids. Members of the organic anion transporting polypeptide (OATP) family are most likely responsible for the hepatic uptake of the unconjugated bile acids and sulphated bile acids [22, 23]. After uptake by the liver, the bile acids can once again be conjugated if needed and secreted into the bile and thereby completing the so called enterohepatic circulation (Figure 2a and 2b).

As explained in the first part of this thesis, the liver plays a central role in the metabolism of bilirubin by accommodating the glucuronidation of

unconjugated bilirubin via UGT1A1 and the hepatic biliary secretion of conjugated bilirubin via MRP2. After entering the intestine conjugated bilirubin is not absorbed in the ileum like bile acids, but instead passes through to the colon. Colonic bacteria metabolise conjugated bilirubin into urobilinogen, which can be further oxidised to urobilin and stercobilin. In contrast to bile acids only about 1% of the urobilinogen is reabsorbed into the enterohepatic circulation, the rest is excreted together with urobilin and stercobilin via the faeces. Reabsorbed urobilinogen can be once again taken up by the liver cells and secreted into bile, but can also be secreted into the urine via the kidneys[24].

Cholestasis

Cholestasis, coming from the Greek words chole (bile) and stasis (standing still), is a pathological condition caused by an impairment in bile formation and flow and thereby interruption of the enterohepatic circulation. The homeostatic formation and secretion of bile is of crucial importance for the physiological function of the liver. The hepatic accumulation of cytotoxic endo- and xenobiotics such as bile acids, (phospho)lipids and drugs, during chronic cholestasis can lead to biliary fibrosis and eventually end stage cirrhosis resulting in liver failure or the development of malignant tumours. Cholestasis is caused by defects in hepatocellular and/or cholangiocellular secretory function, or by obstruction of the intra- or extrahepatic bile ducts. Causes of cholestasis are diverse and include for example drug–induced liver injury, inflammatory bile duct diseases such as primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), or IgG4-associated cholangitis and mechanical obstruction of the bile ducts by choledocholithiasis or pancreatic head and bile duct tumours.

The most common signs and symptoms of cholestatic patients are jaundice, dark urine, pale stools, right upper quadrant abdominal pain, fatigue and pruritus. During cholestasis serum levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin (mostly the conjugated form) and, although rarely measured in the clinical setting, total serum bile acids are often markedly elevated. Serum levels of gamma glutamyl tranferase (γGT), an enzyme localised on the apical membrane of the hepatocytes and cholangiocytes, are elevated under most cholestatic conditions when bile acids are present in bile, but low in rare cases of cholestasis when there is (nearly) absence of biliary bile acid secretion.

The expression and function of enzymes and transporters involved in the enterohepatic circulation are altered during cholestasis due to activation of nuclear receptors. Nuclear receptors act as intracellular ligand-activated transcription factors. After activation nuclear receptors translocate to the nucleus where they bind to specific DNA motives in a gene promoter region and will thereby regulate gene expression. Each nuclear receptor has its own specific set of ligands, which can be both a natural occurring or a pharmacological agent. By regulating hepatic and intestinal gene expression nuclear receptors play a critical role in bile acid homeostasis, bilirubin metabolism, (drug) detoxification, inflammation and the development of fibrosis. A list of the most important hepatic nuclear receptors is provided in table 1. Because of the scope of this thesis two nuclear receptors, FXR and PXR, will be discussed in more detail below.

Nuclear receptor	Natural Ligands	Pharmacological ligands	Therapeutic targets	Target genes
FXR *(NR1H4)*	CDCA, CA, DCA	OCA, PX-102	BA synthesis BA detoxification BA transport Anti-inflammatory	Indirect CYP7A1 repression via SHP and FGF19 Induction: CYP3A4, SULT2A1, UGT2B4, UGT2B7 Induction: BSEP, MRP2, OATP1B3, OSTα/β, MDR3 Repression of NF-kB activity
PXR *(NR1I2)*	Litocholic acid	Rifampicin, St. John's wort, Fibrates, Budesonide	BA synthesis BA detoxification BA transport Drug detoxification Anti-inflammatory	Repression: CYP7A1 Induction: CYP3A4, SULT2A1, UGT1A1, UGT1A3, UGT1A4 Induction: MRP2, MRP3, MDR1 Induction: Several CYP1A/2A/2C/3A serotypes Repression of NF-kB activity
CAR *(NR1I3)*	Bilirubin	Phenobarbital, CITCO, TCPOBOP	BA detoxification BA transport Drug detoxification	Induction: CYP3A4 and UGT1A1 Induction: MRP2 and MRP4 Induction: Several CYP1A/2A/2C/3A serotypes
VDR *(NR1I1)*	1α25-dihydroxy vitamin D3, Litocholic acid	Vitamin D	BA detoxification Anti-microbial Anti-fibrotic Nuclear receptors	Induction: CYP3A4, SULT2A1 Induction: Cathelicidin Inhibition: COL1A1, COL1A2, TIMP1, TGFbeta Inhibition of FXR trans-activation
HNF4α *(NR2A1)*	Linoleic acid	-	BA synthesis BA detoxification Drug detoxification Nuclear receptors	Induction: CYP7A1, CYP8B1, CYP27A1, BAT, BACS Induction CYP3A4, SULT2A1 Induction of CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9, CYP2D6 and UGT1A-family Indirect regulation of PXR and CAR
GR *(NR3C1)*	Glucocorticoids, potential UDCA	Budesonide	BA transport Drug detoxification Nuclear receptors	Induction: ASBT, NTCP, MRP3 Induction: CYP3A4, CYP2C9, CYP2B6 Induction: PXR, CAR, RXRα
PPAR α *(NR1C1)*	Free fatty acids, eicosanoids, leokotriens	Fibrates, statins, NSAIDS, Perfluorononanoic acid	BA synthesis BA detoxification BA transport Nuclear receptors	Repression: CYP7A1 Induction: SULT2A1, UGT2B4, UGT1A3 Induction: ASBT, OSTα/β,MDR3 Modeluates FXR

Table 1: Hepatic nuclear factor, their ligands and effect on expression of genes involved in bile acid (BA) synthesis, phase I and II detoxification enzymes, hepatic transporters, anti-micobitc and anti-fibrotic enzymes and their impact on other nuclear receptors.

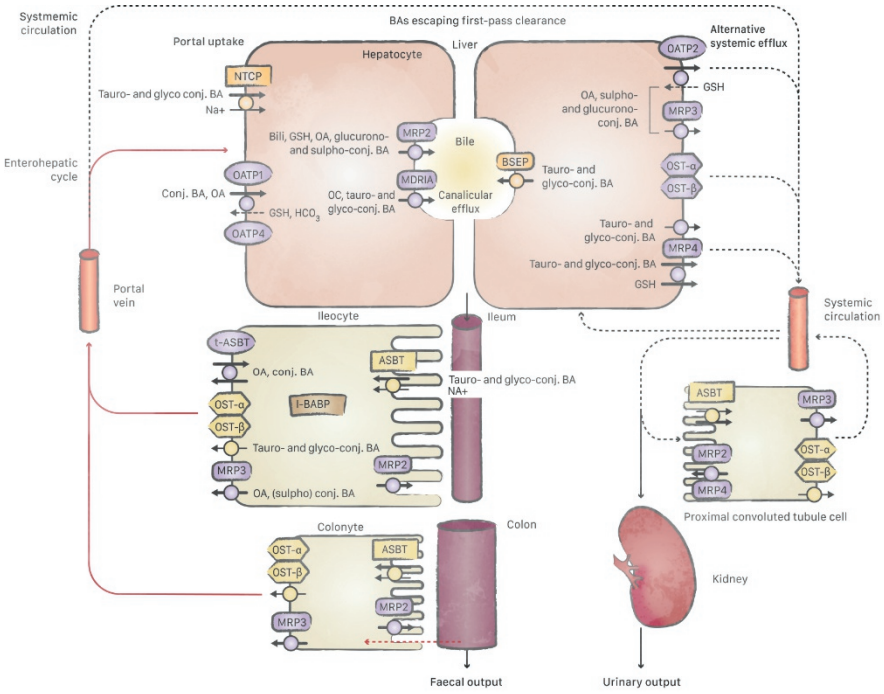

Figure 2 A. Bile acids (BA) are excreted in the bile canaliculi through the canalicular bile-salt export pump (BSEP). Conjugated BAs, organic anions (OAs), organic cations (OCs) and reduced glutathione (GSH) are secreted into bile by the multidrug resistance-associated protein 2 (MRP2) and the multidrug export pump 1a (MDR1A). At the basolateral membrane of hepatocytes, BA efflux is an important spillover route for BA and bilirubin (bili) that has accumulated during BA overload under cholestatic conditions. Basolateral export transport systems are mediated by members of the MRP family including MRP3 and MRP4, but the organic anion transporting polypeptide 2 (OATP2) and the heteromeric organic solute transporter-α/β (OST-α/β) also provide alternative excretion routes for BAs and other OAs into the systemic circulation. Under normal conditions, bile is stored in the gall bladder and released into the intestinal lumen upon feeding. BAs ensure the digestion and absorption of ingested lipids and are afterwards reabsorbed by the terminal ileum via the apical bile-salt transporter (ASBT) and secreted in the systemic circulation by OST-α/β, MRP3 and a truncated form of ASBT (t-ASBT). MRP2 and MRP4, present at the apical membrane of enterocytes and the proximal renal tubules, respectively, may ensure apical excretion of BAs. In the ileum, BAs are recycled to the liver where they are mainly taken up by the Na+-taurocholate cotransporting polypeptide (NTCP) and to a lesser extent by organic anion transporter 1 (OATP1) and OATP4. After the first hepatic pass, BAs that have not been cleared (less than 10%) are filtrated by the renal glomerulus and reabsorbed by epithelial cells of the proximal convoluted tubules of the kidney. Together, these transport systems minimize faecal and urinary loss of BAs *(From Thomas et al[91])*.

The Farnesoid X receptor (FXR/*NR1H4*) is a bile acid sensor which plays a central role in the regulation of bile acid synthesis, conjugation, secretion and uptake [25, 26]. The natural ligands of FXR are CA and CDCA, however the semi-synthetic bile acid obeticholic acid (OCA) has been shown to be a more potent FXR agonist

with a 100 fold greater affinity for FXR in comparison to CDCA. After binding with a ligand FXR forms a heterodimer with the retinoid x receptor (RXR) after which it translocates to the nucleus to regulate gene transcription. During cholestasis the accumulation of bile acids within hepatocytes will result in the activation of hepatic FXR. In the liver, FXR reduces bile acid synthesis via indirect repression of CYP7A1 via the induction of short heterodimer partner (SHP)[27]. Also, the induction of fibroblast growth factor 19 (FGF19) by FXR during cholestasis, in both liver and ileum, results in repression of CYP7A1 expression[28]. On the other hand activation of FXR stimulates bile acid conjugation by directly inducing BAT expression, as well as regulating biliary bile acid transport by inducing BSEP expression and reducing hepatic bile salt uptake due to inhibition of NTCP. All these changes in expression result in reducing hepatic bile acid load, and thereby reducing their hepato-toxic effect during cholestasis. Besides modulation of bile acid metabolism FXR is found to regulate gene expression involved in glucose, cholesterol and lipid metabolism. By inhibiting the pro-inflammatory nuclear receptor NF-κB, FXR has also been shown to exhibit anti-inflammatory properties[29]. By modulating hepatic stellate cell activity, FXR also has potential beneficial effects on the development of liver fibrosis[30].

The Pregnane X receptor (PXR/*NR1I2*) is a nuclear receptor abundantly expressed in the liver and to a lesser extent in the small intestine. PXR plays a critical role in bile acid metabolism and conjugation, drug detoxification and bilirubin metabolism[31]. Just like FXR, PXR translocates to the nucleus to regulate genetic expression after binding with a ligand and forming a heterodimer with RXR. Because of its flexible ligand pocket PXR can bind a wide range of ligands. The secondary bile acid litocholic acid is one of the natural occurring PXR ligands. The antibiotic rifampicin is currently known as the most potent human PXR agonist, other human PXR ligands are fibrates, St John's wort and phenobarbital[32]. The expression of cytochrome P450 CYP3A4, a key enzyme for biotransformation phase 1 reactions in liver and small intestine, is significantly induced by PXR[33]. CYP3A4 is responsible for the hydroxylation and therefore in most cases detoxification of approximately 60 to 80% of all drugs but can also hydroxylate bile acids. By inducing the expression of SULT2A1 and of multiple UGT1A isoforms, PXR also potently stimulates biotransformation phase 2 reactions including the sulfo- and glucuronide conjugation of bile acids, hormones, bilirubin and xenobiotics. Hydroxylation and conjugation render bile acids and other compounds more hydrophilic and, thereby, better secretable into bile and urine. Induction of the expression of hepatic and intestinal apical transporters such as MRP2 and MDR1,

and basolateral transporters such as MRP3 by PXR, facilitates the excretion of conjugated compounds into bile, intestinal lumen, urine and the circulation[34]. As seen upon FXR activation, PXR also inhibits the activity of the pro-inflammatory nuclear receptor NF-κB[35] and shows potential anti-fibrotic effect by inhibiting functional hepatic stellate cells[36].

Because of the beneficial effects on endo- and xenobiotic detoxification, inflammation and fibrosis, FXR and PXR are interesting targets for the treatment of cholestasis and cholestasis-related symptoms such as pruritus.

Treatment for cholestasis

The naturally occurring bile acid ursodeoxycholic acid (UDCA) is the most, and until recently the only, established medical treatment for cholestatic disorders. In PBC, a chronic progressive autoimmune liver disease, UDCA at 13-15 mg/kg daily has been shown to improve biochemical markers of cholestasis, liver histological features and to delay progression to cirrhosis and the time to liver transplantation[37-39]. Today, up to two out of three patients with PBC reach a normal life expectancy when treated with UDCA. However, approximately up to 40% of all PBC patients do only show inadequate biochemical response as defined by surrogate prognostic markers like serum ALP, bilirubin or prognostic scores like the GLOBE score. Especially patients with very high serum ALP activity lack adequate response to UDCA treatment and have been shown to have a worse prognosis[40]. The effect of UDCA at therapeutic doses of 15-20 mg/kg daily on long-term survival is uncertain for the treatment of other cholestatic diseases such as (secondary) sclerosing cholangitis, drug-induced liver injury, sarcoidosis hepatis or bile duct injury after liver transplantation. In contrast, an American study has shown that the use of a very high dose of UDCA (28-30 mg/kg/day) increases the risk for the development of varices and listing for liver transplantation in patients with PSC[41]. Therefore, there is a continuous search for the development of new medical therapeutic options in cholestatic patients.

Because of its strong agonistic effect on FXR activity, OCA has been tested in phase II and phase III trials for the treatment of PBC. A double-blind randomised controlled trial in PBC patients who have an inadequate response to UDCA treatment indeed showed a reduction in serum ALP levels, the biochemical surrogate marker for PBC, after three months of OCA treatment. This was

independent of the dose of 10, 25 or 50 mg per day. However, although 70% of patients in the OCA group showed a reduction of serum ALP levels, only 7% of the patients had a normalisation of ALP. Other surrogate markers such as serum γGT, bilirubin and inflammatory markers C-reactive protein and IgM also improved in the patients treated with OCA in comparison to the placebo group. Increase in serum FGF 19 concentration and decrease in serum C4 levels, a metabolite of bile acid metabolism, and endogenous serum bile acids confirmed the agonistic FXR effect of OCA in these patients[42]. A follow up phase III double-blind randomised controlled trial with a low dose OCA of 5-10mg per day for 12 months confirmed the positive effects of OCA on serum ALP, bilirubin, γGT and inflammatory markers in patients treated with UDCA. No difference was observed in liver stiffness between the OCA and the placebo control group at the end of the study period[43]. However, besides these very promising results, pruritus, the most frequent symptom in PBC patients next to fatigue, was the most common documented adverse event during both trials. The intensity of the pruritus increased in a dose-dependent manner. Also, in a trial for the treatment of nonalcoholic steatohepatitis, a non cholestatic inflammatory disease of the liver, with OCA pruritus was the most common reported adverse event[44]. The exact mechanism behind why OCA induces pruritus has still to be elucidated[42]. OCA is recently approved by de FDA and EMA for adjuvant therapy in PBC.

One of the most recent developments in the treatment for PSC, a chronic inflammation of the large bile ducts resulting in cholestasis, liver fibrosis and cirrhosis, is the clinical testing of the UDCA derivate 24-norursodeoxycholic acid (*nor*UDCA). By lacking a methylene group *nor*UDCA is thought to be passively absorbed by cholangiocytes, therefore instead of completing the enterohepatic circulation *nor*UDCA mostly undergoes 'cholehepatic shunting'. This results in the induction of biliary HCO_3^- secretion and thereby protection against bile acid toxicity as proposed by the 'biliary bicarbonate umbrella hypothesis'[45]. *nor*UDCA also has been shown to have anti-inflammatory, anti-lipotoxic and anti-fibrotic effects in mouse models of obstructive cholestasis and sclerosing cholangitis[46, 47]. Preliminary data of a double-blind randomised controlled phase II trial showed indeed a dose dependent reduction in serum ALP in PSC patients after three months of treatment with *nor*UDCA. No severe adverse events were observed[48]. A phase III trial is about to start.

Besides the promising results for OCA and *nor*UDCA, also other therapeutic options as activation of nuclear receptors such as glucocorticoid receptor (GR) with budesonide, PXR with rifampicin, and PPAR´s with fibrates and

specific designed ligands, in combination with UDCA are currently investigated for their anti-cholestatic and anti-inflammatory effect in PBC, PSC and other chronic cholestatic diseases[49].

Cholestasis-associated pruritus

As mentioned above pruritus is a frequent symptom of cholestasis and, depending on its severity, can have a severe negative impact on the quality of life of the patient. Besides inducing the urge to scratch and causing related scratch marks and lesions on the skin, chronic pruritus can lead to sleep deprivation, lower mood and, although rarely seen in the clinic, it can even lead to suicidal thoughts. Pruritus during cholestasis is typically located on the palms of the hands and soles of the feet, and the intensity is most severe in the evening and early night[50]. Although pruritus has long been acknowledged to be a consequence of cholestasis, both acute and chronic, the exact mechanism behind this remains enigmatic. Only in recent years with the discovery of potentially new pruritogen candidates a better understanding of this mechanism is beginning to arise.

Understandingly bile acids have for long been the focus of research as the cause for cholestasis-induced pruritus. However, a correlation between itch intensity and serum or tissue bile salt concentrations could never be established. Women suffering from intrahepatic cholestasis of pregnancy (ICP), a pathological condition characterised by itch and cholestasis, show only modestly elevated serum bile acid levels[51]. On the other hand, a young patient who presented with extremely high serum bile acid concentrations of above 1500 µmol/L (ref. value <16.3 µmol/L) of mostly the tauro-conjugated form due to a deficiency in the NTCP/*SLC10A1* transporter did not report any pruritus, making conjugated bile acids less likely as the pruritogen in cholestasis-induced pruritus[52]. For other investigated pruritogen candidates such as histamine, serotonin and endogenous µ-opioids a direct correlation with serum concentrations and the itch intensity could never be demonstrated[53]. A recent breakthrough has focused the research on two new potential pruritogens.

A thorough screen revealed that sera of patients with cholestasis-associated pruritus significantly activated neuronal cells *in vitro* more in comparison to control sera of cholestatic patients without pruritus. An intensive analysis of the sera revealed the signalling molecule lysophosphatidic acid (LPA) as possible cause for neuronal cell activation *in vitro.* Indeed serum LPA levels were significantly

elevated in patients with cholestasis-induced pruritus[54]. It is known that multiple neuronal receptors such as TRPV1 and G-coupled receptors are involved in itch sensation and can be activated by LPA[55, 56]. Intradermal injections of LPA in mice surely resulted in increased scratching behaviour in a dose-dependent manner[54].

LPA appeared to be rather an unstable molecule in (stored) serum, therefore the presence and activity of autotaxin (ATX), a lysophospholipase D responsible for LPA formation, was further investigated. ATX generates LPA via cleaving the choline group of its precursor lysophosphatidylcholine (LPC)[57]. Because LPC is present in high micromolar (>100 µmol/L) concentrations in blood, ATX is the rate-limiting factor in the formation of LPA. Indeed both ATX protein concentrations and ATX activity were significantly elevated in cholestatic patients with pruritus in comparison to both healthy controls and cholestatic patients without pruritus. ATX activity also correlated with the itch intensity in cholestatic patients ($r=0.7764$, $p<0,0001$)[54].

ATX is known to exert multiple effects on cell survival, proliferation and migration, and neuronal and vascular development. ATX may also play a role in tumour formation, is highly expressed in hepatocellular carcinoma's and is correlated with liver cirrhosis and inflammation[58]. ATX activity is also associated with disease specific symptoms in PBC and PSC and worsening disease and cirrhosis in patients with PSC and PBC[59]. In patients with viral hepatitis or alcohol-related liver cirrhosis serum ATX levels are increased compared to healthy subjects and ATX levels were correlated with Child-Pugh and MELD scores, two standard scoring systems for prediction of prognosis of patients with liver cirrhosis[59].

The exact source of autotaxin ATX formation during cholestasis is still uncertain. ATX is widely expressed throughout the whole body. Small intestinal entero-endocrine cells stained positive for ATX and it has been hypothesised these cells could be an important source for ATX production during cholestasis, however these biopsies were from non-cholestatic patients and need to be confirmed in a cholestasis-associated pruritus control group[60]. Cytokines such as tumour necrosis factor alpha (TNFα) or interleukin (IL)-6 or certain growth factors can induce ATX expression *in vitro*, whereas interferon-gamma, IL-1, and IL-4 inhibit ATX expression[61]. Cytokines could play a role in ATX induction during cholestasis, although in other inflammatory driven diseases pruritus is not a typical symptom. On the other hand, hormones could be another important factor in the regulation of ATX expression and activity. Experiments in rodents showed that that ATX expression was induced upon oestrogen therapy in ovariectomised animals[62]. In healthy women the use of oral contraceptives increased serum ATX activity[63].

A very recent publication showed that early in the pregnancy, patients who will develop ICP have higher serum concentrations of sulphated progesterone metabolites[64]. These metabolites were shown to be an early prognostic marker for the development of ICP. One specific sulphated progesterone metabolite, 5β-pregnan-3α,-20α-diol-3-sulfate (P3MS), was associated with pruritus in ICP. *In vitro* P3MS was able to activate the TGR5 receptor, a G-protein-coupled steroid and bile acid receptor expressed among others on the dorsal root ganglia in mice. Mice overexpressing Tgr5 treated with P3MS had significantly higher numbers of scratches in comparison to WT and Tgr5 knock out mice[64]. Besides P3MS, ATX has also been shown to be a specific marker for the diagnosis of ICP, distinguishing it from other pruritic disorders of pregnancy[63]. However, P3MS and ATX most likely contribute to development of itch by different, possibly additive mechanisms.

Treatment of cholestasis-associated pruritus

The nonabsorbable bile acid resin cholestyramine is recommended by both the European and American clinical practice guidelines as the first-line treatment option in cholestasis-associated pruritus[65, 66] (figure 3). By binding bile acids in the intestine it interrupts the enterohepatic circulation and lowers serum bile acid concentrations. Cholestyramine is not always effective in relieving pruritus, and due to the frequently reported side effects of bloating and constipation cholestyramine is not always well tolerated[67]. Notably, the more efficient and better tolerated bile acid questran colesevelam did not alleviate pruritus better than placebo, although it significantly reduced serum bile acid levels[68].

The antibiotic and PXR agonist rifampicin is recommend as second-line therapy in the treatment of cholestasis-induced pruritus. A meta-analysis of five prospective randomised controlled trials showed that 77% (n=61) of patients treated with rifampicin had a complete or partial resolution of pruritus, with an odds ratio of 15.2 (CI-95 5.2-45.6)[69]. Although the use of rifampicin is associated with severe adverse events such as worsened hepatic function, acute liver failure, renal impairment and drug interactions [70, 71], no severe irreversible adverse events were observed in the meta-analysis[69]. However, all studies only administered rifampicin for a short period (1-2 weeks) with a short-term follow up. Larger studies with longer study duration are therefore warranted to confirm these results.

Besides rifampicin, (oral) opioid antagonist agents are recommended and used as third line therapy for cholestasis-associated pruritus. It has been

shown in humans that cholestasis is associated with higher plasma levels of endogenous opioids such as enkephalin[72, 73]. Opioids are known to induce itch, which can be reversed by opioid antagonists. Therefore, multiple randomised controlled trials have been performed with parenteral (naloxone) and oral (naltrexone and nalmefene) opioid antagonists for the treatment of cholestasis-induced pruritus[74-78]. Indeed, opioid antagonist were found to induce a significant reduction in pruritus compared with the control group [79]. However, adverse events were common in the opioid antagonist group. Most often patients developed transient symptoms of opioid withdrawal, such as dizziness, nausea, vomiting, headache and abdominal cramping[80]. Close monitoring during the administration of oral opioid antagonists is therefore recommended.

A small, randomised, double-blind controlled trial showed that the selective serotonin reuptake inhibitor sertraline was effective in improving itch score, in comparison to placebo[81]. Although again the exact mechanism is not completely understood, these results suggest that serotonergic pathways are important in central regulation of itch perception during cholestasis.

Case-reports have provided evidence for the use of more experimental approaches such as extracorporeal albumin dialysis[82], plasmapheresis[83], bile duct drainage[84] and nasobiliary drainage[85] in patients not responding to the standard recommended therapies. Together with a reduction in pruritus, nasobiliary drainage also resulted in a decrease in serum autotaxin axctivity[54]. Also a phase II randomised controlled trial in PBC patients showed that by selectively blocking the ileal bile acid transporter (IBAT/ASBT) not only pruritus much improved in these patients, but this correlated with a significant decrease in serum autotaxin activity[86]. Both the effects of nasobiliary drainage and the selective ileal bile acid transporter inhibitor on pruritus and autotaxin activity emphasise the role the enterohepatic circulation and autotaxin play in cholestasis-associated pruritus

The treatment of cholestasis-associated pruritus can be notoriously difficult, with some patients not responding to any of the therapeutic options at all. For those patient's liver transplantation remains the very last option[87, 88].

Except for the treatment of ICP, there is no convincing evidence for the use of UDCA in the treatment of cholestasis-associated pruritus. Anecdotally, the use of UDCA during initiation of treatment worsened pruritus in cholestatic patients leading to the recommendation to start UDCA treatment at very low doses and weekly enhancing the dose in cholestatic patients suffering from itch. It is thought that UDCA exerts its effect in ICP by post-transcriptionally stimulating hepatobiliary secretion[89]. New insights have revealed that UDCA can bind directly

to autotaxin and thereby inhibiting its activity, which could be an additional mechanism of action for the relief of pruritus in ICP[90].

Conclusion

The enterohepatic circulation is of vital importance for bile acid homeostasis and general liver function. Disruption of the enterohepatic circulation due to cholestasis can result in liver fibrosis, cirrhosis, liver failure and the development of malignant hepatic and ductular tumours. Treatment of chronic cholestatic conditions such as primary biliary cholangitis and primary sclerosing cholangitis is currently suboptimal, but new agents are under investigation in clinical trials and show promising results.

Pruritus is a frequent accompanying symptom of cholestasis. Although the exact mechanisms leading to cholestasis-associated pruritus are yet unknown, recent studies have pointed in the direction of the lysophospholipase D enzyme autotaxin and its product lysophosphatidic acid as important players in the induction of pruritus during cholestasis. Cholestasis-associated pruritus is difficult to treat, and the recommended therapies are often inadequate. Further progress in understanding of cholestasis-induced pruritus is therefore highly needed.

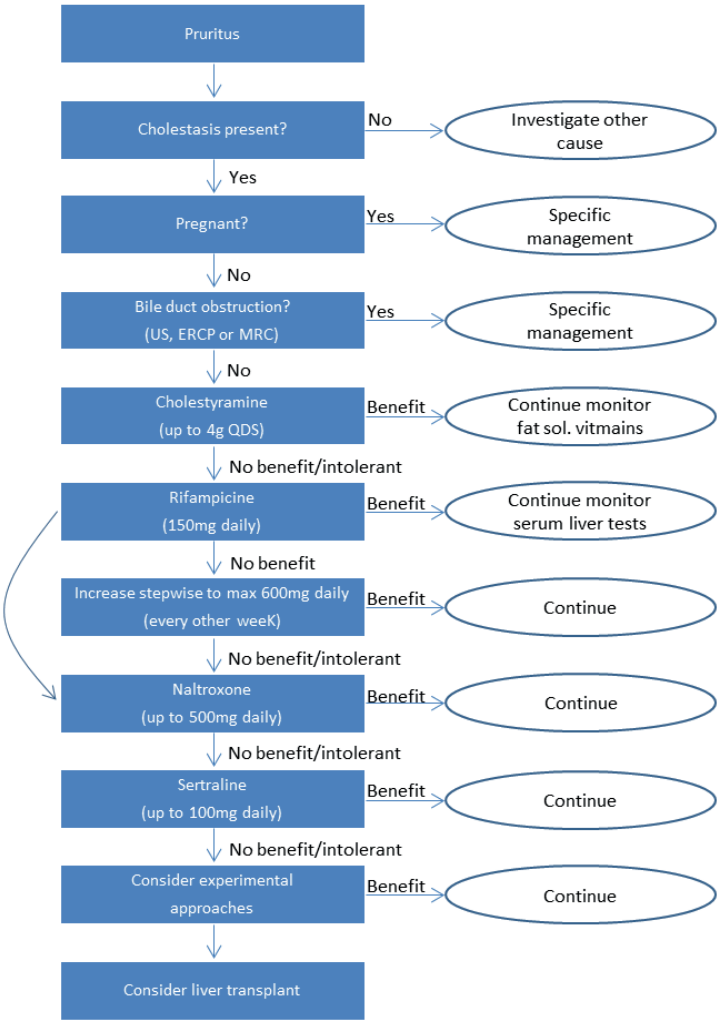

Figure 3: EASL Guidline: Management of pruritus in cholestasis. Adjusted from the EASL guidelines for the management of cholestasis-associated pruritus. Abbreviations: US, ultrasound; ERCP, endoscopic retrograde cholangiopancreatography; MRCP, magnetic resonance cholangiopancreatography

References

1. Cohen JC, Cali JJ, Jelinek DF, et al. (1992) *Cloning of the human cholesterol 7 alpha-hydroxylase gene (CYP7) and localization to chromosome 8q11-q12*. Genomics 14: 153–61
2. Reshetnyak VI. (2013) *Physiological and molecular biochemical mechanisms of bile formation*. World J Gastroenterol 19:7341-60
3. Chiang JY. (2009) *Bile acids: regulation of synthesis*. J. Lipid Res. 50:1955–66
4. Perez MJ, Briz O. (2009) *Bile-acid-induced cell injury and protection*. World J Gastroenterol 15: 1677-1689
5. Hohenester S, Wenniger LM, Paulusma CC, et al. (2012) *A biliary HCO3- umbrella constitutes a protective mechanism against bile acid-induced injury in human cholangiocytes.* Hepatology 55: 173-83
6. Vlahcevic ZR, Pandak WM, Stravitz RT. (1999) *Regulation of bile acid biosynthesis.* Gastroenterol Clin North Am. 28:1-25
7. Gerloff T, Stieger B, Hagenbuch B, et al. (1998) *The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver.* J Biol. Chem. 273: 10046-50
8. Hayashi H, Takada T, Suzuki H, et al. (2005) *Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: a comparison of human BSEP with rat Bsep.* Biochim Biophys Acta 1738: 54-62
9. Van der Bliek AM, Baas F, Ten Houte de Lange T, Kooiman PM, van der Velde-Koerts T, and Borst P (1987) *The human MDR3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver.* The EMBO journal 6: 3325-3331
10. Morita SY, Terada T. (2014) *Molecular mechanisms for biliary phospholipid and drug efflux mediated by ABCB4 and bile salts.* Biomed Res Int. 2014: 954781
11. Berge KE, Tian H, Graf GA, et al. (2000) *Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters*. Science 290: 1771–1775
12. Yu L, Hammer RE, Li-Hawkins J, et al (2002) *Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion.* Proc Natl Acad Sci USA 99: 16237–16242
13. Lawson M, Everson GT, Klingensmith W, Kern F. (1983) *Coordination of gastric and gallbladder emptying after ingestion of a regular meal.* Gastroenterology 85: 866–870
14. Wong MH, Oelkers P, Dawson PA. (1995) *Identification of a mutation in the ileal sodium-dependent bile acid transporter gene that abolishes transport activity.* J Biol. Chem. 270: 27228–27234.
15. Wong MH, Oelkers P, Craddock AL, Dawson PA. (1994). *Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter*. J Biol. Chem. 269: 1340–1347
16. Midtvedt T. (1974) *Microbial bile acid transformation*. Am. J. Clin. Nutr. 27: 1341–1347
17. Gérard P. (2014) *Metabolism of Cholesterol and Bile Acids by the Gut Microbiota.* Pathogens 3: 14-24
18. Hofmann AF. (2004) *Detoxification of lithocholic acid, a toxic bile acid: Relevance to drug hepatotoxicity.* Drug Metab. Rev. 36: 703–722
19. Björkhem I, Einarsson K, Melone P, Hylemon P. (1989*) Mechanism of intestinal formation of deoxycholic acid from cholic acid in humans: evidence for a 3-oxo-delta 4-steroid intermediate.* J Lipid Res. 30: 1033-9

20. Wang W, Seward DJ, Li L, Boyer JL, Ballatori N. (2001) *Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate.* Proc Natl Acad Sci US 98: 9431–9436
21. Ballatori N, Christian WV, Lee JY, et al. (2005) *OSTalpha-OSTbeta: a major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia*. Hepatology 42: 1270–1279
22. Anwer MS, Stieger B. (2014) *Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters.* Pflugers Arch 466: 77-89
23. Dawson PA, Lan T, and Rao A. (2009) *Bile acid transporters.* J Lipid Res. 50: 2340–2357
24. Fevery J, Vanstapel F, Blanckaert N. (1989) *Bile pigment metabolism.* Baillieres Clin Gastroenterol 3: 283-312
25. Chiang JY. (2004) *Regulation of bile acids synthesis: pathways, nuclear receptors and mechanis.* J Hepatol 40: 539-551
26. Matsubara T, Li F and Gonzalez FJ (2013) *FXR signaling in the enterohepatic system.* Mol Cell Endocrinol 368: 17-29
27. Goodwin B, Jones SA, Price RR, et al. (2000) *A regulatory cascade of the nuclear receptors FXR, SHP-1 and LRH-1 represses bile acid synthesis* Mol Cell 6: 517-526
28. Inagaki T, Choi M, Moschetta A et al. (2005) *Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis.* Cell Metal 2: 217-225
29. Lefebre P, Cariou B, Lien F., et al. (2009) *Role of bile acids and bile acid receptors in metabolic regulation.* Physiol Rev. 89: (1) 147–91
30. Fiorucci S, Rizzo G, Antonelli E, et al. (2005) *Cross-talk between farnesoid-x-receptor (FXR) and peroxisome proliferator activated receptor gamma contributes to the anti-fibrotic activity of FXR ligands in rodent models of liver cirrhosis.* J Pharmacol Exp Ther 315: 58-68
31. Timsit YE and Negishi M. (2007) *CAR and PXR: The xenobiotic-Sensing Receptors.* Steroids 72: 231–246
32. Kliewer SA, Goodwin B, Wilson TM. (2002) *The nuclear pregnane x receptor: a key regulator of xenobiotic metabolism.* Endocr. Rev. 23: 687 – 702
33. Bertilsson G, Heidrich J, Svensson K, et al. (1998) *Identification of a human nuclear receptor defines a new signalling pathway for CYP3A induction.* Proc Natl Acad Sci U.S.A. 95: 12208 - 12213
34. Marschall HU, Wagner M, Zollner G, et al. (2005) *Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans.* Gastroenterology 129: 476 – 85
35. Gu X, Ke S, Liu D, et al. (2006) *Role of NF-κB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents.* J Biol. Chem. 281: 17882-9
36. Haughton El, Tucker SJ, Marek CJ, et al (2006) *Pregnane X receptor activators inhibit human hepatic stellate cell transdifferentiation in vitro.* Gastroenterology 131: 194 - 209
37. Poupon RE, Poupon R, and Balkau B. (1994) *Ursodiol for the long-term treatment of primary biliary cirrhosis. The UDCA-PBC Study Group*. N Engl J Med 330: 1342–1347
38. Poupon RE, Bonnand AM, Chretien Y, Poupon R. (1999) *Ten-year survival in ursodeoxycholic acid-treated patients with primary biliary cirrhosis*. The UDCA-PBC Study Group. Hepatology 29: 1668–1671.
39. Pares A, Caballeria L, Rodes J., et al. (2000) *Long-term effects of ursodeoxycholic acid in primary*

biliary cirrhosis: results of a double-blind controlled multicentric trial. UDCA-Cooperative Group from the Spanish Association for the Study of the Liver. J Hepatol 32: 561–566.

40. Leuschner M, Dietrich CF, Youn T, et al. (2000) *Characterisation of patients with primary biliary cirrhosis responding to long term ursodeoxycholic acid treatment.* Gut 46: 121-126
41. Lindor KD, Kowldey KV, Luketic VA, et al. (2009) *High-dose ursodeoxycholic acid for the treatment of primary sclerosing cholangitis.* Hepatology 50:808-8014
42. Hirschfield GM, Mason A, Luketic V, et al. (2015) *Efficacy of obeticholic acid in patients with primary biliary cirrhosis and inadequate response to ursodeoxycholic acid.* Gastroentereology 148: 751-761
43. Nevens F, Andreone P, Mazzella G, et al. (2016) *A placebo controlled trial in obeticholic acid in primary biliary cholangitis.* N. Eng. J. Med. 375: 631-643
44. Mudaliar S, Henry RR, Sanyal AJ., et al. (2013) *Efficacy and safety of the farnesoid x receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease.* Gastroenterology 145: 574-583
45. Beuers U, Hohenester S, de Buy Wenniger LM., et al. (2010) *The biliary HCO_3^-umbrella.* Hepatology 52: 1489-1496
46. Fickert P, Wagner M, Marschall HU, et al. (2006) *24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice.* Gastroenterology 130: 465-481
47. Fickert P, Pollheimer MJ, Silbert P, et al. (2013) *Differential effects of norUDCA and UDCA in obstructive cholestasis in mice.* J. Hepatol 58: 1201-1208
48. Trauner M, Fickert P, Hirschfield G, et al. (2016) *norUrsodeoxycholic acid improves cholestasis in primary sclerosing cholangitis independent of ursodeoxycholic acid pre–treatment and response.* Hepatology A210
49. Beuers U, Trauner M, Jansen P and, Poupon R (2015) *New paradigms in the treatment of hepatic cholestasis: From UDCA to FXR, PXR and beyond*. J Hepatol 62: S25–S37
50. Bergasa NV. (2011) *The itch of liver disease.* Semin Cutan Med Surg 30: 93-95
51. Glantz A, Marschall HU, Lammert F, Mattsson LA. (2005) *intrahepatic cholestatsis of pregnancy: a randomized controlled trial comparing dexamethasone and ursodeoxycholic acid.* Hepatology 42: 1399-405
52. Vaz FM, Paulusma CC, Huidekoper H, et al (2015) *Sodium taurocholate cotransporting polypeptide (SLC10A1) deficiency: conjugated hypercholanemia without a clear clinical phenotype.* Hepatology 61: 260-7
53. Kremer AE, Oude Elferink RP, Beuers U. (2011) *Pathophysiology and current management of pruritus in liver disease.* Clin Res Hepatol Gastroenterol 35:89-97
54. Kremer AE, Martens JJ, Kulik W, et al. (2010) *Lysophosphatidic acid is a potential mediator of cholestatic pruritus.* Gastroenterology 139: 1008-1018
55. Nieto-Posadas A, Picazo-Juarez G, Llorente I, et al. (2012) *Lysophosphatidic acid directly activates TRPV1 through a C-terminal binding site.* Nat Chem Biol 8: 78-85
56. Sun YG, Zhao ZQ, Meng XL., et al (2009) *Cellular basis of itch sensation.* Science 325: 1531-1534
57. Tokumura A, Majima E, Kariya Y, et al (2002) *Identification of human plasma lysophopholipase D, a lysophophatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase.* J Biol CHem 277: 39436 – 39442

58. Wu JM, Xu Y, Skill NJ, et al (2010) *Autotaxin expression and its conncection with the TNF-alpha NF-kappB axis in human hepatocellular carcinoma.* Mol Cancer 31:71
59. Pleli T, Martin D, Kronenberger B, et al (2014) *Serum autotaxin is a parameter for the severity of liver cirrhosis and overall survival in patients with liver cirrhosis - a prospective cohort study.* Plso one 25:9 (7)2
60. Bolier R, Tolenaars D, Kremer AE, et al. (2016) *Endocrine cells are a potential source of serum autotaxin in men.* Biochim Biophys Acta 1862: 696-704
61. Houben AJ, Moolenaar WH, (2011) *Autotaxin and LPA receptor signaling in cancer.* Cancer Metast Rev 30: 557-565
62. Takeo C, Ikeda K, Horie-Inoue K, Inoue S. (2009) *Identification of Igf2, Igfbp2 and Enpp2 as estrogen-responsive genes in rat hippocampus.* Endocr J56: 113-120.
63. Kremer A.E., Bolier R, Dixon P.H, et al. (2015) *Autotaxin activity has a high accuracy to diagnose intrahepatic cholestasis of pregnancy.* 62:897-904
64. Abu-Hayyeh S, Ovadia C, Lieu T, et al. (2016) *Prognostic and mechanistic potential of progesterone sulfates in intrahepatic cholestasis of pregnancy and pruritus gravidarum.* Hepatology 63: 1287-98
65. EASL Clinical Practice Guidelines. (2009) *Management of cholestatic liver diseases.* J Hepatol 51:237-267
66. Lindor KD, Gershwin ME, Poupon R, et al. (2009) *Primary biliary ciirhosis (AASLD Practice Guidelines).* Hepatology 50(1): 291-308
67. Datta DV and Sherlock S. (1966) *Chloestyramine for long term relief of the pruritus complicating intrahepatic cholestasis.* 50: 323-332
68. Kuiper EM, van Erpecum KJ, Beuers U., et al. (2010) *The potent bile acid sequestrant colesevelam is not effective in cholestatic pruritus: results of a double-blind, randomized, placebo-controlled triail.* Hepatology 52: 1334-1340
69. Khurana S and Singh P. (2006) *Rifampicin is safe for treatment of pruritus due to chronic cholestasis: a meta-analysis of prospective randomized controlled trials.* Liver international 26: 943-948
70. Bachs L Pares A, Elena M, et al (1989) *Comparison of rifampicin with phenobarbitone for treatment of pruritus of primary biliary cirrhosis with rifampicin.* Lancet 1: 574-576
71. Prince MI, Burt AD, Jones DE. (2002) *Hepatitis and liver dysfunction with rifampicin therapy for pruritus in primary biliary cirrhosis*. Gut 50: 436-439
72. Thornton JR and Losowsky MS (1989) *Plasma leucine enkephalin is increased in liver disease.* Gut 30: 1392-1395
73. Spivey J, Jorgensen R, Gores G, et al. (1992) *Serum met-enkephalin levels in patients with primary biliary cirrhosis correlate with severity of disease but not pruritus.* Gastroenterology 102:A892
74. Summerfield JA (1980) *Naloxone modulates the perception of itch in man.* Br J Clin Pharmacol 10: 180-183
75. Bergasa NV, Alling DW, Talbot TL, et al. (1995) *Effects of naloxone infusions in patients with the pruritus of cholestasis. A double-blind, randomized, controlled trial.* Ann Intern Med 123: 161-167
76. Bergasa NV, Alling DW, Talbot TL, et al. (1999) *Oral nalmefene therapy reduces scratching activity due to the pruritus of cholestasis: A controlled study.* J Am Acad Dermatol 41: 431-44
77. Wolfhagen FH, Sternieri E, Hop C, et al. (1997) *Oral naltrexone treatment for cholestatic pruritus:*

A double-blind placebo controlled study. Gastroenterology 113: 1264-1269

78. Terg R, Coronel E, Sorda J, et al. (2002) *Efficacy and safety of oral naltrexone treatment for pruritus of cholestasis, a crossover, double-blind, placebo-controlled study.* J Hepatology 37: 717-722
79. Tandon P, Rowe BH, Vandermeer B, et al. (2007) *The efficacy and safety of bile acid binding agents, opioid antagonists, or rifampicin in the treatment of cholestasis-associated pruritus.* Am J of Gastroenterology 102: 1528-1536
80. Shawcross DL and Jalan R. (2001) *Delayed opioid withdrawal-like reaction in primary biliary cirrhosis following naloxone therapy.* Gastroenterology 121: 743-744
81. Mayo MJ, Handem I, Sladana S, et al. (2007) *Sertraline as a first-line treatment for cholestaic pruritus.* Hepatology 45: 666-674
82. Pares A, Cisneros L, Salmeron JM, et al. (2004) *Extracorporeal albumin dialysis: a procedure for prolonged relief of intractable pruritus in patients with primary biliary cirrhosis.* Am J Gastroenterol. 98: 1105-1110
83. Pusl T, Denk GU Parhofer KG (2006) *Plasma separation and anion adsorption transiently relieve intractable pruritus in primary biliary cirrhosis.* J Hepatololgy 45: 887-891
84. Stapelbroek JM, van Erpecumm KJ, Klomp LW, et al. (2006) *Nasobiliary drainage induces long-lasting remission in bening recurrent intrahepatic cholestasis.* Hepatology 43: 51-53
85. Beuers U, Gerken G, Pusl T. (2006) *Biliary drainage transiently relieves intractable pruritus in primary biliary cirrhosis.* Hepatology 44: 280–281.
86. Hegade VS, Kendrick S, Dobbins R, et al. (2017) *Effect of ileal bile acid transporter inhibitor GSK2330672 on pruritus in primary biliary cholangitis: a double-blind, randomised, placebo-controlled, crossover, phase 2a study.* Lancet 389: 1114-1123
87. Markus BH, Dickson ER, Grambsch PM, et al. (1989) *Efficacy of liver transplantation in patients with primary biliary cirrhosis.* N Eng J Med 320: 1709-1713
88. Weisner R, Porayko M, Dickson R, et al. (1992) *Selection and timing of liver transplantation in primary biliary cirrhosis and primary sclerosing cholangitis.* Hepatology 16: 1290-1299
89. Beuers U. (2006*) Drug insight: mechanisms and sites of action of ursodeoxycholic acid in cholestasis.* Nat Clin Pract Gastroenterol Hepatol 3: 318–328
90. Keune WJ, Hausmann J, Bolier R, et al. (2016) *Steroid binding to autotaxin links bile salts and lysophosphatidic acid signaling.* Nature Communications 14: 11248
91. Thomas C, Pellicciari R, Pruzanski M et al (2008) *Targeting bile-acid signalling for metabolic diseases.* Nat Rev Drug Discov 7: 678-93

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CHARACTERIZATION AND TREATMENT OF PERSISTENT HEPATOCELLULAR SECRETORY FAILURE

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Abstract

Background & Aims: Hepatocellular secretory failure induced by drugs, toxins or transient biliary obstruction may sometimes persist for months after removal of the initiating factor and may then be fatal without liver transplantation. We characterized patients with severe persistent hepatocellular secretory failure (PHSF) and treated them with the pregnane X receptor (PXR) agonist, rifampicin. We also studied the effect of rifampicin on PXR dependent expression of genes involved in biotransformation and secretion in vitro. Methods: Thirteen patients (age 18–81 years, 6 male) with hepatocellular secretory failure that persisted after removal of the inducing factor (drugs/toxin: 9) or biliary obstruction (4) were identified over 6 years. Six of these patients were screened for ATP8B1 or ABCB11 mutations. All were treated with rifampicin (300 mg daily) for 1–10 weeks. Expression of genes involved in biotransformation and secretion was determined by rtPCR in human hepatocytes and intestinal cells incubated with rifampicin (10 µmol/ L). Results: Serum bilirubin of patients with PHSF ranged from 264 to 755 µmol/L. Normal γGT was found in 10/13 patients of whom 3/6 tested positive for ATP8B1/ABCB11 mutations. Serum bilirubin declined to <33 µmol/L after 1–10 weeks of rifampicin treatment. In vitro, rifampicin PXR-dependently upregulated biotransformation phase 1 (CYP3A4), phase 2 (UGT1A1) and phase 3 (MRP2) enzymes/carriers as well as the basolateral bile salt exporter OSTβ. Conclusion: Persistent hepatocellular secretory failure may develop in carriers of transporter gene mutations. In severe cases, rifampicin may represent an effective therapeutic option of PHSF. PXR dependent induction of CYP3A4, UGT1A1, MRP2 and OSTβ could contribute to the anticholestatic effect of rifampicin in PHSF.

Introduction

Persistent hepatocellular secretory failure (PHSF) after exposure to drugs, toxins, or short-term mechanical biliary obstruction with deep jaundice and progression after removal of the underlying cause is a rare, but often detrimental, event. The molecular mechanisms leading to PHSF under these conditions are not understood. Hepatocellular secretion recovers in the majority of patients within days to weeks after stopping the causative drugs or toxins, or removal of the mechanical obstruction.

Medical interventions for PHSF are lacking: Ursodeoxycholic acid (UDCA) has anticholestatic properties in various cholestatic disorders and represents the only approved drug for the treatment of primary biliary cirrhosis (1, 2). However, UDCA is ineffective in PHSF when serum bilirubin exceeds 170-255 µmol/L (10-15 mg/dl) (1, 3). The ligand-activated nuclear receptor, pregnane X receptor (PXR; NR1I2), modulates expression of genes involved in detoxification and elimination of bile salts and other endo- and xenobiotics (4, 5). We postulate that treatment with a strong agonist of this transcriptional activator could possibly reverse PHSF.

We defined PHSF as (i) serum bilirubin >255 µmol/L (>15 mg/dl); (ii) persistence or increasing elevated bilirubin serum levels (>1 week) after removal of the underlying trigger; (iii) exclusion of obstructive cholestasis by imaging techniques; and (iv) no evidence of chronic liver disease before the initiating event (i.e. drug or toxin exposure or transient biliary obstruction by stones or tumour). As therapeutic intervention, we chose the pregnane X receptor agonist, rifampicin, which is recommended by European and American guidelines as a second-line treatment of severe cholestasis- associated pruritus (1, 2).

Here, we describe thirteen consecutive patients who fulfilled the diagnosis of PHSF. We searched for a possible genetic background of PHSF in those patients from whom DNA was available. We also investigated the effect of PXR activation by rifampicin on genes involved in biotransformation and secretion (biotransformation phase 1–3) of potential toxins in primary human hepatocytes and human HepG2 hepatoma cells, HepG2 cells overexpressing PXR (HepG2PXR), HepG2 cells after PXR knock down and the human colon adenocarcinoma HT-29 cell line, for a better molecular understanding of the effects of rifampicin treatment in patients with PHSF.

Patients and methods

Human subjects

Between 2007 and 2013, thirteen consecutive patients who fulfilled the criteria for persistent hepatocellular secretory failure [(i) serum bilirubin >255 µmol/L (>15 mg/dl); (ii) persistence or increasing elevated serum bilirubin levels (>1 week) after removal of the underlying trigger; (iii) exclusion of obstructive cholestasis by imaging techniques; and (iv) no evidence of chronic liver disease before the initiating event] were treated with rifampicin (300 mg per day) for 1–10 weeks. Patients with a drug-/toxin-induced PHSF (n = 9) were at least 2 weeks off the PHSF-inducing drug/toxin before start of rifampicin treatment. In transient biliary obstruction-induced PHSF (n = 4), patients were treated with (multiple) stents, or ongoing biliary obstruction and dilated bile ducts were excluded by ultrasound, MRCP and/or ERCP before start with rifampicin. Other causes of (chronic) liver disease, such as viral, autoimmune, hereditary metabolic or vascular liver diseases, were excluded by biochemical, imaging and histological approaches before start of rifampicin treatment. During and after rifampicin therapy, serum liver tests (ALT, AST, γGT, alkaline phosphatase, and bilirubin) were closely monitored to foresee possible hepatotoxicity. The patients were informed about the possible side effects of rifampicin. As this is an FDA- and EMA approved and guideline-recommended drug for use in cholestasis, no formal informed consent had to be signed according to the local Medical Ethics Committee's advice.

Mutation analysis of ATP8B1 and ABCB11

All coding exons with flanking intronic sequences of the ATP8B1 and ABCB11 genes were sequenced after PCR amplification of DNA from peripheral blood mononuclear cells of six patients with normal γGT after informed consent and were compared with references sequences.

Materials for in vitro experiments

Rifampicin, dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Steinheim, Germany). Forskolin was purchased from Ascent Science (Cambridge, UK). 6-Ethyl chenodeoxycholate (6-ECDC; INT-747; obeticholic acid) was purchased from Intercept Pharmaceuticals (New York, NY, USA). All other chemicals were of the highest purity available.

Primary human hepatocyte isolation

Mature primary human hepatocytes were isolated from liver tissue specimens of three female patients (age between 32 and 40 years) undergoing partial hepatectomy for large liver adenoma. Macroscopic adenoma free liver tissue was used to isolate primary hepatocytes. Tissue weight ranged from 1 to 4 g. This procedure was approved by the Medical Ethical Committee of the Academic Medical Center Amsterdam. Before every procedure, informed consent was obtained from each patient. Hepatocytes were isolated from small resection samples of human liver by a two-step collagenase treatment, as described by Seglen (6). Cells were cultured in complete Williams E Medium, containing 10% foetal bovine serum, 2 mmol/L L-glutamine, 1 µmol/L dexamethason- di-sodiumphosphate, 20 mU/L insulin, 100 U/ml penicillin, 100 U/ml streptomycin, 0.25 µg/ml Fungizon, and 1 mmol/L ornithine hydrochloride. Cells were seeded in Primiria 6-wells plates (BD bioscience) at a density of 1 x 10^6 cells/well; after 4 h, medium was refreshed. The next day, subconfluent cells were incubated for 24 h in complete Williams E medium containing 10 µmol/L rifampicin or the solvent 0.1% DMSO only (vehicle control).

Cell culture

Human HepG2 hepatoma cells overexpressing PXR and PXR knock down HepG2 cells were generated using lentiviral transduction as reported previously (7, 8). These and control HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza BioWhittaker, Cologne, Germany) supplemented with 10% foetal calf serum, 4 mmol/L L-glutamine and a mixture of antibiotics (5 mg/ml penicillin, 5 mg/ml streptomycin). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. For studying the effect of rifampicin, cells were seeded in 6-well plates at a density of 8 x 10^5 cells/well until reaching 80% confluence. Subconfluent cells were cultured overnight in serum-free medium containing 0.2% BSA. Following brief washing, cells were incubated for 24 h in DMEM/0.2% BSA containing 10 µmol/L rifampicin, 10 µmol/L 6-ECDC (INT-747) or rifampicin plus 6-ECDC (10 µmol/L, each). As a solvent control, 0.1% DMSO was added to control cells. The human colon adenocarcinoma HT-29 cell line was grown in DMEM supplemented with 10% foetal calf serum, 4 mmol/L L-glutamine and a mixture of antibiotics (5 mg/ml penicillin, 5 mg/ml streptomycin). Cells were incubated at 37°C in a humidified atmosphere containing 10% CO2. HT-29 cells were differentiated and polarized as described by Cohen (9). Briefly, HT-29 cells were seeded in 6-well plates at a density of 8 x 10^5 cells/well and were grown up to 20 days and then treated with forskolin for 20 h. After forskolin was removed,

the cells were rested for 24 h in supplemented DMEM. Following brief washing, cells were incubated for 24 h in DMEM/0.2% BSA containing 10 µmol/L rifampicin or 0.1% DMSO.

RNA isolation and quantification of transcript levels
Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and used to generate cDNA with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed at 60°C in a Lightcycler apparatus (Roche, Mannheim, Germany) with Lightcycler Faststart DNA Master Plus CYBR Green I (Roche). Transcript levels were normalized to the housekeeping gene 36B4 (acidic ribosomal phosphoprotein P0). Primers used for quantitative PCR experiments are listed in Table S1.

Statistical analysis
Statistical differences were evaluated for two groups by Student's t-test and for three or more groups by one-way ANOVA with Bonferroni correction using SPSS (version 18.0). All data are expressed as means ± standard deviations (SD).

Results

Rifampicin normalizes serum bilirubin in patients with persistent hepatocellular secretory failure of different aetiology
Between 2007 and 2013, we treated thirteen consecutive patients with rifampicin who presented with PHSF that was progressive after stopping the suspected toxins, drugs, or total parenteral nutrition or after resolving of a mechanical obstruction by stone removal or stenting. The patients diagnosed with PHSF comprised 7 females and 6 males (Table 1). The age at time of onset ranged from 18 to 81 years. Various events contributed to the development of PHSF. One patient developed PHSF after exposure to high amounts of volatile spray-paint (patient A), two patients after antibiotic treatment with flucloxacillin or clavulanic acid (B and C), two (American) patients after abusing anabolic steroids (D and E), three patients after the use of oestradiol supplementation (F, G and H) and one patient after start of total parenteral nutrition because of short bowel syndrome (I). Four patients developed PHSF after successful removal of obstruction of the common bile duct because of benign (J, K and L) or malignant obstruction (patient M) (Fig. 1; Table 1).

Table 1: Patient characteristics of 13 consecutive patients with severe persistent hepatocellular secretory failure (PHSF) successfully treated with rifampicin

Patient	Sex	Age	Underlying cause of cholestasis	Lab tests at the start of rifampicin treatment	Obstructive cholestasis excluded by	Liver histology	Treatment
A	Male	54	Toxic hepatocellular injury: Obese man (BMI 40 kg/m2), developed jaundice and pruritus weeks after paintbrushing 20–25 motorbikes without adequate airway protection (identical episode with identical history 5 years before)	Bilirubin 264 lmol/L, AP 216 U/L, c-GT 107 U/L, AST 162 U/L, ALT 97 U/L, ANA, AMA, LKM neg., HAV, HBV, HCV neg.	2x US	Extensive steatosis, steatohepatitis and marked intracellular cholestasis	(Pretreatment with cholestyramine, colesevelam and naltrexon without effect on pruritus) Rifampicin 300 mg od for 25 days; complete recovery
B	Female	53	DILI: Flucloxacillin-induced liver and bile duct injury; jaundice and pruritus started 1 week after a 3-week course of flucloxacillin	Bilirubin 278 lmol/L, AP 298 U/L, c-GT 39 U/L, AST 43 U/L, ALT 51 U/L; IgG and IgM normal, ANA, ANCA neg., AMA pos. HAV, HBV, HCV neg.	US MRC	Mild portal inflammation, portal fibrosis and ductopenia, as well as extensive cholestasis compatible with flucloxacillin-induced liver injury, but not PBC	UDCA (450 mg od for 4 weeks without success) Rifampicin, 300 mg od for 4 weeks followed by budesonide 3 mg bid; Incomplete biochemical recovery possibly because of ductopenia (flucloxacillin)
C	Male	76	DILI: Clavulanate-induced liver and bile duct injury after cholecystitis	Bilirubin 487 lmol/L, AP 183 U/L, c-GT 42 U/L, AST 201 U/L, ALT 186 U/L, ANA, AMA, ANCA neg., HAV, HBV, HCV neg.	US CT MRC	Mild cholangitis, extensive cholestasis, compatible with clavulanate-induced hepatopathy	(UDCA 300 mg tid without effect) Rifampicin 150 mg bid for 10 weeks; complete recovery

Patient	Sex	Age	Underlying cause of cholestasis	Lab tests at the start of rifampicin treatment	Obstructive cholestasis excluded by	Liver histology	Treatment
D	Male	19	DILI: Anabolic steroid-induced liver injury with jaundice and pruritus	Bilirubin 755 lmol/L, AP 290 U/L, c-GT 28 U/L, AST 162 U/L, ALT 65 U/L	US MRC HIDA	Marked non-inflammatory canalicular cholestasis suggestive of anabolic steroid ingestion	Rifampicin 150 mg od for 3 days, then increased to 300 mg od for 3 weeks; complete recovery
E	Male	19	DILI: Anabolic steroid-induced liver injury with jaundice and pruritus	Bilirubin 503 lmol/L, AP 577 U/L, AST 46 U/L, ALT 36 U/L, c-GT 25 U/L, HAV, HBV, HCV, EBV neg., ANA, AMA, IgG neg.	US MRC	-	Rifampicin 300 mg od for 3 weeks, then for 1 week 150 mg/od. After rapid recovery, rifampicin treatment was stopped.
F	Female	50	DILI: Oestradiol-induced liver injury with jaundice and pruritus	Bilirubin 383 lmol/L, AP 556 U/L, AST 45 U/L, ALT 33 U/L, c-GT 64 U/L, HAV, HBV, HCV, EBV neg., AMA neg., ANA slightly pos.	US CT	-	Rifampicin 300 mg od for 4 weeks. After rapid recovery, rifampicin treatment was stopped
G	Female	18	DILI: Oestradiol-induced liver injury with jaundice and pruritus	Bilirubin 593 lmol/L, AP 178 U/L, AST 87 U/L, ALT 186 U/L, c-GT 27 U/L, HAV, HBV, HCV, HEV, EBV, CMV, HIV neg., AMA, ANA, ANCA, SMA neg.	US	Marked non-inflammatory canalicular and cytoplasmatic cholestasis, suggestive of DILI	(Pretreatment with cholestyramine and naltrexon without effect on pruritus) Rifampicin 300 mg od for 70 days; complete recovery

Patient	Sex	Age	Underlying cause of cholestasis	Lab tests at the start of rifampicin treatment	Obstructive cholestasis excluded by	Liver histology	Treatment
H	Female	31	DILI: Oestradiol-induced liver injury with jaundice and pruritus	Bilirubin 565 lmol/L, AP 347 U/L, c-GT 23 U/L, AST 70 U/L, ALT 55 U/L HAV, HBV, HCV, EBV neg.	US MRC	-	Rifampicin, 150 mg bid for 2 weeks followed by UDCA 900 mg bid with complete recovery
I	Female	50	Total parenteral nutrition-induced hepatocellular cholestasis; renal failure with dialysis-associated sclerosing peritonitis and short bowel syndrome	Bilirubin 268 lmol/L, AP 302 U/L, AST 94 U/L, ALT 131 U/L, c-GT 148 U/L HAV, HBV, HCV neg.	US	-	Rifampicin 300 mg od; after 4 days of therapy also switch to fatreduced TPN; rapid recovery
J	Male	57	Transient biliary obstruction because of choledocholithiasis with colicky RUQ pain and acholic stool	Bilirubin 577 lmol/L, AP 298 U/L, c-GT 164 U/L, AST 62 U/L, ALT 81 U/L; HAV, HBV, HCV neg.	2 x US ERC	Colestasis and mild nonspecific portal alterations	Rifampicin, 150 mg bid for 2 weeks followed by UDCA 300 mg bid and cholecystectomy; complete recovery
K	Male	57	Transient biliary obstruction because of choledocholithiasis with colicky RUQ pain and acholic stools	Bilirubin 358 lmol/L, AP 314 U/L, c-GT 43 U/L, AST 114 U/L, ALT 128 U/L; SMA, AMA, LKM-1, SLA, ANA neg., Anti-HBs pos., HBs-Ag, HBe-Ag neg., HAV, HCV, HIV neg.	4 x US	Extensive cholestasis and degenerative alterations	(UDCA 300 mg tid and Colestyramine 4 g od without effect) Rifampicin 300 mg od for 4 weeks followed by cholecystectomy; complete recovery

Patient	Sex	Age	Underlying cause of cholestasis	Lab tests at the start of rifampicin treatment	Obstructive cholestasis excluded by	Liver histology	Treatment
L	Female	34	Transient bile duct stenosis after hemi- hepatectomy for colon ca metastasis; biloma after bile leakage (index patient)	Bilirubin 455 lmol/L, AP 183 U/L, c-GT 33 U/L, AST 186 U/L, ALT 115 U/L	US CT ERC PTC	-	Rifampicin 150 mg bid for 10 weeks (intermittently stopped for 18 days after 2 weeks of therapy due to a single elevated serum creatinine level (incorrect measurement); complete recovery
M	Female	81	Transient biliary obstruction because of pancreatic carcinoma	Bilirubin 436 lmol/L, AP 183 U/L, c-GT 33 U/L, AST 186 U/L, ALT 115 U/L, IgG neg	US CT 3 x ERC (with patent stents)	-	Rifampicin 150 mg bid for 3 weeks. After 4 weeks pylorus-preserving pancreato-duodenectomy with complete recovery

All patients remained severely jaundiced or even showed increasing serum bilirubin levels despite removal of the initiating cause of cholestasis. Imaging techniques including ultrasound, CT, MRCP and ERCP were used to rule out biliary obstruction in patients. Other causes of (chronic) liver disease were excluded by biochemical and histological markers (Table 1) before treatment with rifampicin (300 mg/day) was initiated. Eleven patients were treated in the Netherlands and two patients were treated in the United States (patients D and E). At the start of rifampicin therapy, serum total bilirubin levels ranged from 264 to 751 µmol/L (normal 0-17 µmol/L) (Fig. 1), serum transaminases and alkaline phosphatase ranged from 1.5 to 5 times the upper limit of normal (ULN). Ten patients showed normal serum levels of γGT at start of rifampicin treatment. Serum bile salt levels could only be determined in two patients (patient A and K), and were markedly elevated (268 and 225 µmol/L respectively) at the start of rifampicin treatment.

Duration of rifampicin therapy ranged from 1 to 10 weeks (Table 1). After the start of rifampicin treatment, serum total bilirubin declined rapidly in all patients (Fig. 1), as did serum alkaline phosphatase in the majority of patients (Figure S1). During and shortly after rifampicin treatment, patients were regularly checked for signs and symptoms of adverse events of rifampicin. No side effects were noted. Rifampicin therapy was interrupted in patient L after 3 weeks and was restarted when total serum bilirubin increased again. This led to normalization of serum bilirubin within 8 weeks (patient L Fig. 1B).

Taken together, rifampicin dramatically improved hepatobiliary secretion in all thirteen patients with PHSF. No side effects were observed.

Genetic background of PHSF: sequencing analysis of ATP8B1 and ABCB11

Cholestasis with normal serum γGT activity is characteristic for progressive familial intrahepatic cholestasis types 1 and 2 (10). We were able to retrieve genomic DNA from six patients of our PHSF cohort for diagnostic sequencing of both genes. In 3/6 patients, mutations were found.

In patient F, sequencing analysis revealed a homozygous mutation in ATP8B1, c.1982T>C leading to an amino acid change Ile661Thr1. This mutation has been described to cause benign recurrent intrahepatic cholestasis type 1 (BRIC1) (11) the symptoms of which had not been observed before and after the episode of PHSF in our patient F.

Two heterozygous mutations in ABCB11 were disclosed in patients G and H respectively (Table 2). Inpatient G, a known PFIC type 2 mutation, c.890A>G leading to a Glu297Gly amino acid change was found (12). In patient H, the

mutation c.2809G>A, resulting in a Gly937Arg amino acid change, has not been reported so far in PFIC or BRIC type 2 patients. Recent publications have shown that heterozygous mutations and polymorphisms of ABCB11 may play a role in the development of drug-induced liver injury (13–15).

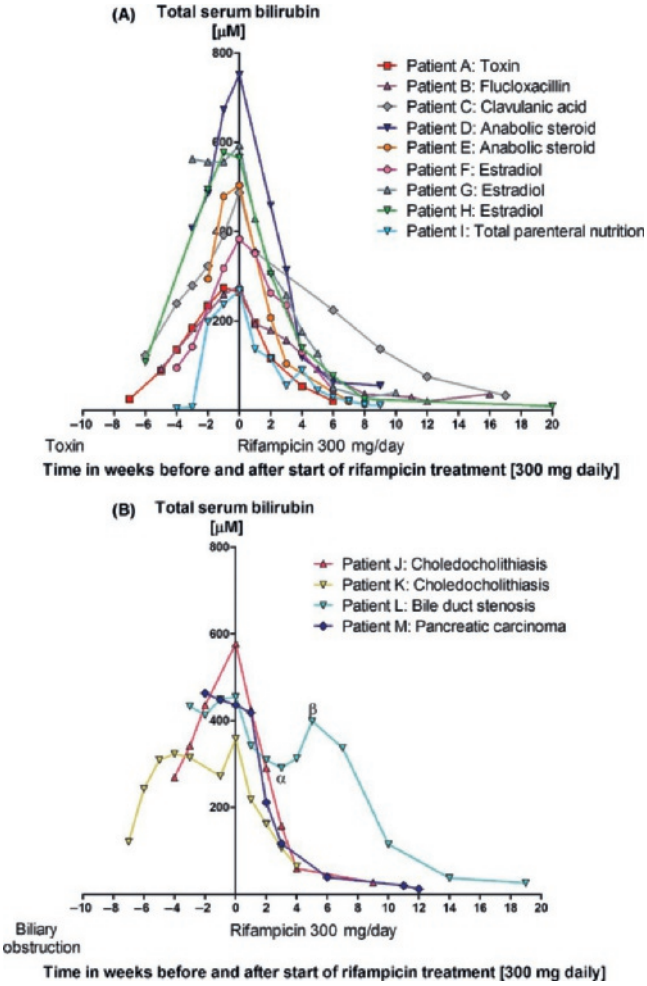

Figure 1. Rifampicin improves hepatobiliary secretion in patients with persistent hepatocellular secretory failure. Total serum bilirubin (µmol/L; after removal of the cause of liver injury) before, during and after treatment with rifampicin in (A) patients with toxin-/drug-/total parenteral nutrition-induced persistent hepatocellular secretory failure and (B) patients with persistent hepatocellular secretory failure induced after short-term biliary obstruction. Week 0 indicates start of rifampicin treatment. α: End of first period of treatment with rifampicin in patient L. β: Beginning of second period of treatment with rifampicin in patient L.

Table 2 ATP8B1 and ABCB11 (BSEP) mutations in patients with PHSF

Patient	Undelying cause of cholestasis	ATP8B1	ABCB11 (BSEP)
F	DILI: oestardiol	c.1982T>C Ile661THr*	
G	DILI: oestardiol		c.890A>G p.Glu297Gly (heterozygote)†
H	DILI: oestardiol		c.2809G>A p.Gly937Arg (heterozygote) ‡

* Folmer DE, van der Mark VA, Ho-Mok KS, Oude Elferink RP, Paulusma CC. *Differential effects ofprogressive familial intrahepatic cholestasis type 1 and bening recurrent intrahepatic cholestasis type 1 mutations on canalicular localization of ATP8B1*. Hepatology 2009; 50 1597- 605
† Kubitz R, Dröge C, Stindt J, Weissenberger K, Häussinger D. *The bile salt export pomp (BSEP) in health and disease.* Clin Res Hepatol gastroenterol. 2012 36; 536-53
‡ Mutation not earlier published

Rifampicin induces expression of genes involved in transport and detoxification in the enterohepatic circulation by PXR-dependent mechanisms

To understand the effect of the PXR activator rifampicin in PHSF, we investigated its effect on the expression of genes involved in biotransformation and secretion (biotransformation phase 1–3) of potential toxins in liver and intestine-derived cell lines. In primary human hepatocytes, rifampicin stimulated the expression of PXR sensitive genes: Cytochrome P450 3A4 (CYP3A4; phase 1), responsible for the hydroxylation of bile salts and drugs, and UDP-glucuronosyltransferase 1A1 (UGT1A1; phase 2), responsible for glucuronidation of bilirubin. After 24 h, both were induced by 10 µM (Fig. 2) confirming data previously reported (16, 17). Also, the expression of the apical ATP-binding cassette (ABC) transporter MRP2 (ABCC2), responsible for the biliary secretion of bilirubin glucuronides and other conjugated organic anions (phase 3), was 1.5-fold induced by this concentration of rifampicin (Fig. 2) as described previously (18).

The organic solute transporter (OSTα-OSTβ) is expressed on the basolateral membrane of epithelial cells mainly of the ileum and colon, liver, kidney and testis where it functions as a transporter of bile acid and steroid conjugates (19, 20). Both OSTα and OSTβ are upregulated by farnesoid X receptor (FXR)-dependent mechanisms (21). Notably, OSTβ expression was 67- fold induced in liver tissue of patients with severe obstructive cholestasis, while OSTα expression was induced 3-fold under these conditions (22). This suggests that modulation of OSTβ expression seems to play a critical role in the protection of a cholestatic

liver. Here, we found that OSTβ, but not OSTα, expression was 2.5-fold induced in primary human hepatocytes exposed to rifampicin (Figure 2).

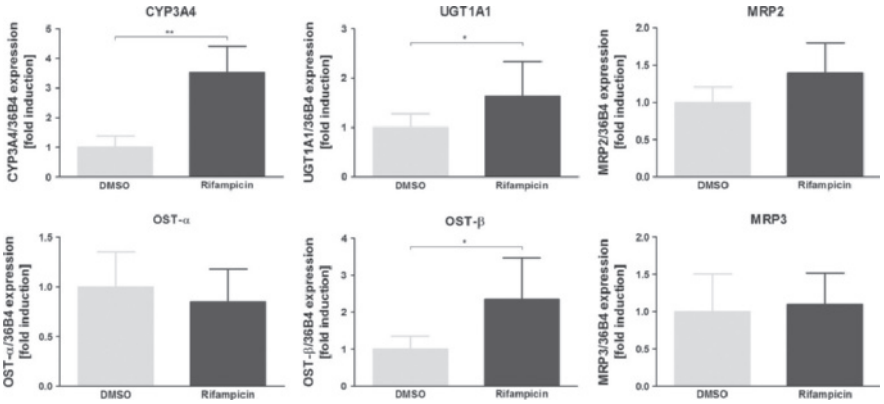

Figure 2. Rifampicin induces CYP3A4, MRP2 and OSTβ in primary human hepatocytes. Primary human hepatocytes were incubated with rifampicin (10 µM) (dark greybars) or DMSO (0.1%, v/v) for 24 h (n = 3). Total RNA was isolated to generate cDNA and CYP3A4, UGT1A1, MRP2, MRP3, OSTα and OSTβ mRNA levels were determined using qPCR suing 36B4 as a reference gene. Figure is representative of 3 independent primary human hepatocyte isolations. Data are presented as mean ± SD; *P < 0.05, **P < 0.01.

To prove that rifampicin induces gene expression via the pregnane X receptor (PXR), we subsequently studied its effect in the human HepG2 hepatoma cell line, a HepG2PXR cell line overexpressing PXR, and a HepG2 cell line in which PXR was knocked down. The difference in CYP3A4, UGT1A1, MRP2 and OSTβ expression seen between HepG2 (over)expressing PXR and HepG2 lacking it indicated that rifampicin indeed induces these genes via PXR-dependent mechanisms (Figure S2A–C). PXR is also expressed in the human intestinal tract. To assess the effect of rifampicin on intestinal cells, we chose the polarized and differentiated HT-29 cell line. Again, rifampicin induced CYP3A4, MRP2 and OSTβ expression and tended to induce UGT1A1 expression (P = 0.08) (Figure S2D). In contrast to a previous report (23), PXR activation by rifampicin did not lead to induction of the basolateral exporter MRP3 (ABCC3) in primary human hepatocytes and HepG2 cells, whereas a trend towards induction of MRP3 (P = 0.06) was observed in HepG2PXR cells (Figure S4).

OSTα-OSTβ serves as an escape cellular efflux path. OSTα-OSTβ is controlled by the nuclear bile salt receptor FXR. In cholestatic hepatocytes, its expression prevents intracellular accumulation of toxic hydrophobic bile salts. To investigate whether PXR and FXR may have co-operative effects on OSTα-

OSTβ expression, we incubated HepG2 cells with rifampicin and the potent FXR agonists, 6-ECDC (INT-747), a well-known inducer of OSTα-OSTβ mRNA expression. As reported previously, after 24h, the expression of both OSTα and OSTβ was significantly induced by 10 μM of 6-ECDC (6.2- and 39.0-fold, respectively, Figure S3A and B). Addition of the PXR agonist, rifampicin, had a substantial additive effect on OSTβ expression, leading to a 49.8-fold induction in comparison with vehicle control. This additional effect was abolished in HepG2 cells after knock down of PXR (Figure S3D).

Discussion

Persistent hepatocellular secretory failure (PHSF) is a rare, but life-threatening complication of acute liver injury induced by drugs, toxins, or short-term mechanical obstruction for which no effective treatment is known. In the present study, we demonstrate that thirteen consecutive patients who fulfilled the criteria for PHSF were successfully treated with the potent PXR agonist rifampicin. Our findings in human liver and intestinal cells in vitro suggest that rifampicin exerts these beneficial effects at least in part by upregulating key detoxification enzymes and export pumps including apical MRP2 and basolateral OSTβ by PXR-dependent mechanisms. Induction of genes involved in detoxification and elimination of bilirubin, bile salts and other endo- and xenobiotics may represent a novel therapeutic strategy in PHSF.

In nine patients, PHSF was observed after toxin- (ill protected volatile paint-brushing; patient A), drug- (DILI induced by flucloxacillin, clavulanate, anabolic steroid abuse, and oestradiol; patients B–H), or total parenteral nutrition-induced liver injury (patient J) (Fig. 1A). Although only 1 in 10.000–100.000 patients usually develops DILI because of a specific drug, DILI accounts for 5–10% of patients hospitalized for jaundice (24). A recent retrospective study showed that 10% of patients with DILI either died or underwent a liver transplantation (25). Serum bilirubin was significantly increased in deceased patients or those who underwent liver transplantation, and was an independent predictor of mortality (25, 26).

In four patients, PHSF developed after previous mechanical obstruction of the biliary tract (choledocholithiasis; transient bile duct stenosis after hemihepatectomy; bile duct stenosis because of pancreatic carcinoma; patients J–M; Fig. 1B). Treatment of the initial cause of cholestasis in these patients by removal of the underlying stenosis, and afterwards conformation of a

non-obstructive biliary tract by various imaging techniques, did not result in improvement of the jaundice.

Ten of thirteen severely cholestatic patients showed normal serum γGT during the course of PHSF, a clinical characteristic of PFIC and BRIC type 1 and 2. This observation suggested a role for ATP8B1 and/or ABCB11 (BSEP) in the development of PHSF. Therefore, sequence analysis of both genes was performed in six of the PHSF patients who were able and willing to provide blood for analysis and had a normal γGT. A homozygous mutation was identified in ATP8B1, c.1982T>C, leading to an amino acid change Ile661Thr1 in patient F, which had been reported in BRIC1 patients (11). BRIC1 is a rare hereditary disease caused by a mutation in ATP8B1 characterized by recurrent episodes of jaundice with pruritus and normal γGT levels (27). Patient F had no clinical or biochemical history of BRIC1, but developed PHSF after the start of a course of oestradiol, which has been reported to induce a cholestatic episode in BRIC1 patients (27). In the past, rifampicin was used in the treatment for both low γGT PFIC and BRIC patients. Although rifampicin relieved pruritus in patients with low γGT-PFIC, it had only a temporary effect on serum bilirubin levels. In contrast, rifampicin was shown to be effective in BRIC, resolving eighteen of twenty-two cholestatic episodes in seven patients (28). Rifampicin induces hydroxylation and conjugation of bile acids because of upregulation of CYP3A4 and UGT1A1. This results in an increased urinary bile acid excretion. Patient F rapidly responded to rifampicin with regard to total serum bilirubin and pruritus, and recovered completely from her cholestatic episode.

Heterozygous mutations in ABCB11 were found in two of six patients analysed (G, H). Notably, heterozygous mutations of ABCB11 are known to be associated with drug-induced cholestasis (14).

We treated our patients with rifampicin (300 mg/day), a potent activator and high-affinity ligand of the pregnane X receptor (PXR). PXR acts as a broad-specificity sensor of xeno- and endobiotics and regulates expression of various target genes predominantly in liver and intestine involved in uptake, detoxification and excretion of endo- and xenobiotics (biotransformation phase 0–3) and bile salt synthesis (29, 30). Here, we confirmed the effect of rifampicin in vitro on key detoxification enzymes and exporters including CYP3A4, UGT1A1 and MRP2 in different human liver and intestinal cell lines.

The organic solute transporter α-β(OSTα-OSTβ) is responsible for secretion of bile salts and conjugated steroids across the basolateral membrane of hepatocytes into the portal circulation (31). OSTβ is critically required for

transport activity of the OSTα-OSTβ heterodimer (32). The nuclear receptor FXR is considered the main regulator of OSTa and OSTb expression (21). Here, we demonstrate that rifampicin induces OSTβ in both human liver and intestinal cell lines in a PXR dependent manner. Notably, rifampicin had an additive effect on OSTβ expression during potent FXR activation. In human liver, a 7-fold higher expression of OSTα than OSTβ has been described. Upregulation of OSTβ will affect the transport activity of the heterodimer and help in the removal of cholestatic toxins from the hepatocyte. Rifampicin also reduces the expression of cholesterol 7α-hydroxylase (CYP7A1) thereby reducing the de novo synthesis of bile salts (33).

Prolonged treatment with rifampicin for 4–12 weeks is associated with hepatotoxicity particularly under cholestatic conditions in up to 12% (34), whereas treatment periods of <2 weeks appear safe in patients with chronic cholestasis (35). No rifampicin-related hepatotoxicity was observed in our cohort.

In conclusion, we show that short-term treatment with the PXR agonist rifampicin appears to be safe and effective in patients with PHSF. PHSF is a new disease entity defined by persistence of cholestasis for >1 week after stopping the causative drug or toxin or removal of the extrahepatic biliary blockade. In some of these patients, we found genetic defects of hepatocellular transport. We are aware that these are uncontrolled observations, but the effect of rifampicin in these patients seems robust enough to warrant a large placebo-controlled study with crossover design.

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References

1. EASL Clinical Practice Guidelines. Management of cholestatic liver diseases. J Hepatol 2009; 51: 237–67
2. Lindor KD, Gershwin ME, Poupon R, et al. Primary biliary cirrhosis. Hepatology 2009; 50: 291–308
3. Beuers U. Drug insight: mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol 2006; 3: 318–28
4. Zollner G, Trauner M. Nuclear receptors as therapeutic targets in cholestatic liver diseases. Br J Pharmacol 2009;156: 7–27
5. Maillette de Buy Wenniger LJ, Oude Elferink RP, Beuers U. Molecular targets for the treatment of fibrosing cholangiopathies. Clin Pharmacol Ther 2012; 92: 381–7
6. Seglen PO. Hepatocyte suspensions and cultures as tools in experimental carcinogenesis. J Toxicol Environ Health 1979; 5: 551–60
7. Kremer AE, Dijk RV, Leckie P, et al. Serum autotaxin is increased in pruritus of cholestasis, but not of other origin and responds to therapeutic interventions. Hepatology 2012; 56: 1391–1400
8. Nibourg GA, Huisman MT, van der Hoeven TV, et al. Stable overexpression of pregnane X receptor in HepG2 cells increases its potential for bioartificial liver application. Liver Transpl 2010; 16: 1075–85
9. Cohen E, Ophir I, Shaul YB. Induced differentiation in HT29, a human colon adenocarcinoma cell line. J Cell Sci 1999; 112(Pt 16): 2657–66
10. Morotti RA, Suchy FJ, Magid MS. Progressive familial intrahepatic cholestasis (PFIC) type 1, 2, and 3: a review of the liver pathology findings. Semin Liver Dis 2011; 31:3–10
11. Folmer DE, van der Mark VA, Ho-Mok KS. Oude Elferink RP, Paulusma CC. Differential effects of progressive familial intrahepatic cholestasis type 1 and benign recurrent intrahepatic cholestasis type 1 mutations on canalicular localization of ATP8B1. Hepatology 2009; 50: 1597–605
12. Kubitz R, Droge C, Stindt J, Weissenberger K, Haussinger D. The bile salt export pump (BSEP) in health and disease. Clin Res Hepatol Gastroenterol 2012; 36: 536–53
13. Moghadamrad S, Montani M, Weimann R, De GA. Cholestasis in a patient with gallstones and a normal gammaglutamyl transferase. Hepatology 2013; 57: 2539–41
14. Lang C, Meier Y, Stieger B, et al. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. Pharmacogenet Genomics 2007; 17: 47–60
15. Meier Y, Zodan T, Lang C, et al. Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump. World J Gastroenterol 2008; 14: 38–45
16. Enoru-Eta J, Yengi LG, He X, et al. Development of a UGT1A1 reporter gene assay for induction studies: correlation between reporter gene data and regulation of UGT1A1 in human hepatocytes. Drug Metab Lett 2010; 4:31–8
17. Luo G, Cunningham M, Kim S, et al. CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. Drug Metab Dispos 2002; 30: 795–804
18. Marschall HU, Wagner M, Zollner G, et al. Complementary stimulation of hepatobiliary transport

and detoxification systems by rifampicin and ursodeoxycholic acid in humans. Gastroenterology 2005; 129: 476–85

19. Ballatori N. Pleiotropic functions of the organic solute transporter Ostalpha-Ostbeta. Dig Dis 2011; 29: 13–7
20. Dawson PA, Hubbert ML, Rao A. Getting the mOST from OST: role of organic solute transporter, OSTalpha-OSTbeta, in bile acid and steroid metabolism. Biochim Biophys Acta 2010; 1801: 994–1004
21. Boyer JL, Trauner M, Mennone A, et al. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. Am J Physiol Gastrointest Liver Physiol 2006; 290: G1124–30
22. Schaap FG, van der Gaag NA, Gouma DJ, Jansen PL. High expression of the bile salt-homeostatic hormone fibroblast growth factor 19 in the liver of patients with extrahepatic cholestasis. Hepatology 2009; 49: 1228–35
23. Teng S, Jekerle V, Piquette-Miller M. Induction of ABCC3 (MRP3) by pregnane X receptor activators. Drug Metab Dispos 2003; 31: 1296–9
24. Bjornsson E, Ismael S, Nejdet S, Kilander A. Severe jaundice in Sweden in the new millennium: causes, investigations, treatment and prognosis. Scand J Gastroenterol 2003; 38: 86–94
25. Bjornsson E, Olsson R. Outcome and prognostic markers in severe drug-induced liver disease. Hepatology 2005; 42:481–9
26. Bjornsson E, Davidsdottir L. The long-term follow-up after idiosyncratic drug-induced liver injury with jaundice. J Hepatol 2009; 50: 511–7
27. van der Woerd WL, van Mil SW, Stapelbroek JM, et al. Familial cholestasis: progressive familial intrahepatic cholestasis, benign recurrent intrahepatic cholestasis and intrahepatic cholestasis of pregnancy. Best Pract Res Clin Gastroenterol 2010; 24: 541–53
28. Stapelbroek JM, van Erpecum KJ, Klomp LW, Houwen RH. Liver disease associated with canalicular transport defects: current and future therapies. J Hepatol 2010; 52:258–71
29. di MA, De ME, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: molecular, functional, and biomedical aspects. Mol Aspects Med 2009; 30: 297–343
30. Staudinger JL, Goodwin B, Jones SA, et al. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A 2001; 98:3369–74
31. Dawson PA, Hubbert M, Haywood J, et al. The heteromeric organic solute transporter alpha-beta, Ostalpha-Ostbeta, is an ileal basolateral bile acid transporter. J Biol Chem 2005; 280: 6960–8
32. Christian WV, Li N, Hinkle PM, Ballatori N. beta-subunit of the Ostalpha-Ostbeta organic solute transporter is required not only for heterodimerization and trafficking but also for function. J Biol Chem 2012; 287: 21233–43
33. Li T, Chiang JY. Mechanism of rifampicin and pregnane X receptor inhibition of human cholesterol 7 alpha-hydroxylase gene transcription. Am J Physiol Gastrointest Liver Physiol 2005; 288: G74–84
34. Bachs L, Pares A, Elena M, Piera C, Rodes J. Effects of long-term rifampicin administration in primary biliary cirrhosis. Gastroenterology 1992; 102: 2077–80
35. Khurana S, Singh P. Rifampin is safe for treatment of pruritus due to chronic cholestasis: a meta-analysis of prospective randomized-controlled trials. Liver Int 2006; 26:943–8

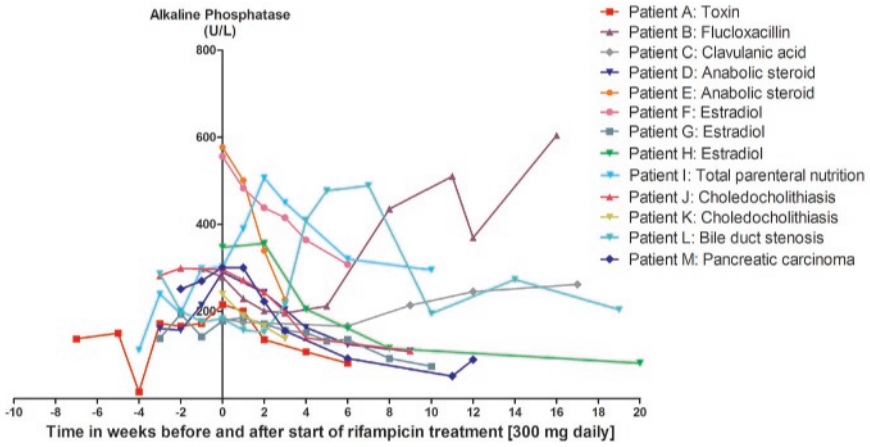
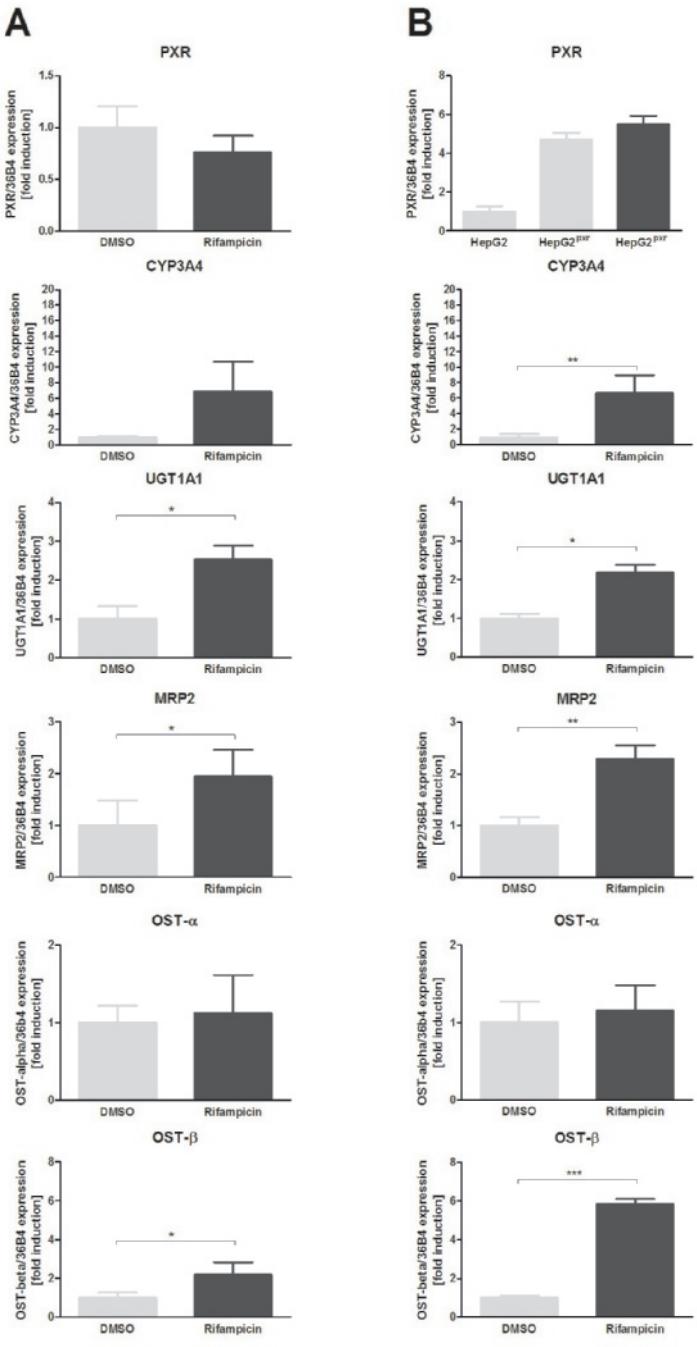


Figure S1. Serum Alkaline Phosphatase in patients with persistent hepatocellular secretory failure. Serum alkaline phosphatase (U/L; after removal of the cause of liver injury) before, during and after treatment with rifampicin in patients with persistent hepatocellular secretory failure. Week 0 indicates start of rifampicin treatment. Patient B developed histology-proven ductopenia with an increase of alkaline phosphatase over months and recovery after combined treatment with UDCA and budesonide. For patient L, see Fig. 1.


A
PXR
PXR/36B4 expression [fold induction]
DMSO
Rifampicin
CYP3A4
CYP3A4/36B4 expression [fold induction]
UGT1A1
UGT1A1/36B4 expression [fold induction]
MRP2
MRP2/36B4 expression [fold induction]
OST-α
OST-alpha/36b4 expression [fold induction]
OST-β
OST-beta/36B4 expression [fold induction]
B
PXR
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MRP2
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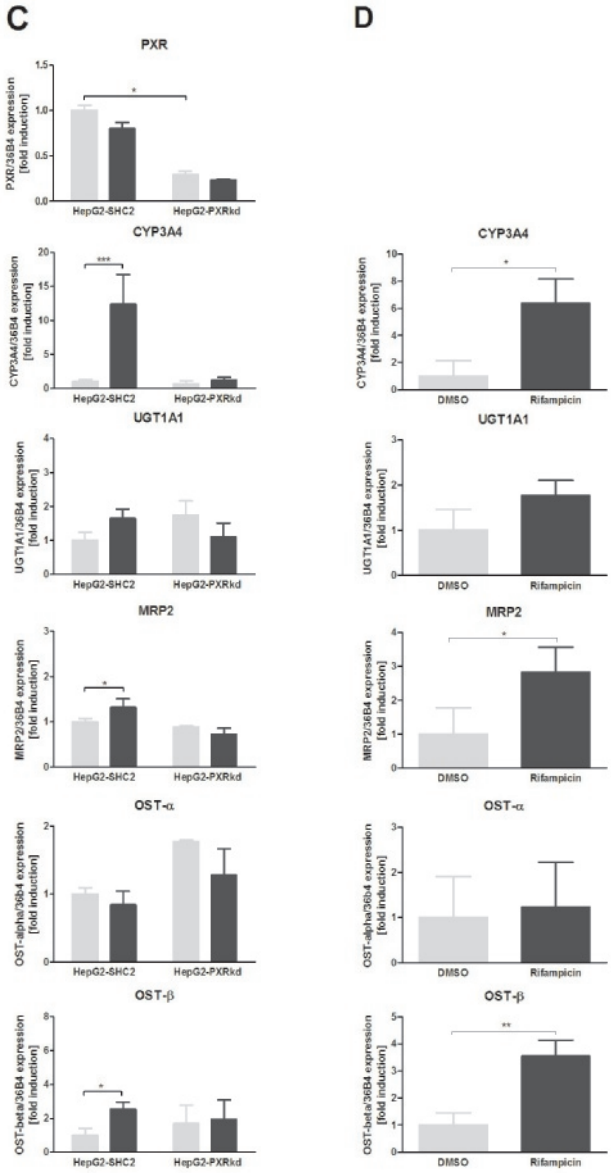

Figure S2. Rifampicin induces CYP3A4, UGT1A1, MRP2 and OST-β in human HepG2 hepatoma cells and human intestinal HT-29 cells in a PXR-dependent manner. (A) Human HepG2 hepatoma cells, (B) human HepG2 cells overexpressing PXR, (C) human HepG2 cells after successful knock down of PXR with short-hairpin RNA lentiviral transduction and (D) human colonic HT-29 cells were incubated with rifampicin (10 μM, dark greybars) or DMSO (0.1%, v/v) for 24 h (n = 3–6). Total RNA was isolated to generate cDNA and CYP3A4, UGT1A1, MRP2, MRP3, OST-α and OST-β mRNA levels were determined using qPCR using 36B4 as a reference gene. Data are presented as mean ± SD; *P < 0.05, **P < 0.01.

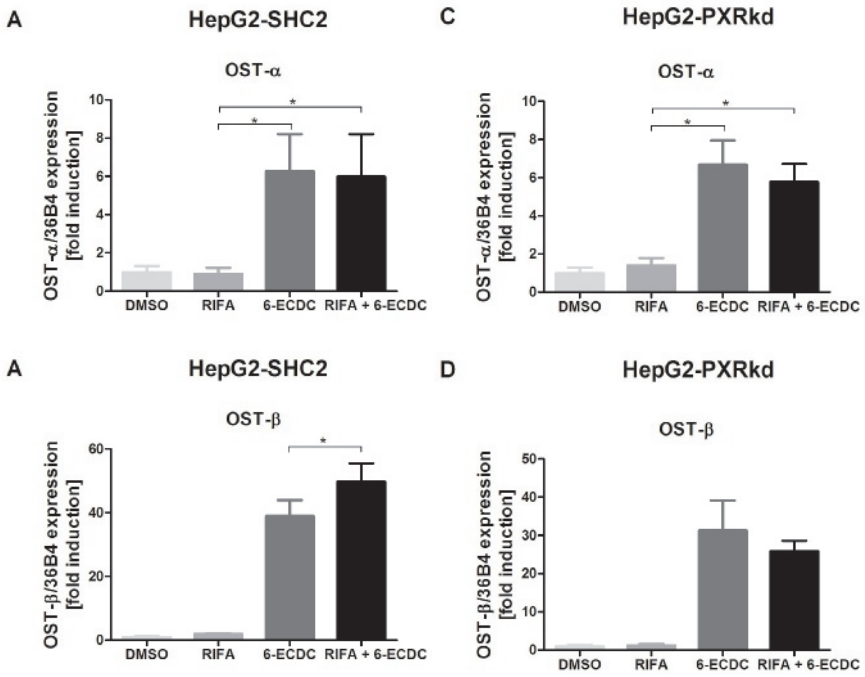

Figure S3. PXR and FXR co-activation induces OST-β expression in human HepG2 hepatoma cells. (A) OST-α and OST-β (B) mRNA levels were determined in human HepG2 hepatoma cells after incubation with rifampicin (10 μM), 6-ECDC (10 μM) or co-incubation of rifampicin and 6-ECDC for 24 h. (C) OST-α and OST-β (D) mRNA levels were determined in human HepG2 hepatoma with knock down of PXR after incubation with rifampicin (10 μM), 6-ECDC (10 μM) or co-incubation of rifampicin and 6-ECDC for 24 h. (n = 3) Data are presented as mean ± SD; *P < 0.05, ** P < 0.01.

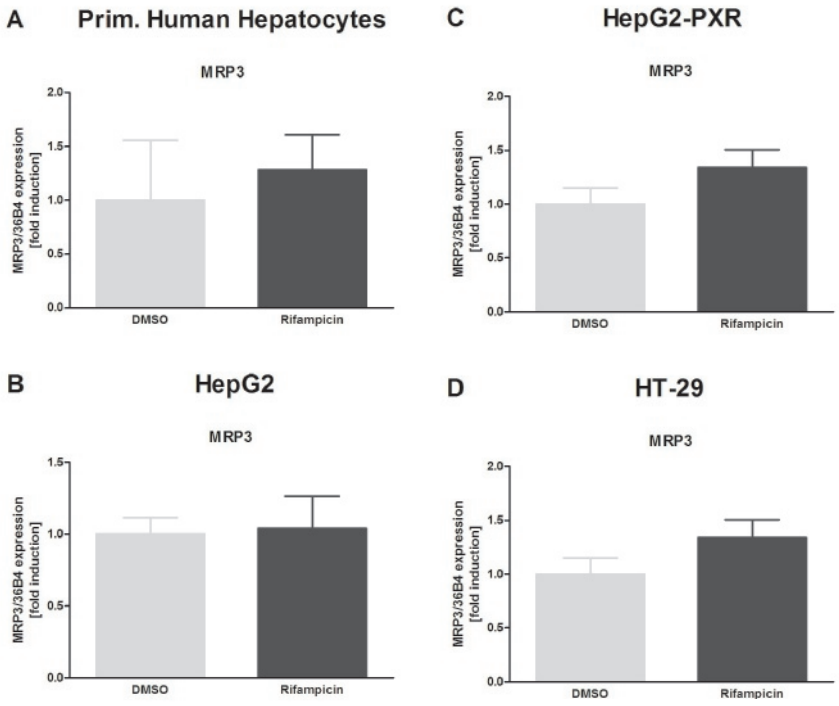

Figure S4. MRP3 expression is marginally affected by rifampicin in human liver cells. Expression of the basolateral organic anion transporter MRP3 (ABCC3) is not altered in (A) primary human hepatocytes, (B) the human hepatoma cell line HepG2 and (D) the differentiated intestinal HT-29 cell line, but tends to increase (P = 0.06) in (C) HepG2-PXR cells after PXR activation with rifampicin (10 µmol/L, 24 h). Mean + SD, n = 3.

Supplementary Table 1. Quantitative PCR primers

Name	Direction	Sequence
36B4	Forward Reverse	TCATCAACGGTACAAACGA GCCTTGACCTTTTCAGCAAG
PXR	Forward Reverse	CTGTGTGGATGCTGAGCTGT TGATTGTCAGCGTAGCCTTG
CYP3A4	Forward Reverse	TGTTTTCAGCCCATCTCCTT CATTGCATCGAGACAGTTGG
UGT1A1	Forward Reverse	TGCAGATGGTTGCAAATTGAT CATTCTTTTTCCCCAAAGCA
MRP2	Forward Reverse	GACGACCATCCAAAACGAGT TTCTCAATGCCAGCTTCCTT
MRP3	Forward Reverse	CCGATTCCCAACTGAGTGTT ACCCAGCAGCTGTCTTCTGT
OST-α	Forward Reverse	AGATAACGCTGACCCTGGTG AATTTGGCTCCCATGTTCTG
OST-β	Forward Reverse	ATCCAGGCAAGCAGAAAAGA CTGGTACATCCGGAAGGAAA

8

SERUM AUTOTAXIN IS INCREASED IN PRURITUS OF CHOLESTASIS, BUT NOT OF OTHER ORIGIN AND RESPONDS TO THERAPEUTIC INTERVENTIONS

Kremer AE, van Dijk R, Leckie P, Schaap FG, Kuiper EM, Mettang T, Reiners KS, Raap U, van Buuren HR, van Erpecum KJ, Davies NA, Rust C, Engert A, Jalan R, Oude Elferink RP, Beuers U

Hepatology 2012; 56:1391-1400

Abstract

Pruritus is a seriously disabling symptom accompanying many cholestatic liver disorders. Recent experimental evidence implicated the lysophospholipase autotaxin (ATX) and its product, lysophosphatidic acid (LPA), as potential mediators of cholestatic pruritus. In this study we highlight that increased serum ATX levels are specific for pruritus of cholestasis but not pruritus of uremia, Hodgkin's disease or atopic dermatitis. Treatment of cholestatic patients with the bile salt sequestrant colesevelam, but not placebo effectively reduced total serum bile salts and fibroblast growth factor 19 levels, but only marginally altered pruritus intensity and ATX activity. Rifampicin significantly reduced itch intensity and ATX activity in pruritic patients not responding to bile salt sequestrants. In vitro, rifampicin inhibited ATX expression in human HepG2 hepatoma cells and hepatoma cells over-expressing the pregnane X receptor (PXR), but not in hepatoma cells in which PXR was knocked down. Treatment of severe, refractory pruritus by Molecular Adsorbents Recirculation System or nasobiliary drainage improved itch intensity which again correlated with the reduction of ATX levels. Upon reoccurrence of pruritus ATX activity returned to pretreatment values. In conclusion, serum ATX activity is specifically increased in patients with cholestatic, but not other forms of systemic pruritus and closely correlates with effectiveness of therapeutic interventions. The beneficial antipruritic action of rifampicin may be explained, at least partly, by PXR-dependent transcriptional inhibition of ATX expression. Thus, ATX likely represents a novel therapeutic target for pruritus of cholestasis.

Introduction

Chronic pruritus can be a seriously debilitating symptom accompanying various cutaneous and systemic disorders.[1] It represents one of the most prominent clinical features in numerous liver disorders such as primary biliary cirrhosis, primary sclerosing cholangitis, cholangiocarcinoma, inherited forms of cholestasis and intrahepatic cholestasis of pregnancy.[2] This form of itching is designated cholestatic pruritus as impaired bile flow is a common denominator in these disorders.[3] The molecular mechanisms involved in the pathogenesis of cholestatic pruritus remain enigmatic and treatment of these patients often represents a clinical challenge due to limited therapeutic options. The current guidelines for the treatment of cholestatic pruritus recommend the use of bile salt sequestrants such as cholestyramine or colesevelam as first line therapy and rifampicin as second line treatment.[2,4] If patients do not respond to these drugs, experimental approaches may be applied. The Molecular Absorbance Recirculating System (MARS) is an extracorporeal liver dialysis system that is capable of removing mainly albumin-bound molecules such as bile salts, bilirubin, ammonia and other amphiphilic toxins. MARS therapy has been shown to effectively alleviate intractable pruritus of cholestasis in patients who do not respond to any medicinal therapy.[2,4,5] Nasobiliary drainage transiently relieves severe pruritus in BRIC[6] and PBC patients[7] who did not respond to standard anti-pruritic treatment.[2,4] However, pruritus may even become refractory to all medical treatments and can be an indication for liver transplantation, even in the absence of liver failure.[4]

By functional screening of sera of cholestatic patients suffering from pruritus on neuronal cells we recently identified lysophosphatidic acid (LPA) as a potent neuronal activator.[8] Serum levels of this phospholipid were increased in cholestatic patients that suffered from pruritus. Circulating LPA is formed by a lysophospholipase D called autotaxin (ATX) which hydrolyses the choline group from lysophosphatidylcholine.[9] In mice, the amount of circulating LPA depends on serum ATX activity.[10] In line with the observed increase in LPA, ATX activity was higher in sera of pruritic patients with cholestatic disorders compared to those without pruritus. Furthermore, itch intensity highly correlated with ATX activity. Intradermal injection of LPA caused a dose-dependent scratch response in mice.[8]

ATX was initially identified as a cell motility factor, that is over-expressed in various tumors and involved in proliferation and generation of metastases.[11] The effects of autotaxin are largely mediated by the enzymatic formation of LPA which activates at least six different G-protein-coupled receptors (GPCRs).[9,11] ATX is also essential

for angiogenesis, neuronal development and lymphocyte homing[10] and LPA mediates initiation of neuropathic pain, hair growth and embryo implantation.[9]
Here, we studied whether increased serum ATX activity is specific for pruritus of cholestasis. We also aimed to investigate the effect of various therapeutic interventions such as treatment with colesevelam, rifampicin, MARS and nasobiliary drainage on ATX activity. Finally, the effects of rifampicin on ATX expression were studied *in vitro*.

Materials and Methods

Human subjects
Peripheral venous blood was obtained from healthy donors and patients with cholestatic disorders, uremia, Hodgkin's disease, and atopic dermatitis after informed consent according to the Declaration of Helsinki. The study was approved by the local Medical Ethical Committees. Treatment interventions such as colesevelam,[12] rifampicin, MARS therapy, and nasobiliary drainage[7] were conducted, recorded, and reported in compliance with the International Conference on Harmonisation Good Clinical Practice and national regulations. Blood samples were allowed to clot for an hour before they were centrifuged at 4°C and serum was cryo-preserved in aliquots at -80°C. Itch intensity was quantified in all patients at the time point of blood drawing using a VAS ranging from 0 (no pruritus) to 100 (unbearable pruritus). In the colesevelam study[12] 35 patients were evaluable of whom 17 patients received colesevelam 1875 mg twice daily and 18 patients were treated with an identical placebo for three weeks. The study population consisted of 22 female and 13 male patients being mainly diagnosed for primary biliary cirrhosis (N=14) or primary sclerosing cholangitis (N=14). MARS treatment was performed in 10 patients (8 female / 2 male) with intractable pruritus due to PBC (n=6), PSC (n=2) or other liver disorders (n=2; Supplementary Table 4).

Materials
Choline oxidase, horseradish peroxidase, homovanillinic acid, dimethyl sulfoxide (DMSO), bovine serum albumine (BSA) and rifampicin were purchased from Sigma-Aldrich (Steinheim, Germany); Stearoyl-lysophosphatidic acid (LPA 18:1) and Myristoyl-lysophosphatdiylcholine (LPC 14:0) were from Avanti Lipids (Alabaster, AL).

Cell culture

Human HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza BioWhittaker, Cologne, Germany) supplemented with 10% fetal calf serum, 4 mM L-glutamine and a mixture of antibiotics (5 mg/mL penicillin, 5 mg/mL streptomycin). Cells were incubated at 37 ° C in a humidified atmosphere containing 5% CO_2. For studying the effect of rifampicin, cells were seeded in 6-well plates at a density of 8×10^5 cells/well until reaching 80% confluence. The subconfluent cells were cultured overnight in serum-free medium containing 0.2% BSA. Following brief washing, cells were incubated for 24 hours in DMEM/0.2% BSA containing 10 µM rifampicin. As a solvent control 0.1% DMSO was added to control cells. HepG2 cells over-expressing PXR and PXR knock-down HepG2 cells (see below) were identically analyzed.

Lentiviral Transduction

Short hairpin RNAs for PXR (TRCN0000021623) and plasmids encoding non-target control (SHC002) as control were obtained from Sigma-Aldrich in a lentiviral vector system. Lenti-viral supernatant was generated from HEK 293T cells as packaging cells calcium phosphate based transfection (ClonTech, Mountain View, CA). HepG2 cells were grown to 50–60% confluence and incubated with virus-containing supernatants/DMEM (1:1) supplemented with 10 µg/mL diethylaminoethyl-dextrane for 24 hours. Selection of transduced cells was achieved by addition of puromycin 30 µg/mL and knock-down of PXR was verified by quantitative PCR (see below). HepG2 cells over-expressing PXR were generously provided by Dr. R. Hoekstra (Academic Medical Center, University of Amsterdam).[13]

RNA isolation and quantification of transcript levels

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen). cDNA was synthesized from total RNA with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Realtime PCR measurements were performed at 60°C in a Lightcycler apparatus (Roche) with Lightcycler Faststart DNA Master Plus CYBR Green I (Roche). Transcript levels were normalized to the housekeeping gene 36b4 (acidic ribosomal phosphoprotein P0). For quantitative PCR experiments the following primer sequences were used: ATX Forward: TGCAATAGCTCAGAGGACGA; ATX Reverse: AGAAGTCCAGGCTGGTGAGA; CYP3A4 Forward: TGTTTTCAGCCCATCTCCTT; CYP3A4 Reverse: CATTGCATCGAGACAGTTGG; 36B4 Forward: TCATCAACGGTACAAACGA; 36B4 Reverse: GCCTTGACCTTTTCAGCAAG.

Autotaxin activity assay

ATX activity was quantified in diluted sera as recently described.[8] Briefly, serum samples were incubated with a buffer containing 500 mmol/L NaCl, 5 mmol/L $MgCl_2$, 100 mmol/L Tris (pH = 9.0) and 0.05% Triton X-100 for 60 min at 37°C. Parallel incubations were performed in the presence and absence of 1 mmol/L of LPC 14:0. The lysophospholipase activity of ATX was determined by the amount of liberated choline, as detected by enzymatic fluorimetry using choline oxidase (2 U/mL), horseradish peroxidase (1.6 U/mL), and homovanillinic acid as substrate for peroxidase. After addition of both enzymes in a buffer (consisting of 20 mmol/L $CaCl_2$, 2 mmol/L homovanillinic acid, 50 mmol/L 3-(N-morpholino)propanesulfonic acid (pH = 8.0) and 0.1% Triton X-100) the increase in fluorescence was monitored at 37°C on a Novostar analyzer (excitation 320 nm, emission 405 nm). The (endogenous) amount of choline present in the sample without addition of LPC was subtracted from the amount measured in the presence of LPC. The inter-assay variance of the assay was less than 15%, the intra-assay variance of the assay was below 10%. For studying the effect of rifampicin on *in vitro* ATX activity, healthy control serum was incubated using above mentioned buffers containing different concentrations of rifampicin. Stock solutions of rifampicin were dissolved in PBS and diluted 100-fold in above mentioned buffer. PBS was used as a solvent control.

Determination of FGF19

Serum FGF19 levels were determined using a sandwich enzyme-linked immunosorbent assay specific for FGF19 as described recently.[14]

Determination of bile salts

Total serum bile salt levels were quantified using Diazyme total bile salts kit (Diazyme Laboratories, Poway, CA) according to the manufacturer's instructions.

SDS-PAGE and Western Blotting

Serum samples or albumin dialysates were diluted and incubated for 10 min at 37°C with SDS-PAGE loading buffer containing β-mercapto-ethanol. Amounts corrected for protein content were separated by SDS-PAGE, blotted on PVDF membranes, blocked with 5% skim milk in phosphate-buffered saline, and incubated with a rat anti-human ATX antibody (mAb 4F1, 1:10000; kindly provided by J. Aoki)[15] and appropriate secondary detection reagents. Immunoreactive bands were visualized by enhanced chemoluminescence (Roche, Amersham, Buckinghamshire, UK).

Statistical analysis

Statistical differences were evaluated for two groups by Student's *t*-test and for three or more groups by one-way ANOVA with Bonferroni correction using SPSS (version 18.0). A paired t-test was used if values before and after therapy were compared. Pearson's correlation coefficient and corresponding p-values were calculated to assess the relationship between tested parameters. A multivariable test score was constructed from a logistic regression model with disease status as the dependent and ATX as the independent variable. Test performance was then assessed by calculating c-statistic (area under the receiver operating characteristic ROC). All data are expressed as means ± standard deviations (SD).

Results

Increased ATX activity is specific for pruritus of cholestasis

Compared to healthy controls, ATX activity was slightly, but significantly increased in patients with atopic dermatitis and Hodgkin lymphoma and strongly in patients with cholestatic liver diseases (Figure 1A). However, the strong elevation in ATX activity seen in cholestatic patients with pruritus compared to non-pruritic cholestatic controls was not observed in age- and gender-matched cohorts of Hodgkin's lymphoma and uremia with vs. without pruritus (Figure 1A, Supplementary Tables 1–3). Since all patients with atopic dermatitis suffer from itch, this comparison could not be made for this disease group. Strongly increased ATX activity appears therefore specific for pruritus of cholestasis.

Our cohort of patients with chronic liver diseases suffering from pruritus consisted of primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), benign recurrent intrahepatic cholestasis (BRIC), progressive familial intrahepatic cholestasis (PFIC), chronic viral hepatitis C infection (HCV), cholangiocarcinoma (CCC), hepatic sarcoidosis, liver cirrhosis, and drug- or toxin-induced intrahepatic cholestasis (Figure 1B). Irrespective of the underlying cause of cholestasis, ATX activity was increased in all patients suffering from cholestatic pruritus. Enzymatic activity and itch intensity correlated linearly in this large group of patients (Supplementary Figure 1). In contrast, neither total bile salts nor FGF19 levels did show any correlation with itch intensity in our patient cohort (data not shown).

Using a cut-off level of 8.5 $nmol \cdot mL^{-1} \cdot min^{-1}$ ATX activity had a sensitivity of 71%, a specificity of 91% and a positive predictive value of 70% to diagnose pruritus due to liver disorders in comparison to atopic dermatitis, uremia or Hodgkin

lymphoma (Figure 1C). Thus, in patients with pruritus of unknown origin (PUO) or in case of coexistence of two or more potentially pruritus-inducing disorders ATX activity might be a useful diagnostic tool to identify patients suffering from a yet undiagnosed liver disorder.

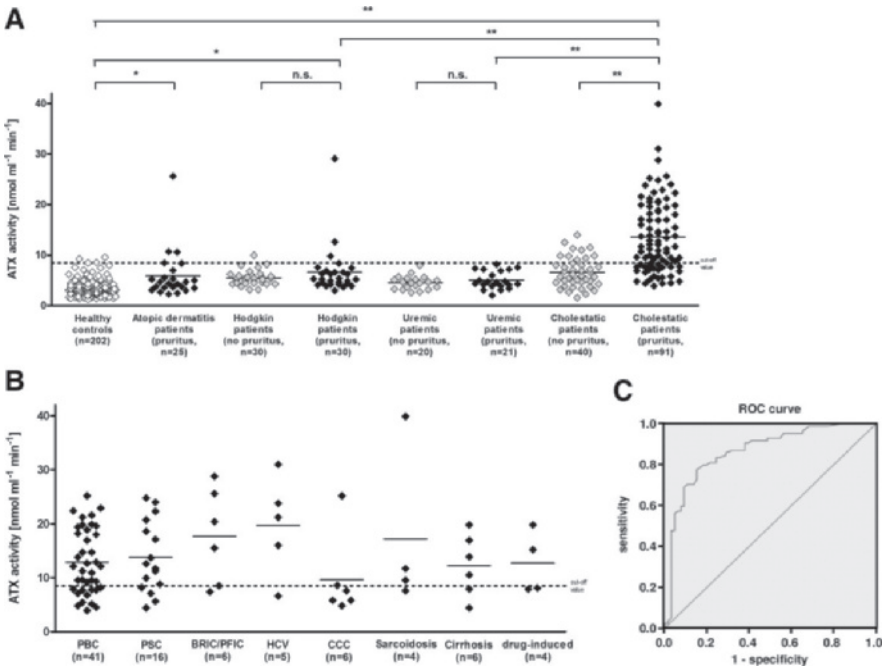

Figure 1: *Elevated serum ATX activity is specific for pruritus of cholestasis.* (A): Patients with atopic dermatitis, Hodgkin's Disease, or cholestasis had a significantly increased ATX activity compared to healthy controls. In patients with Hodgkin's disease and uremia there was no significant difference in serum ATX activity between pruritic and non-pruritic patients. In contrast, all cholestatic patients with pruritus had a significantly increased serum ATX activity compared to cholestatic patients without pruritus. *p<0.05, ***p<0.001 (ANOVA). (B): Increased ATX activity in pruritic patients with cholestatic liver disorders was observed irrespective of the underlying disease. (C): Receiver operator characteristic curve distinguishing patients with cholestatic pruritus from patients with pruritus due to atopic dermatitis, Hodgkin lymphoma or uremia. Sensitivity, specificity and positive predictive value were calculated using a cut-off value of 8.5 nmol·mL^{-1}·min^{-1}.

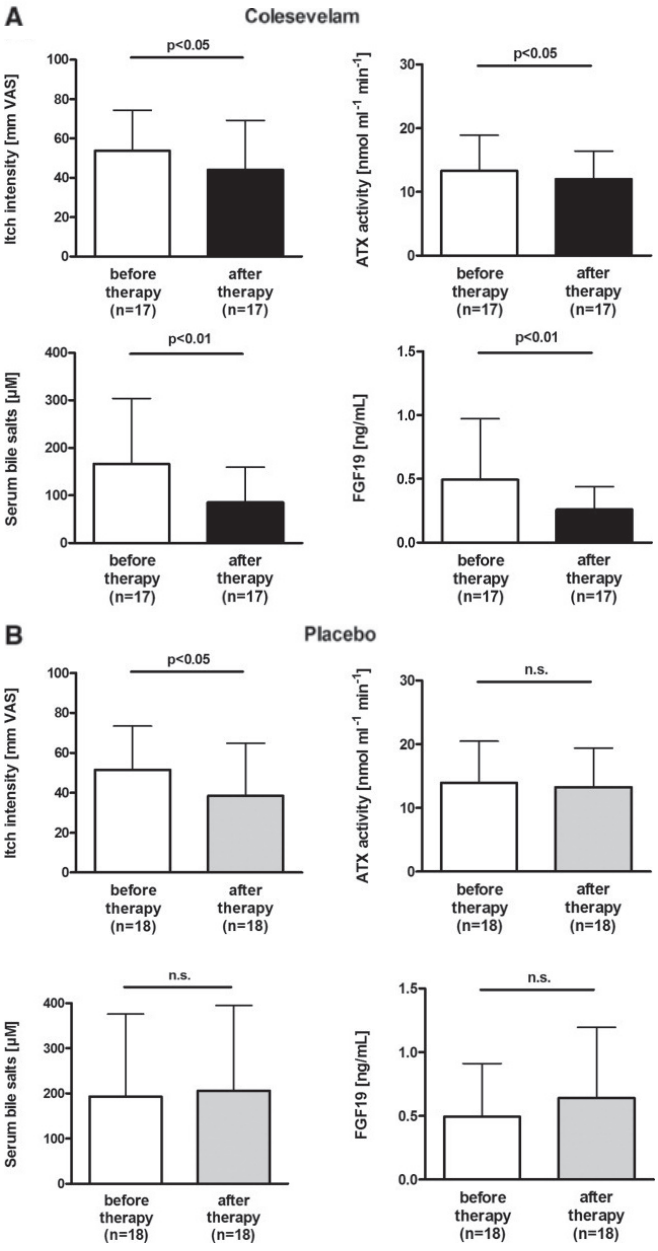

Figure 2: *Marginal effects of colesevelam on ATX activity and itch intensity.* Patients receiving either colesevelam (A) or placebo (B) were analyzed for itch intensity, ATX activity, TBS, and FGF19 levels before and after three weeks of treatment. Colesevelam effectively reduced TBS and FGF19 levels by approximately 50%, but pruritus was similarly reduced compared to the placebo group.[12] ATX activity slightly dropped in the colesevelam group.

Colesevelam marginally lowers ATX activity and itch intensity

The current guidelines for the treatment of cholestatic pruritus recommend the use of bile salt sequestrants as first line therapy.[2,4] In a recent double-blind, randomized, placebo-controlled multicenter study[12], however, colesevelam had only a mild effect in alleviating pruritus of cholestasis and was not more effective than placebo (Figure 2A&B). As expected, bile salt levels were lowered in patients taking colesevelam (-49%; $p<0.01$; Figure 2A). This alteration was physiologically relevant as shown by a similar reduction in circulating levels of FGF19, the product of the bile salt receptor FXR-stimulated FGF19 gene (-47%; $p<0.01$; Figure 2A). ATX activity was slightly reduced (-13%) in the verum group (13.3±5.6 $nmol \cdot mL^{-1} \cdot min^{-1}$ at baseline vs. 11.6±4.4 $nmol \cdot mL^{-1} \cdot min^{-1}$ after treatment, $p<0.05$; Figure 2B), whereas in the placebo group ATX, TBS and FGF19 levels all remained unchanged (Figures 2B).

Rifampicin attenuates itch intensity and ATX activity

When bile salt sequestrants are ineffective, rifampicin is recommended as second line therapy of cholestatic pruritus.[2,4] Six patients who did not experience improvement of pruritus using bile salt sequestrants were treated with 150 mg rifampicin twice daily. Itch intensity improved within two weeks of rifampicin treatment (-65%, $p<0.01$; Figure 3) which was accompanied by a concomitant significant decrease of ATX activity (-32%, $p<0.05$; Figure 3). TBS and FGF19 levels remained unaltered during this treatment (Figure 3).

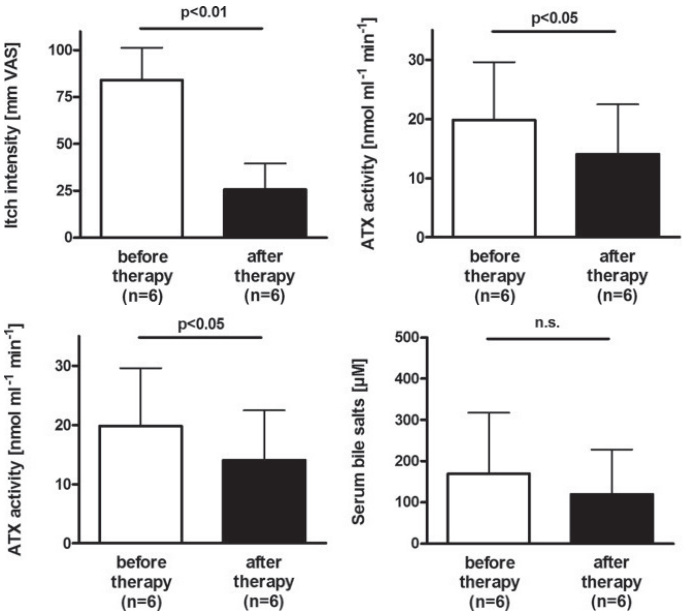

Figure 3: *Rifampicin therapy attenuated pruritus and reduced ATX activity.* Patients with cholestatic pruritus not responding to colesevelam were treated with rifampicin for two weeks. Serum samples taken before and after treatment were analyzed for itch intensity, ATX activity, TBS, and FGF19 levels. Rifampicin attenuated itch severity and reduced ATX activity whereas TBS and FGF19 levels remained unaffected.

Rifampicin does not affect ATX activity, but reduces ATX expression in human hepatoma cells in vitro

To elucidate the molecular mechanism of the antipruritic properties of rifampicin we analyzed the effects of rifampicin on ATX activity and expression *in vitro*. Rifampicin at concentrations up to 100 µmol/L did not modify ATX activity in serum (data not shown). Using HepG2 cells, however, rifampicin attenuated ATX gene expression in HepG2 cells ($p<0.01$; Figure 4A). As rifampicin exerts its transcriptional effects via the Pregnane X Receptor (PXR), we further analyzed its effect in HepG2 cells overexpressing PXR or after knock-down of PXR. In PXR-overexpressing cells rifampicin caused a stronger inhibition of ATX transcription ($p<0.02$; Figure 4B), whereas this effect was lost in HepG2 cells after knock-down of PXR using shRNA (Figure 4C). For all experiments CYP3A4 gene expression served as a positive control to verify the action of rifampicin (Supplementary Figures 4A–C). These data show that expression of autotaxin is reduced at the transcriptional level by rifampicin and that this mechanism is mediated via PXR.

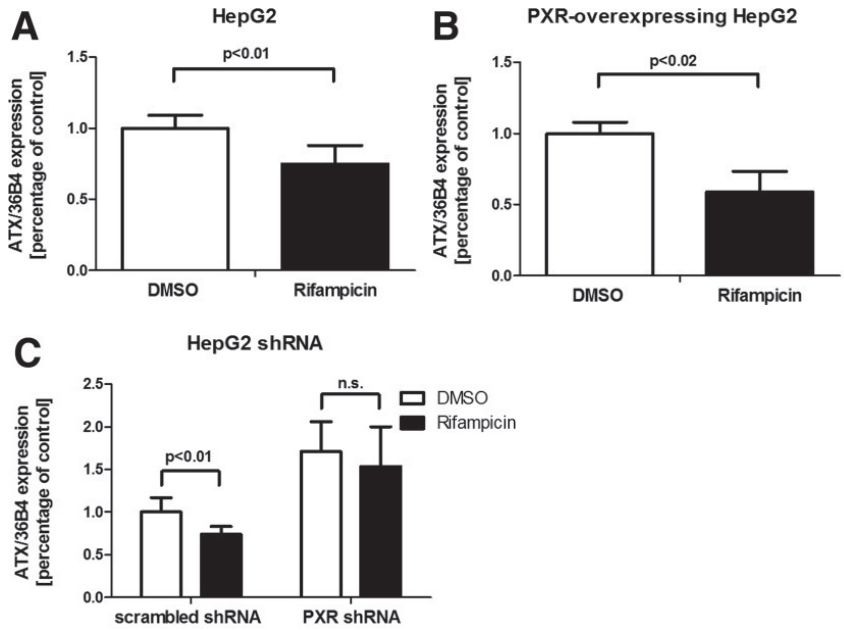

Figure 4: *Rifampicin diminished ATX mRNA expression in vitro via a PXR-dependent mechanism.* ATX mRNA expression was reduced (-25%, p<0.01; n=6) in HepG2 cells after incubation with 10 µmol/L rifampicin for 24 hours (A). This inhibitory effect of rifampicin was increased (-40%, p<0.02; n=3) in HepG2 cells overexpressing PXR (B), whereas it was lost in PXR knock-down cells, but not cells being transduced with scrambled shRNA (C).

MARS responders show diminished ATX activity in parallel with reduced itch severity

Severity of pruritus was evaluated in patients undergoing MARS therapy using VAS and a recently published itch severity score (ISS).[16] The ISS showed a strong linear correlation with VAS (r=0.92, p<0.001; Supplementary Figure 2A). Eight patients had a marked improvement in itch intensity on VAS (-63.6%; p<0.01) and ISS (-60.9%; p<0.01, Supplementary Figure 2B) after MARS therapy and were designated 'responders', whereas two 'non-responders' showed no change in severity of pruritus on VAS (-4.2%) or ISS (-2.2%) (Figure 5 A&B). A mean reduction of ATX activity of -29% (p<0.01) was seen in responders, whereas non-responders remained unchanged (Figure 5A&B, Supplementary Figure 2C). The change in ATX activity directly correlated with the reduction in ISS (r=0.71; p<0.01, Supplementary Figure 2D) and VAS (r=0.61; p<0.03, Supplementary Figure 2E). TBS concentrations and FGF19 levels (Figures 5A) dropped in responders without reaching significance, whereas an apparent increase was observed in the two

Non-Responders (Figure 5B). Neither ATX activity nor ATX protein was detectable in the albumin dialysate (Figures 5 C&D), in line with the MARS membrane pores having a Molecular Weight cut-off of 50 kD which is approximately half the size of autotaxin. Intriguingly, ATX levels returned to pre-treatment values with relapse of itching which occurred in responders between 6 weeks and 4 months. Two patients underwent a second MARS treatment upon relapse of pruritus. During the 2nd intervention pruritus improved again accompanied by a concomitant reduction of ATX activity (Figures 5E).

Nasobiliary drainage strongly reduced pruritus and ATX levels

Nasobiliary drainage effectively alleviated intractable pruritus in PBC patients not responding to standard treatment.[7] Simultaneously with the improvement of itch severity (-85%; Figure 6A), ATX serum activity dropped in these patients to approximately half of the baseline values (-50%; Figure 6A), whereas TBS initially dropped but rose back to baseline values already during nasobiliary drainage, as in part reported previously[7,8] (Figure 6A, Supplementary Figure 3A). Circulating FGF19 levels were strongly diminished one day after start of treatment, indicating effective external biliary drainage (-50%; Figure 6A). Our observation that ATX activity closely correlated with improved itch intensity in patients undergoing nasobiliary drainage[8] is strengthened by the reproducibility in one PBC patient who underwent this procedure twice (Figure 6B). As neither ATX protein nor ATX activity were detected in bile,[8] the reduction in circulating ATX levels cannot be explained by biliary clearance of autotaxin.

In summary, itch severity and ATX serum activity were barely reduced by colesevelam, moderately diminished by rifampicin and MARS therapy and markedly diminished by nasobiliary drainage. The improvement of pruritus showed a linear correlation with the reduction in ATX serum activity for all treatment groups (Figure 7A), whereas no correlation was found for the change in serum TBS concentrations (Figure 7B).

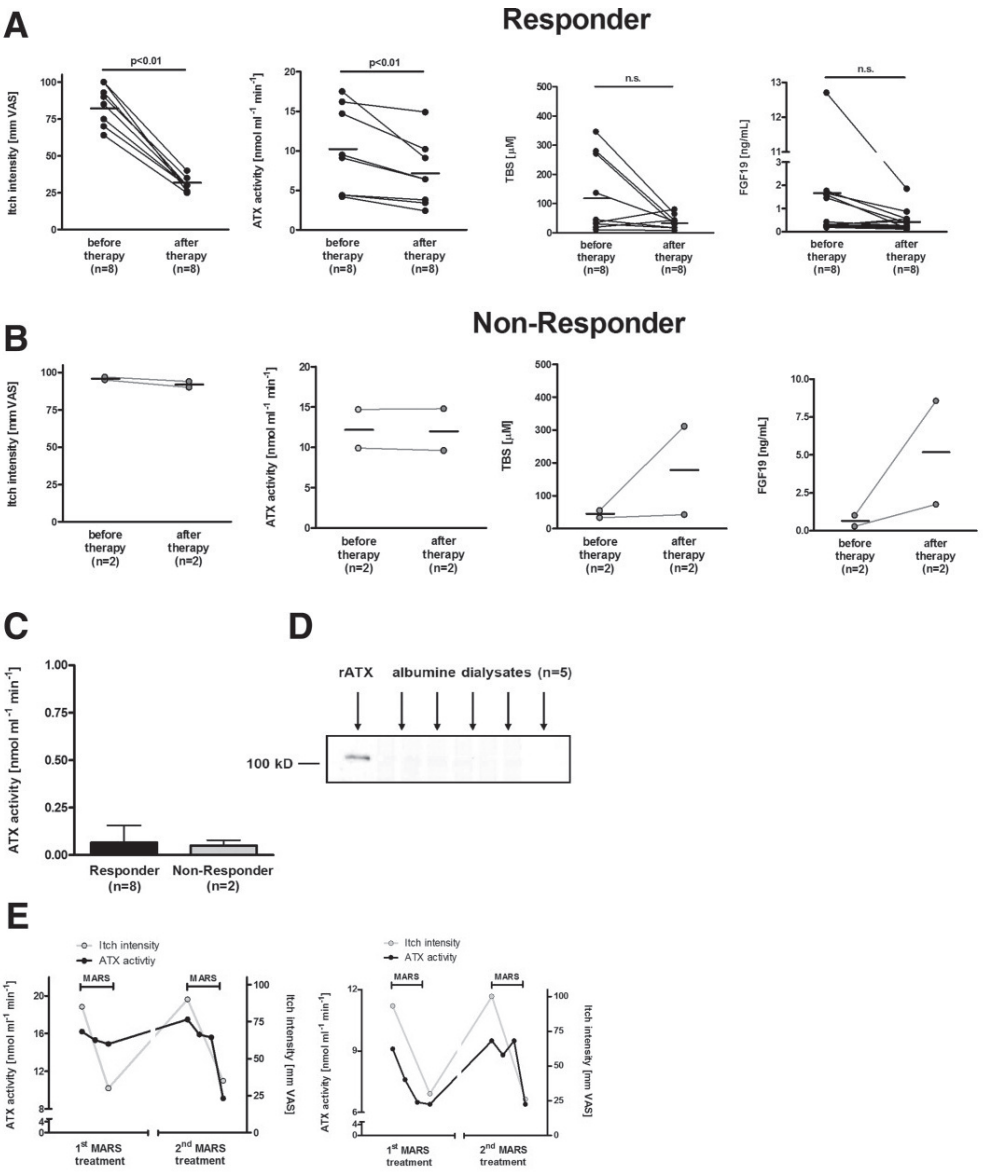

Figure 5: *MARS therapy diminished itch intensity in most patients with refractory pruritus and caused a concomitant reduction in ATX activity.* Patients undergoing MARS were divided into responders (A) and non-responders (B) on the basis of VAS and itch severity score (Supplementary Figure 2). Pruritus and ATX dropped significantly in responders and remained unchanged in non-responders. Neither ATX activity (C) nor ATX protein (D) could be detected in the albumin dialysate of MARS patients. Panel E represent individual courses of itch intensity and ATX activity in two patients undergoing sequential MARS therapy to treat otherwise intractable pruritus. Improvement of pruritus correlated with the reduction of ATX levels. Upon reoccurrence of pruritus ATX levels had returned to pre-treatment values.

Discussion

In the present study we demonstrate that elevated serum ATX activity has a high specificity for pruritus of cholestasis and might therefore serve as a diagnostic marker in cases of pruritus of unknown origin (PUO) or multiple underlying diseases. A strong correlation between ATX activity and efficacy of pruritus treatment further strengthens the role of ATX in the pathogenesis of cholestatic pruritus. The beneficial effect of rifampicin on cholestatic pruritus may be explained at least in part by PXR-dependent inhibition of ATX expression as observed *in vitro*.

LPA is generated by ATX and serum levels of both correlate with the occurrence of cholestatic itch.[8] Quantification of LPA can be artificially altered after blood sampling through release by platelets and levels may vary dependent on processing and storage.[17] To circumvent these potential artifacts, we analyzed ATX activity as a reliable parameter for LPA formation. The source of the increased circulating ATX levels remains elusive but might either be due to reduced clearance, increased expression, or a combination of both. A reduced clearance may result from decreased uptake by liver sinusoidal endothelial cells.[18] Despite their completely different mechanisms of action rifampicin, MARS treatment, and nasobiliary drainage all markedly reduced ATX serum levels, whereas ATX protein was neither directly drained into bile[8] nor removed in the albumin dialysate. We hypothesize that a factor which is capable of increasing ATX expression (or reducing its clearance) is removed by these treatments. This yet to be identified factor might accumulate in the circulation during cholestasis and might be metabolized in the liver and/or the gut, followed by biliary secretion and reabsorption via enterohepatic circulation. The different therapeutic approaches might intervene at different stages in this cycle.

Colesevelam binds various amphiphilic substances in the gut lumen and was believed to effectively improve pruritus in cholestatic patients. The binding capacity of colesevelam for the ATX-inducing factor might be minimal as opposed to that for bile salts, which is underlined by only a small, though significant, decrease in ATX activity. As cholestyramine has been reported in uncontrolled trials to attenuate pruritus, it might be that cholestyramine could bind the ATX-inducing factor better than colesevelam which was not superior to placebo in diminishing pruritus.[12]

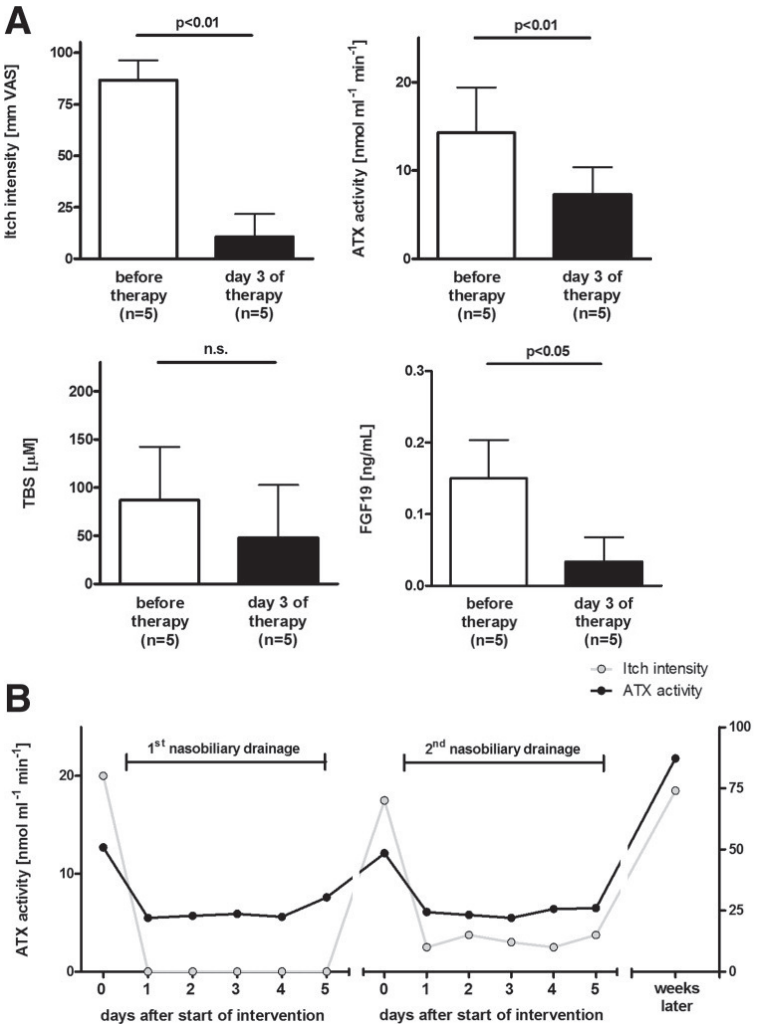

Figure 6: *Refractory pruritus and ATX activity were strongly diminished by nasobiliary drainage.* Panel A: Treatment significantly reduced pruritus and ATX activity, whereas TBS were hardly changed. FGF19 levels dropped significantly indicating efficacy of the drainage procedure. Panel B illustrates ATX activity and itch intensity in one patient undergoing successive treatment with nasobiliary drainage.

Rifampicin alleviates pruritus in cholestasis by so far unknown molecular mechanisms. Our *in vitro* data suggest that the antipruritic action of rifampicin in cholestasis can be explained at least in part by transcriptional inhibition of ATX expression in a PXR-dependent fashion. This may explain why rifampicin is effective in pruritus of cholestasis, but not in pruritus of other origin such as

uremia, Hodgkin's disease or atopic dermatitis where systemic ATX does not play a major pathogenetic role. One could speculate that, in addition, rifampicin may reduce an ATX-inducing factor by modulation of PXR-regulated genes involved in hepatic and intestinal detoxification and secretion and/or by alteration of the gut flora.[4] Determination of plasma ATX activity and LPA levels in animal models of cholestasis in the presence and absence of an effective PXR agonist may teach us more about ATX and LPA turnover under these pathological conditions in the future. Alternatively, one has to consider that rifampicin might exert antipruritic effects, at least in part, by PXR-independent mechanisms. An experimental approach to test this option could be to compare the scratch response of mice towards injection of LPA with or without prior administration of rifampicin - a PXR agonist in men, but not in mice.

MARS therapy removes countless undefined substances from the circulation[5] possibly including the ATX-inducing factor. Nasobiliary drainage removes secreted bile from the body and thereby possibly also removes the ATX-inducing factor from the enterohepatic circulation. Further *in vitro* analyses in cell culture systems of bile or albumin dialysates of pruritic patients undergoing nasobiliary drainage or MARS treatment, respectively, could possibly help to identify the ATX inducing factor in cholestatic pruritus.

It is of note that in as much as 10–35% of patients presenting with chronic generalized pruritus an internal disease can be determined as underlying cause.[19] Despite extensive diagnostic examination, the cause of itching could not be identified in 8–20% of patients with generalized pruritus.[20-22] Eisendle et al. reported in a study with 117 patients with pruritus of unknown origin that almost 30% of these patients had elevated TBS concentrations without any evidence for liver disease.[22] Identifying the underlying disease causing pruritus apparently is a clinical challenge and diagnostic parameters are warranted to make a differential diagnosis. ATX may represent such a novel marker for pruritus of cholestasis. In this study an increased enzymatic activity above 8.5 $nmol \cdot mL^{-1} \cdot min^{-1}$ had a positive predictive value of 70% in differentiating cholestatic pruritus from pruritus associated with atopic dermatitis, uremia and Hodgkin lymphoma. Determination of ATX serum activity in PUO or, more importantly, in cases of coexistence of two or more potentially pruritus-inducing disorders might help clinicians in choosing a targeted therapeutic regimen.

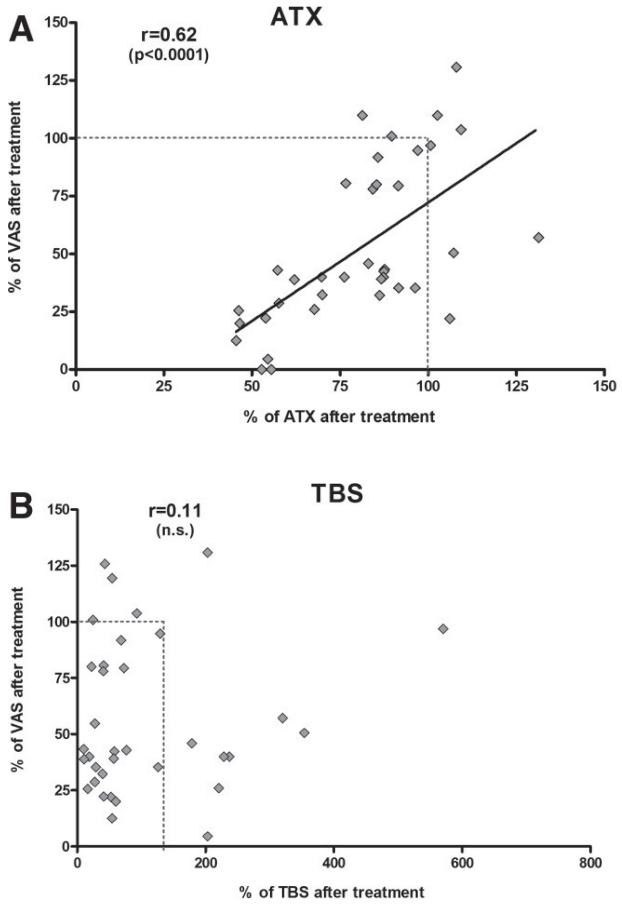

Figure 7: *Overview of therapeutic interventions in patients with chronic cholestasis suffering from pruritus.* Effect on itch intensity and ATX activity is shown for colesevelam, RMP, MARS, and nasobiliary drainage. Individual changes of itch intensity and ATX activity (A), but not TBS (B), after therapy (related to the pretreatment value) showed a linear correlation (Pearson's correlation coefficient: r¼ 0.62; P<0.0001). Dashed linerepresents no change in itch intensity and ATX activity, respectively.

Slightly increased serum ATX activities were observed in patients with atopic dermatitis and Hodgkin lymphoma compared to healthy controls in our cohort. A local overproduction of ATX with only marginal increases in the systemic circulation could be a conceivable mechanism causing itch perception in these patients. In line with our results slightly enhanced ATX levels have been reported in a small cohort of 11 Hodgkin lymphoma patients compared to healthy controls.[23] In relation

to uremia it has been reported that patients with renal failure have three-fold elevated circulating LPA levels compared to control subjects.[24] More recently, this was confirmed in a rat model of unilateral urethral obstruction.[25] Strikingly, in this study the elevated plasma LPA was accompanied by increased autotaxin activity in renal effluent rather than in plasma. It could be hypothesized that in renal failure autotaxin is primarily secreted into primary urine but its product LPA may also end up in the plasma. Hence, this leaves open the possibility that LPA plays a role in itch perception also in atopic dermatitis, Hodgkin's disease and uremia.

Several experimental and clinical observations favored increased levels of bile salts as causative pruritogens in hepatobiliary disorders in the past.[26] However, no correlation between the level of any naturally occurring bile salt in the circulation or skin and severity of pruritus could be proven.[26] In addition, several observations in the present study argue further against a direct causal role of bile salts in pruritus: *(i)* colesevelam halved TBS levels without being more effective than placebo regarding improvement of itch intensity; *(ii)* rifampicin or MARS therapy did not significantly reduce bile salt levels, yet strongly diminished itch severity; *(iii)* in patients undergoing nasobiliary drainage TBS levels dropped initially but returned to baseline values during the treatment long before pruritus re-occurred, and *(iv)* the lack of correlation between TBS concentrations and itch perception.

In our patient cohorts, markedly elevated ATX activity was specific for pruritus of cholestasis. Thus, ATX might represent a useful diagnostic tool for those cases in whom chronic pruritus remains unclassified. In addition, our study provides further clinical and experimental evidence that ATX inhibitors and LPA receptor blockers may have potential as future therapeutic agents to effectively treat pruritus in cholestatic liver disorders.

Acknowledgements

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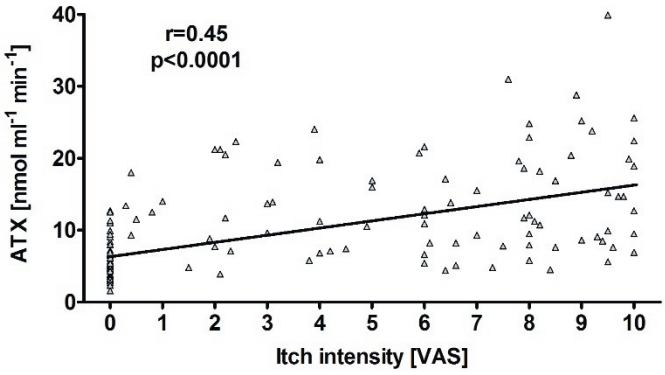
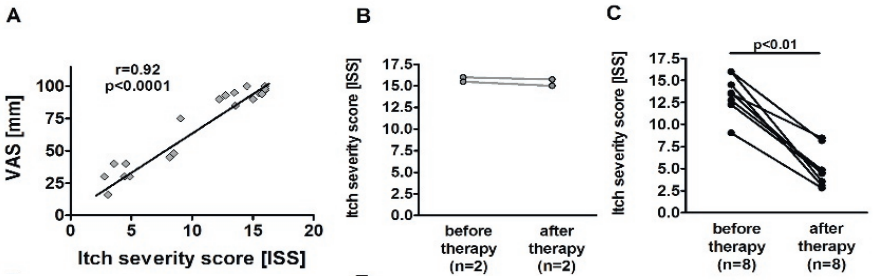
References

1. Ikoma A, Steinhoff M, Stander S, Yosipovitch G, Schmelz M. The neurobiology of itch. Nat Rev Neurosci. 2006;7:535-54
2. Kremer AE, Beuers U, Oude-Elferink RP, Pusl T. Pathogenesis and treatment of pruritus in cholestasis. Drugs 2008;68:2163-2182.
3. Beuers U. Drug insight: Mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol 2006;3:318-328.
4. EASL Clinical Practice Guidelines: Management of cholestatic liver diseases. J Hepatol 2009;51:237-267.
5. Pares A, Herrera M, Aviles J, Sanz M, Mas A. Treatment of resistant pruritus from cholestasis with albumin dialysis: combined analysis of patients from three centers. J Hepatol 2010;53:307-312.
6. Stapelbroek JM, van Erpecum KJ, Klomp LW, Venneman NG, Schwartz TP, van Berge Henegouwen GP, et al. Nasobiliary drainage induces long-lasting remission in benign recurrent intrahepatic cholestasis. Hepatology. 2006;43:51-53.
7. Beuers U, Gerken G, Pusl T. Biliary drainage transiently relieves intractable pruritus in primary biliary cirrhosis. Hepatology 2006;44:280-281.
8. Kremer AE, Martens JJ, Kulik W, Rueff F, Kuiper EM, van Buuren HR, et al. Lysophosphatidic acid is a potential mediator of cholestatic pruritus. Gastroenterology 2010;139:1008-1018.
9. Nakanaga K, Hama K, Aoki J. Autotaxin--an LPA producing enzyme with diverse functions. J Biochem 2010;148:13-24.
10. van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA, Pradere JP, et al. Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Mol Cell Biol 2006;26:5015-5022.
11. Mills GB, Moolenaar WH. The emerging role of lysophosphatidic acid in cancer. Nat Rev Cancer 2003;3:582-591.
12. Kuiper EM, van Erpecum KJ, Beuers U, Hansen BE, Thio HB, de Man RA, et al. The potent bile acid sequestrant colesevelam is not effective in cholestatic pruritus: Results of a double-blind, randomized, placebo-controlled trial. Hepatology 2010;52:1334-1340.
13. Nibourg GA, Huisman MT, van der Hoeven TV, van Gulik TM, Chamuleau RA, Hoekstra R. Stable overexpression of pregnane X receptor in HepG2 cells increases its potential for bioartificial liver application. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society 2010;16:1075-1085.
14. Schaap FG, van der Gaag NA, Gouma DJ, Jansen PL. High expression of the bile salt-homeostatic hormone fibroblast growth factor 19 in the liver of patients with extrahepatic cholestasis. Hepatology 2009;49:1228-1235.
15. Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, et al. Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. J Biol Chem 2006;281:25822-25830.
16. Leckie P, Tritto G, Proven A, Cheshire L, Mookerjee RP, Jones DE, et al. "Outpatient" albumin dialysis for intractable itch in chronic cholestatic liver disease patients is a feasible, safe and efficacious alternative to liver transplantation Hepatology 2009;50:376A.

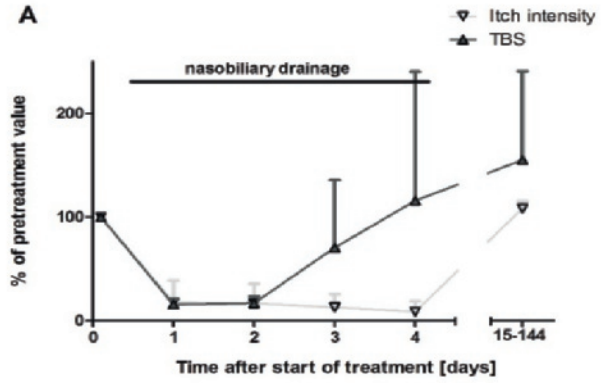
17. Nakamura K, Ohkawa R, Okubo S, Tozuka M, Okada M, Aoki S, et al. Measurement of lysophospholipase D/autotaxin activity in human serum samples. Clin Biochem 2007;40:274-277.
18. Jansen S, Andries M, Vekemans K, Vanbilloen H, Verbruggen A, Bollen M. Rapid clearance of the circulating metastatic factor autotaxin by the scavenger receptors of liver sinusoidal endothelial cells. Cancer Lett 2009;284:216-221.
19. Weisshaar E, Dalgard F. Epidemiology of itch: adding to the burden of skin morbidity. Acta Derm Venereol 2009;89:339-350.
20. Zirwas MJ, Seraly MP. Pruritus of unknown origin: a retrospective study. J Am Acad Dermatol 2001;45:892-896.
21. Sommer F, Hensen P, Bockenholt B, Metze D, Luger TA, Stander S. Underlying diseases and co-factors in patients with severe chronic pruritus: a 3-year retrospective study. Acta Derm Venereol 2007;87:510-516.
22. Eisendle K, Muller H, Ortner E, Talasz H, Graziadei I, Vogel W, et al. Pruritus of unknown origin and elevated total serum bile acid levels in patients without clinically apparent liver disease. J Gastroenterol Hepatol 2011;26:716-721.
23. Masuda A, Nakamura K, Izutsu K, Igarashi K, Ohkawa R, Jona M, et al. Serum autotaxin measurement in haematological malignancies: a promising marker for follicular lymphoma. Br J Haematol 2008;143:60-70.
24. Sasagawa T, Suzuki K, Shiota T, Kondo T, Okita M. The significance of plasma lysophospholipids in patients with renal failure on hemodialysis. Journal of nutritional science and vitaminology 1998;44:809-818.
25. Tsutsumi T, Adachi M, Nikawadori M, Morishige J, Tokumura A. Presence of bioactive lysophosphatidic acid in renal effluent of rats with unilateral ureteral obstruction. Life sciences 2011;89:195-203.
26. Kremer AE, Oude Elferink RP, Beuers U. Pathophysiology and current management of pruritus in liver disease. Clin Res Hepatol Gastroenterol 2011;35:89-97.



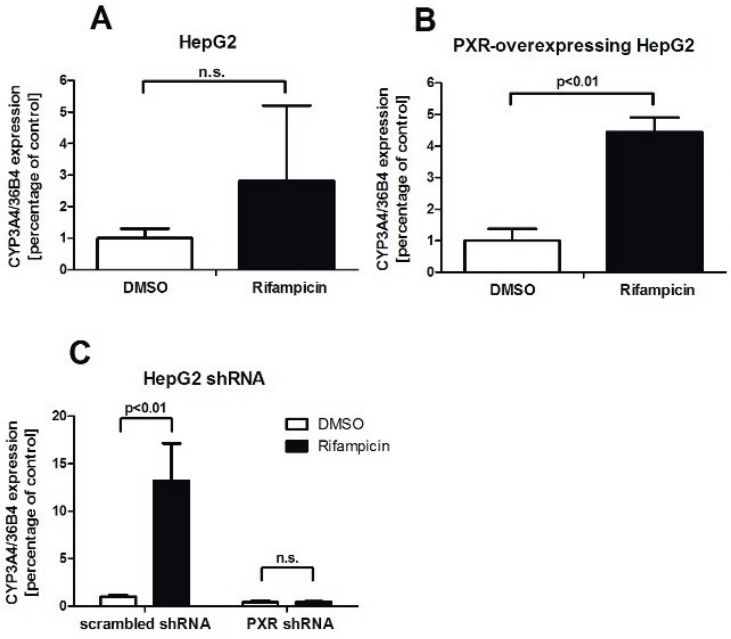
Supplementary Figure 1: ATX activity showed a linear correlation with the itch intensity represented on a VAS ranging from 0 (no pruritus) to 100 (unbearable pruritus). Pearson's correlation coefficient: r=0.45, p<0.0001.



Supplementary Figure 2: (A): Itch severity score (ISS) showed a linear correlation with the VAS in patients undergoing MARS therapy (Pearson's correlation coefficient: r=0.92, p<0.0001). (B): Improvement in ISS in patients responding (Responders) to MARS therapy. (C): No change in ISS was observed in Non-Responders. (D/E): Changes of itch intensity (ISS and VAS) and ATX activity after therapy (related to the pre-treatment value) showed a linear correlation (Pearson's correlation coefficient: ISS: r=0.71, p<0.01, VAS: r=0.61, p<0.03)



Supplementary Figure 3: Change in total serum bile salt (TBS) levels and itch intensity during the course of nasobiliary drainage. After an initial drop on the first and second day of the intervention, TBS rose back to baseline values long before pruritus re-occurred in these patients.



Supplementary Figure 4: Rifampicin induced CYP3A4 in a PXR-dependent manner. CPY3A4 gene expression tended to be increased (n=6; not significant) after incubation with 10 µM rifampicin for 24 hours (A). This induction was increased (p<0.01; n=3) in HepG2 cells overexpressing PXR (B), whereas it vanished in PXR knock-down cells, but not cells being transduced with scrambled shRNA (C).

Supplementary Table 1: Characteristics of cholestatic patients without and with pruritus.

	Patients without pruritus (n=40)	Patients with pruritus (n=91)	P-value
Male/Female	10/30	35/56	0.13
Age (yrs)	54.8±18.0	51.5±12.8	0.36
Disease (PBC/PSC/other)	27/2/11	41/17/33	0.07
AP (IU/L)	289.7±203.8	321.4±239.2	0.54
γGT (IU/L)	160.6±191.4	187.2±233.0	0.61
Bilirubin (mg/dL)	3.1±5.5	3.7±6.3	0.65
TBS (µmol/L)	46.9±60.1	135.9±122.9	0.01
Albumin (g/dL)	4.4±1.1	4.6±1.2	0.48
ALT (IU/L)	71.9±95.0	72.6±55.3	0.96
AST (IU/L)	73.7±75.5	118.6±150.9	0.15
CRP (mg/dL)	0.5±0.3	0.4±0.5	0.87
ATX (nmol ml-1 min-1)	6.6±2.9	13.6±7.2	< 0.001

Supplementary Table 1: Characteristics and serum chemistry of cholestatic patients with and without pruritus. All values are expressed as means ± SD. P-values are for comparison between the subgroups of cholestatic patients with and without pruritus. Abbreviations: AP = alkaline phosphatase, γGT = γ-glutamyltransferase, TBS = total serum bile salts, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRP = C-reactive protein.

Supplementary table 2: Characteristics of Hodgkin lymphoma patients without and with pruritus.

	Patients without pruritus (n=29)	Patients with pruritus (n=30)	P-value
Male/Female	18/11	13/17	0.16
Age (yrs)	39.0 ± 13.0	32.5 ± 10.3	0.13
Stage of disease early/intermittent/advanced	16/13/0	4/19/7	0.01
ESR (mm/h)	26.7 ± 26.9	39.2 ± 24.2	0.07
Erythrocytes (109/L)	4.9 ± 0.5	4.7 ± 0.4	0.23
Haemoglobin (g/dL)	14.1 ± 1.5	12.7 ± 1.4	0.02
Leucocytes (109/L)	8.2 ± 2.5	11.7 ± 4.1	0.01
Albumin (g/dL)	4.3 ± 0.6	4.1 ± 0.9	0.22
TBS (µmol/L)	5.2 ± 5.9	5.6 ± 4.1	0.78
ATX (nmol ml-1 min-1)	5.5 ± 1.5	6.7 ± 4.9	0.23

Supplementary Table 2: Characteristics and serum chemistry of Hodgkin lymphoma patients with and without pruritus. All values are expressed as means ± SD. P-values are for comparison between the subgroups of patients with and without pruritus. Abbreviations: ESR = erythrocyte sedimentation rate, TBS = total serum bile salts.

Supplementary Table 3: Characteristics of uremic patients without and with pruritus.

	Patients without pruritus (n=20)	Patients with pruritus (n=21)	P-value
Male/Female	9/11	14/7	0.22
Age (yrs)	67.9 ± 11.8	63.5 ± 17.1	0.34
Time of dialysis (months)	53.8 ± 34.2	36.3 ± 41.6	0.14
Calcium	2.2 ± 0.2	2.1 ± 0.5	0.57
Phosphat	1.6 ± 0.4	1.7 ± 0.7	0.49
PTH	383.2 ± 400.6	260.6 ± 276.6	0.26
1α-OH-Vit D3	0.6 ± 0.5	0.7 ± 0.5	0.84
25-OH-Vit D3	14.5 ± 8.3	15.5 ± 8.5	0.70
Albumin (g/dL)	3.7 ± 0.5	3.6 ± 0.9	0.59
CRP (mg/dL)	5.6 ± 7.9	8.7 ± 8.4	0.24
TBS (µmol/L)	4.9 ± 3.8	4.2 ± 3.2	0.83
ATX (nmol mL-1 min-1)	4.6 ± 1.3	5.1 ± 1.7	0.33

Supplementary Table 3: Characteristics and serum chemistry of uremic patients with and without pruritus. All values are expressed as means ± SD. P-values are for comparison between the subgroups of patients with and without pruritus. Abbreviations: PTH = parathyroid hormone, CRP = C-reactive protein, TBS = serum bile salts.

Supplementary Table 4: Characteristics of Responders and Non-responders of MARS therapy

	Responder (N=8)		Non-Responder (N=2)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Male/Female	7/1		1/1	
Disease (PBC/PSC/Other)	5/1/2		1/1/0	
MELD score	10.5 ± 6.6		6.0	
AP (IU/L)	505.1 ± 168.4	427.7 ± 111.2	319.5	251.5
ALT (IU/L)	103.9 ± 78.1	85.0 ± 52.2	76.5	64.5
Bilirubin (µmol/L)	28.6 ± 26.7	21.7 ± 16.9	22.5	28.0
TBS (µmol/L)	133.1 ± 140.4	43.6 ± 13.3	43.6	176.7
Albumin (g/dL)	3.9 ± 0.5	3.9 ± 0.6	4.4	4.3
Creatinine (mg/dL)	75.0 ± 34.4	61.3 ± 14.0	54.0	42.0
INR (IU/L)	0.96 ± 0.05	0.94 ± 0.03	1.00	1.06
Platelets (109/L)	223.3 ± 87.4	148.4 ± 52.7	306.0	229.0
Haemoglobin (g/L)	11.5 ± 1.6	10.0 ± 1.1	13.3	12.1
ATX (nmol mL-1 min-1)	10.8 ± 6.5	7.8 ± 4.0	12.3	12.2

Supplementary Table 4: Characteristics and serum chemistry of cholestatic patients undergoing MARS therapy. All values are expressed as means ± SD. P-values are for comparison between the subgroups of patients with and without pruritus. Abbreviations: AP = alkaline phosphatase, ALT = alanine aminotransferase, TBS = serum bile salts, INR = prothrombin time.

9

SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

Part 1: Severe unconjugated hyperbilirubinemia

In 1952 Dr John Fielding Crigler and Dr Victor Assad Najjar described six patients with non-haemolytic unconjugated hyperbilirubinemia which all died at young age[1]. After four decades of research it was found that reduced or complete lack of UGT1A1 activity, the hepatic and intestinal enzyme responsible for bilirubin glucuronidation, is the cause for Crigler-Najjar syndrome[2]. The introduction of phototherapy in the late 1950's and 1960's[3, 4] allowed patients with Crigler-Najjar syndrome (CNS) reaching adulthood without any neurological damage[5, 6]. However, the long exposure time of up to 12-14 hrs/day for patients with the severe type I Crigler-Najjar syndrome[7], gradual loss of efficacy over time and the risk of life-threatening spikes in serum unconjugated bilirubin levels due to e.g. sepsis or haemolysis[8] makes phototherapy far from being the ideal treatment. Orthotopic and auxiliary liver transplantation is currently the only available curative therapeutic option for CNS, but this comes with gross limitations such as the need for life-long immunosuppression (and related side-effects), procedural risks (including mortality) and a shortage of liver donors[9]. Patients with residual UGT1A1 activity, the less severe type II Crigler-Najjar syndrome, can be treated with phenobarbital which reduces serum bilirubin levels by >30%, but this can cause common adverse effects such as dizziness and drowsiness[10]. Experimental treatments such as donor hepatocyte transplantation did only show transient partial reductions of serum bilirubin, with gradual loss of efficacy over several months[11, 12]. In the view of the limitations of current available therapies, we looked in the first part of this thesis into new therapeutic options for CNS.

The first clinical trials applying viral gene therapy to correct monogenetic diseases in young patients with severe combined immunodeficiency (SCID) showed very promising results, with long-term immunological reconstitution in the majority of the patients. This work showed the way for the development of viral gene therapy for other (monogenetic) diseases such as Crigler-Najjar syndrome.

However, the death of one patient due to induction of a massive innate immune response against the used adenovirus[13], as well to the development of haematological malignancies in some young patients treated with an integrating murine leukemia viral vector[14] severely slowed down further development. These events emphasise the importance of developing the most suitable viral vector for a specific disease, patient and administration at the most appropriate time and way.

Because Crigler-Najjar syndrome is a monogenetic disease which only needs correction in one organ and has a clear therapeutic endpoint (lowering serum unconjugated bilirubin levels), it makes an excellent candidate for *in vivo* viral gene therapy. As introduced in chapter 1, different types of viral vectors have been used for the treatment of a variety of diseases. Adeno-associated virus (AAV) is a non-pathogenic virus, which depends on a helper virus such as adeno or herpes virus for replication, does not integrate in the host genome (remains episomal), has several serotypes which are able to transduce hepatocytes *in vivo* resulting in long-term stable expression of a target gene, and induces only relatively mild immune responses upon administration[15]. A recent clinical trial showed the safety and long-term therapeutic expression of factor IX in haemophilia B patients via the use of AAV8 mediated gene transfer[16]. Also an AAV viral product (Glybera) was the first gene therapy vector registered by the European Medicines Agency for the treatment of lipoprotein lipase deficiency[17]. It is therefore our understanding that AAV is the most appropriate candidate as delivery system for the human UGT1A1 gene to the liver for the treatment of Crigler-Najjar syndrome. Indeed already in 2006 our group showed that a single portal vein injection of an AAV vector encoding the human UGT1A1 gene provided life-long therapeutic correction of serum unconjugated bilirubin levels in the Gunn rat, the rat model for CNS[18]. However, further pre-clinical studies for optimization of the vector and the promoter, modification of the immune system and testing of different administration sites (such as administration via a peripheral vein) are warranted before it can be implicated in a phase I/II clinical trial.

Optimized AAV-hUGT1A1 vectors

To enhance the therapeutic efficacy and safety of AAV vector mediated therapy we performed a systematic evaluation of a novel human UGT1A1 expression cassette with an optimization of the UGT1A1 cDNA and the HBB2 intron sequence. Increasing the efficacy of transgene expression could not only increase therapy efficacy, but would also allow the use of a lower AAV vector dose, which would reduce the risk of a vector-associated cytotoxic immune response observed in haemophilia patients treated with the highest vector dose[16]. It has previously been shown that optimization of the transgene cDNA can indeed increase the expression in hepatocytes of the secreted clothing factors VIII and IX both *in vitro* and *in vivo*[19-21]. Not only secreted proteins, but also the expression of the cytosolic expressed muscular protein such as dystrophin can be increased via codon optimization. This resulted both in an increased *in vivo* expression of dystrophin and an improved physiological outcome in treated dystrophic mice[22]. As described in **Chapter 2** we generated two new human UGT1A1 cDNA sequences using codon optimization algorithms**.** *In vitro* transfection of the human hepatoma Huh7 cell line revealed an approximately two-fold induction of UGT1A1 mRNA and protein expression levels for both codon optimized expression cassettes in comparison to the wild-type UGT1A1 expression cassette. For all constructs correct intracellular localization of the UGT1A1 protein was confirmed with co-staining of the endoplasmic reticular specific GRP-78 protein. We also showed that removal of cryptic start codons present in the HBB2 intron sequence improved the mRNA processing and stability as well as prevented the formation of alternative splicing forms *in vitro.* Correct and efficient splicing of the intron sequence is of importance not only because it enhances expression of the therapeutic protein but also because it reduces the risk of an immune response towards the proteins encoded by these splice variants[23].

AAV 2/8 vectors containing the wild-type (wtUGt1A1), codon optimized version 1 (v1UGT1A1) or version 2 (v2UGT1A1) behind a liver specific hAAT promoter and the modified HBB2 intron were tested in the Gunn rat at two different vector doses ($5*10^{10}$ vg/kg resp. $5*10^{11}$ vg/kg). Unexpectedly the cDNA with the highest codon adaptation index (v2UGT1A1), a predictor for the most optimized coding region[24], provided the lowest correction *in vivo*. Besides the highest codon adaptation index the v2UGT1A1 codon also contained the highest GC content, which is expected to result in a more stable mRNA product[25]. Thus, although the v2UGT1A1 looked very promising *in vitro*, it is not clear why the *in vivo* results

did not reflect this. The v1UGT1A1 vector had a codon adaptation index and GC content closer to the native UGT1A1 codon. In both mouse and rat models for Crigler-Najjar syndrome this v1UGT1A1 AAV vector was able to provide therapeutic levels of hepatic UGT1A1 expression and activity, which resulted in correction of total serum bilirubin levels. The unexpected difference seen *in vivo* could be due to misfolding of UGT1A1 in the endoplasmic reticulum (ER). As for most enzymes, proper folding in the ER is important for UGT1A1 activity[26]. Overexpression due to a very efficient translation could lead to misfolding of the UGT1A1 in the ER which could explain the observed low activity *in vivo*. Limiting factors in bilirubin metabolism, i.e. unconjugated bilirubin uptake by the hepatocyte, could also be an explanation for the difference of *in vitro en in vivo* results. Unfortunately, UGT1A1 activity was only indirectly determined via measurement of serum total bilirubin levels.

Because of the early age of onset, AAV-mediated gene therapy in CNS is preferably initiated at a young age and should result in life-long and stable correction of serum bilirubin levels. However, in **Chapter 2** we noticed a gradual loss of the correction of serum bilirubin levels over time in 3 of the 10 Gunn rats treated with a single dose of AAV-hUGT1A1. Loss of serum bilirubin correction correlated with the absence of vector genome copies within these livers at the end of the study. Interestingly, all three rats who lost their serum bilirubin correction were male. It was found that at the moment of vector administration (between 6-8 week of age) the livers of the male rat were still growing, while the livers of the female rats had already reached their final size. AAV integrates into the host genome at a very low frequency [27], however most of the vector DNA remains episomal[28]. It is therefore thought that the observed loss of efficacy of AAV-mediated gene transfer in the male Gunn rat is could be due to vector dilution in proliferative hepatocytes. The principle of vector dilution for both single-stranded and self-complementary AAV vectors in proliferative tissue, including the liver, is previously demonstrated by other groups after administration of AAV vectors to neonatal mice[29-31]. The extensive loss of efficacy observed upon hepatocyte division, indicates that degradation of the AAV vectors most likely contributes to the loss of correction[31]. Because of the continued liver growth in children AAV-mediated gene therapy is expected to provide a less stable correction in young patients in comparison to adults. One could therefore argue to postpone AAV-mediated gene therapy in patients with Crigler-Najjar until adolescence when the final size of the liver is reached. However, other therapies such as phototherapy should be

available to prevent irreversible brain injury to occur during childhood. To make liver directed AAV gene therapy possible in pediatric patient, other strategies such as vector re-administration, gene editing tools and promoterless integrative AAV vectors are investigated to overcome this problem. Ideally in the future there is a range of different viral vectors available (integrating or non-integrating vectors, different AAV serotypes adjusted to the patient neutralizing antibody profile, vectors containing the UGT1A1 gene, or an editing tool to repair the mutation etc) for CNS patients to choose from for a curative treatment on the day of diagnosis.

Adeno-associated viral gene therapy and the innate immune system

In vivo administration of a viral vector induces interactions with the immune system. An immune response after vector administration can be directed against the viral vector, the transgene product or both. Compared to other viral vectors such as lenti- and adenovirus, AAV vectors show only a mild pro-inflammatory profile after *in vivo* administration[32]. However, interactions between AAV and the immune system have been shown to have a significant impact on the outcome of AAV-mediated viral gene therapy therapy[33].

The first line defence against viral infections is the innate immune system. Besides their target cell (in our case the hepatocyte), AAV vectors can also be taken up by cells of the innate immune system such as macrophages and Kupffer cells within in the liver[34]. Depletion of Kupffer cells in mice resulted in a reduced innate immune response after systemic AAV administration[35]. For adenoviral vectors it was demonstrated that Kupffer cell depletion not only resulted in a reduced immune response, but also increased the efficacy of hepatocyte transduction by adenoviral vectors. Work by the group of Haisma showed that the scavenger receptor A (SR-A) is involved in adenoviral vector uptake by Kupffer cells and that blocking of this receptor with polyinosinic acid (poly[i]) increased *in vivo* adenoviral vector-mediated transgene expression in hepatocytes[36]. In serum the AAV capsid binds to the proteolytically inactive complement factor iC3b, a factor which can bind to the SR-A[37]. The scavenger receptor could therefore also be responsible for the macrophagic uptake of AAV particles. We demonstrated in **Chapter 3** that the scavenger receptor A can indeed mediate the endocytosis of AAV serotype 8 in human macrophage like cells *in vitro* and blocking of this receptor with poly[i] increased the *in vivo* efficacy of AAV-mediated viral gene therapy in the Gunn rat. Interestingly, we observed that blocking of SR-A had a stronger effect in animals

treated with the lowest viral dose. Most likely saturation of the scavenger receptor occurs in animals treated with the highest viral vector dose. Our study has added more evidence underlining the important role the innate immune system plays in the outcome of AAV mediated gene therapy to treat hepatic autosomal recessive liver diseases, and that by over saturating the system or by blocking the scavenger receptor A the efficacy of AAV *in vivo* hepatocyte transduction can be increased.

Humoral immunity response

Besides the innate immune system, the adaptive immune system also functions as a barrier to successful viral gene therapy. The humoral immunity composed of neutralizing antibodies (Nab) against AAV vectors can have a severe negative impact on the outcome of liver-directed AAV-mediated gene therapy. This was shown in the first haemophilia B trial where the AAV vector was administrated systemically[38]. This study provided the first evidence that even low titres of Nab (1:17) can completely inhibit AAV liver transduction in human subjects. Indeed previous studies in mice and non-human primates observed that low AAV Nab titres (1:5) can completely inhibit AAV liver transduction[39, 40]. In current clinical trials patients with detectable levels of Nabs are excluded from participation. Unfortunately a significant portion of the population develops humoral immunity against AAV, mostly during their childhood[41, 42]. The prevalence of Nabs against AAV2 is between 30 and 60%, which is significantly higher than the prevalence of Nabs to the other AAV serotypes. The frequency of Nabs against AAV8, the vector we plan to use in our proposed clinical trial to treat Crigler-Najjar syndrome, is around 30%[43]. In a collaboration between our group and research groups in France and Italy all patients with Crigler-Najjar syndrome are currently tested for the presence of AAV Nabs, especially Nabs against AAV8. Although the prevalence of Nabs is depending on the age, gender and geographical region of the patient, a significant percentage of all CN patients will not be eligible for AAV8 mediated gene therapy.[43] New strategies are therefore under development to ensure patients with detectable Nabs can be included in clinical trials in the future. Some of those new strategies to overcome humoral immunity to AAV are removal of the Nabs via plasmapheresis[44] or B-cell depletion with the use of the CD20 monoclonal antibody rituximab[45]. Other options that are investigated are the use of new AAV variants which are less well bound by Nabs[46] present in the circulation or to avoid systemic administration via isolated perfusion of the target organ with the viral vector[47].

Immune response against the transgene product

Successful transduction of the target tissue will ideally result in life-long expression of the transgene product. In patients with null-mutations, the newly expressed therapeutic protein can be recognized as a foreign antigen which will trigger an immune response resulting in the production of transgene-specific neutralizing antibodies or triggering a T-cell response directed against the transgene expressing cell[48, 49]. Both immune reactions can have a negative impact on the long-term outcome of AAV-mediated gene therapy.

Vector mediated transfer of the antigen presenting dendritic cells is necessary for the induction of the immune response against the transgene product. Although other viruses such as adenovirus are much more potent in transducing dendritic cells[50], recombinant AAV vectors including serotypes 1 and 2 can also transduce dendritic cells (DCs) and generate immune responses to transgene products[51]. However, other studies have demonstrated that humoral and cellular immune responses to transgene products can be avoided by the use of AAV serotype 8, this is partly because AAV8 is unable to transduce dendritic cells[52, 53]. An important and unique feature of the liver as a target organ is, that in contrast to other tissues such as the muscle, it can induce transgene-specific tolerance upon sufficiently high expression. This effect has been observed both in rodent and canine animal models and is most likely mediated by regulatory T-cells[54-56]. The formation of transgene specific antibodies can be avoided by selecting hepatotropic AAV serotypes, and by restricting transgene expression via the use of a liver-specific promoter [18].

Although we used the hepatotropic recombinant AAV serotype 8 vector with a liver specific promoter in **Chapter 2**, Gunn rats treated with this vector still developed antibodies against the human UGT1A1. However, the presence of these antibodies did not result in loss of efficacy of the therapy. Interestingly we did not see any neutralizing antibody development in treated mice, indicating that this can be species specific. Indeed it is commonly seen that immune response to a transgene product is weaker in mouse models than in other animal models[57].
The risk of an unwanted immune response against UGT1A1 in Crigler-Najjar patients will be minimized by using AAV serotype 8 with a hepatocyte specific promoter controlling the hUGT1A1 gene expression. To minimize the risk for a T-cell response to occur after AAV administration the clinical use of the corticosteroid prednison could be advocated.

New strategy to inhibit bilirubin production

Although the pre-clinical studies of AAV8 mediated gene therapy for Crigler-Najjar syndrome have shown very promising results and preparations for the first European multi-centre clinical trial are in a final stage, we also looked into other possible future strategies to treat unconjugated hyperbilirubinemia.

Current therapies are directed at eliminating the toxic unconjugated bilirubin from the body, rather than preventing its production. However, all these therapies have their limitations. Phototherapy becomes less effective over time and can take up to 12 hours in the most severely affected patients. Phenobarbital can cause side effects such as dizziness and drowsiness and can only be used in patients with Crigler-Najjar syndrome type 2. Patients who undergo a liver transplantation have to take life-long immunosuppression. Thrombosis and lethal electrolyte disorders can occur during exchange transfusions. We therefore investigated if instead of elimination of unconjugated bilirubin, preventing the production of (unconjugated) bilirubin could be a potential target in Crigler-Najjar syndrome or unconjugated hyperbilirubinemia in general.

The possibility to reduce serum bilirubin concentrations by inhibiting its production was proven when it was shown that inhibition of haem oxygenase by metalloporphyrins reduced serum bilirubin levels in neonatal jaundice in small clinical trials[58]. However, due to the side effect profile (phototoxicity, inhibition of CYP_{450} enzymes and anaemia) and the fact that inhibition of haem oxygenase induces HO expression, the long-term use of metalloporphyrins in the clinic has never been approved. In **Chapter 4** we looked if instead of inhibiting haem oxygenase, we could reduce serum bilirubin levels by inhibiting biliverdin reductase (BVRA) activity. By inhibition of BVRA the non-toxic hydrophilic compound biliverdin would be the end product of the bilirubin metabolism.

After a semi-high-throughput screen of over 1200 compounds, Disulfiram and Montelukast were selected for further testing in the Gunn rat. Unfortunately, Disulfiram turned out to be toxic in Gunn rats and only low serum Montelukast concentrations could be achieved via oral administration. To prove the concept of BVRA inhibition as therapeutic option for unconjugated hyperbilirubinemia Montelukast was administered via intraperitoneal injections to reach high serum levels. However, with even serum concentrations up to 100 µmol/L, well above the required concentration for complete *in vitro* inhibition of BVRA activity, there

was no effect on the serum bilirubin levels in the Gunn rat.

The macrophages and Kupffer cells of the reticuloendothelial system are responsible for erythrocyte degradation and are therefore most likely the site of haem, biliverdin and bilirubin production. However, BVRA is expressed ubiquitously in most tissues. A potential BVRA inhibitor should therefore have high bioavailability within all tissues. Two biliverdin reductase isoforms are known in mammals, BVRA and BVRB. Although BVRB is only expressed in the fetus[59], it is unknown if BVRB can be expressed and active if the activity of BVRA is inhibited. Finally, it can be speculated that a currently unknown enzyme can take over the function of biliverdin reduction. This is supported by the fact that bilirubin was found in two patients with a non-sense mutation in the BVRA gene[60]. Although BVRA seemed an attractive target for the treatment of severe unconjugated hyperbilirubinemia, pharmacological inhibition did not result in decreased serum bilirubin levels in the Gunn rat. Future research should focus on the development of highly potent, and selective, BVRA inhibitors, with a broad systemic bioavailability without any serious side effects.

Crigler-Najjar syndrome and the important role of the UGT1A1 promoter

In vitro data have shown that HFN1α plays a major role in the UGT1A1 expression. In **Chapter 5** we show the first clinical data which reveals the essential role of HFN1α for *in vivo* UGT1A1 activity. We report a patient who had serum bilirubin levels characteristic for CNS type II (unconjugated bilirubin levels around 300 µmol/L and conjugated bilirubin levels around 15 µmol/L) but was non-responsive to Phenobarbital therapy. UGT1A1 exon sequencing did not reveal any mutations in the UGT1A1 gene. However, analysis of the UGT1A1 promoter showed two common polymorphisms in the gtPBREM and the TATA box, and a 3 nucleotide insertion in the HFN1α binding site. We showed that the presence of the two polymorphisms results in a 50% reduction in UGT1A1 promoter activity, clinically such a reduction would result in a Gilbert syndrome[61], the benign form of CNS. However, the presence of the 3 nucleotide insertion in the HFN1α binding site resulted in a 95% reduction of UGT1A1 promoter activity, the 5% remaining activity explaining the CNS type II phenotype of our patient. Also we showed that due the disruption of the HFN1α binding site the UGT1A1 promoter could not be activated by CAR and PXR, explaining why our patient was unresponsive to Phenobarbital. This case report unravelled the important role HNF1α has in the

control of UGT1A1 gene expression and *in vivo* enzyme activity. Thus, if patients present with CNS but have no mutations in the UGT1A1 gene a further sequence analysis of the UGT1A1 promoter should be considered in these patients. This case report also clearly shows that response to Phenobarbital therapy cannot be used to distinguish between CNS type I and type II patients.

Part 2: Severe conjugated hyperbilirubinemia and pruritus

In the second part of this thesis we focused on severe cholestasis and cholestasis-associated pruritus. First, we looked at patients who developed severe and progressive conjugated hyperbilirubinemia after short-term exposure to drugs, toxins or short-term mechanical biliary obstruction. In **Chapter 6** we refer to this condition as 'persistent hepatocellular secretory failure' (PHSF). We defined PHSF by (i) serum bilirubin > 255 µmol/L (> 15mg/dL), (ii) persistent or increasing elevated serum bilirubin serum levels (> 1 week) after removal of the underlying trigger, (iii) exclusion of obstructive cholestasis by cholangiography and other imaging techniques and (iv) no evidence of liver disease before the initiating event. Currently there is no therapy available to treat PHSF. Ursodeoxycholic acid (UDCA) is the preferred drug in the treatment of cholestatic diseases, however when serum bilirubin levels exceed 170 µMol/L UDCA is regarded as ineffective[62]. In **Chapter 6** we describe thirteen consecutive patients with PHSF whom we successfully treated with the pregnane X receptor (PXR) agonist rifampicin with the intention to transcriptionally improve their strongly impaired hepatocellular secretory capacity. Serum bilirubin levels ranged between 430 and 750 µMol/l at the start of rifampicin treatment and all decreased and normalised rapidly after the start of the treatment. Duration of treatment ranged between four days and ten weeks. Although prolonged treatment with rifampicin during cholestasis has been reported to be associated with hepatotoxic adverse events in up to 12%[63], no adverse events were observed in our cohort. Thus, we described a novel treatment option for patients with severe conjugated hyperbilirubinemia of unknown origin classified as PHSF.

In vitro assays of hepatic and intestinal cell lines suggest that the positive effect of rifampicin observed in PHSF patients is, partly, due to induction of genes involved in the conjugation of bilirubin (UGT1A1), bilirubin secretion (MRP2),

and bile salt export (OSTβ) by rifampicin in a PXR-dependent manner. Although the effect of rifampicin on UGT1A1 and MRP2 has been described before[64], we were the first to demonstrate that rifampicin induces OSTβ expression in a PXR-dependent manner. It has been demonstrated that under cholestatic conditions OSTβ expression is induced by FXR after activation by bile salts[65]. Here, we showed that PXR activation by rifampicin can have an additional effect on OSTβ induction during cholestasis.

Notably, ten of thirteen patients showed normal levels of γGT levels during the course of PHSF, which is a characteristic of patients with progressive familial intrahepatic cholestasis (PFIC) and benign recurrent intrahepatic cholestasis (BRIC) type 1 and type 2. PFIC/BRIC type 1 is caused by dysfunction of the phospholipase FIC1/*ATP8B1*. PFIC/BRIC type 2 is caused by dysfunction of the hepatic bile salt export pump BSEP/*ABCB11*[66]. Sequence analysis of the *ATP8B1* and *ABCB11* gene in six of the patients revealed that one patient had a homozygous mutation in the *ATP8B1* gene (c.1982>C), a known mutation causing BRIC type 1. In two other patients a heterozygous mutation was found in *ABCB11*. The first mutation (c.890A>G) has been described before to cause PFIC type 2. The second *ABCB11* mutation, c.2809G>A, results in a Gly937Arg amino acid change. Although the second mutation has not been reported before in PFIC and BRIC type 2 patients, it has been shown before that heterozygous mutations in *ABCB11* may play a role in the development of drug induced liver injury (including oral contraceptive drugs) and intrahepatic cholestasis of pregnancy[67, 68]. All three patients developed PHSF after the use of the female hormone estradiol, and all had severe pruritus which recovered with rifampicin treatment. It has previous been described that rifampicin can indeed be successful to relieve pruritus in low γGT-PFIC patients and resolving cholestatic episodes in BRIC patients[69]. Before rifampicin can be implicated as the standard therapy for PHSF, future clinical research should aim at initiating a double-blind, randomized controlled trial (e.g. with cross-over design) to prove to efficacy and safety of rifampicin in patients with PHSF. This trial would be challenging as PHSF is apparently a very rare disorder. Also further research into the pathophysiology of PHSF beyond sequencing of the BRIC/PFIC genes and investigating PXR activation in PHSF patients would be of eminent interest.

In 2010 our group showed that serum levels of the lysophospholipase autotaxin (ATX) and its product, lysophosphatidic acid (LPA), are elevated in patients with cholestasis and that ATX and LPA could be potential mediators of cholestasis-

induced pruritus as ATX serum activity correlated with itch intensity[70]. In **Chapter 7** we demonstrate that elevated serum ATX activity is significantly higher in patients with cholestatic pruritus than in healthy controls and cholestatic patients without pruritus, and that increased serum ATX activity is specific for cholestatic itch in comparison to patients with pruritus due to other conditions such as atopic dermatitis, Hodgkin's disease and chronic kidney disease. We also show that the effectiveness of therapeutic interventions for cholestatic pruritus correlate with the reduction in serum ATX activity. The anion exchange resin colesevalam, recommended as first line treatment in the management of pruritus in cholestasis by the EASL clinical practice guidelines for the management of cholestatic liver diseases, had only a marginal effect on both itch intensity and ATX activity. In contrast, rifampicin significantly alleviated itch intensity in six cholestatic patients who did not experience any improvement under colesevelam treatment. Rifampicin treatment also significantly reduced serum ATX activity in these patients. *In vitro* assays showed that ATX expression was reduced by rifampicin in a PXR-dependent manner, which could in part explain the positive effect of rifampicin on cholestasis-induced pruritus. Alternatively, via the induction of genes involved in hepatic and intestinal detoxification, such as CYP3A4, UGT1A1 and MRP2, an unknown ATX-inducing factor could be modulated and removed from the systemic circulation. As today the gut microbiota represents a popular suspect for the cause of a wide range of diseases and rifampicin is known to influence the gut flora, we cannot exclude that alterations of the gut microbiota could play an additional part in the mechanism by which rifampicin relieves pruritus in cholestatic patients.

Two more experimental therapeutic interventions, MARS therapy and nasobiliary drainage both strongly reduced itch intensity temporarily in cholestatic patients. This relief clearly correlated with the reduction of serum autotaxin activity in these patients. Notably, neither ATX protein nor ATX activity were detected in the drained bile or the MARS dialysates, suggesting that both therapies remove an ATX inducing factor rather than removing ATX from the systemic circulation.
Although the mechanism behind cholestasis-associated pruritus is still not completely understood, autotaxin seems to have an important role in the development of cholestasis-associated pruritus, and therefore could be a target in the diagnosis and treatment.

In conclusion, in the **first** part of this thesis we show that adeno-associated virus mediated gene therapy is a viable option for the curative treatment of patients with

Crigler-Najjar syndrome. Still, a careful patient selection and consideration of the impact of the immune system during and after the therapy are warranted before the initiation of a clinical trial. We also demonstrate that biliverdin reductase is a potential new target in the treatment of severe unconjugated hyperbilirubinemia. In addition, we showed that HNF1α plays an important role in modulating UGT1A1 activity *in vivo*.

In the second **part** of the thesis we show that the potent PXR agonist rifampicin is effective and safe for the treatment of patients with persistent hepatocellular secretory failure, and that mutations in PFIC and BRIC genes could be part of the underlying cause of the development of PHSF. We also show that increased serum ATX activity is associated with cholestasis-associated pruritus, but not with other forms of pruritus and that it closely correlates with effectiveness of antipruritic treatment strategies. Notably, *in vitro* findings show that the antipruritic activity of rifampicin may be explained, at least in part, by PXR-dependent transcriptional inhibition of ATX expression.

References

1. Crigler JF, Najjar VA (1952) *Congenital familial nonhemolytic jaundice with kernicterus.* Pediatrics 10:169–180
2. Ritter JK, Yeatman MT, Ferreira P., et al (1992) *Identification of a genetic alteration in the code for bilirubin UDP-glucuronosyltransferase in the UGT1 gene complex of a Crigler- Najjar type I patient.* J Clin Invest 90:150–155
3. Cremer RJ, Perryman PW, Richards DH (1958). *Influence of light on the hyperbilirubinaemia of infants.* The Lancet 271 (7030): 1094–1097
4. Lucey J, Ferriero M, Hewitt J (1968) *Prevention of hyperbilirubinemia of prematurity by phototherapy.* Pediatrics 41(6):1047–1054
5. Van Der Veere CN, Sinaasappel M, McDonagh AF., et al (1996) *Current therapy for Crigler-Najjar syndrome type 1: report of a world registry.* Hepatology 24:311–315
6. Jansen PL (1999) *Diagnosis and management of Crigler-Najjar syndrome.* Eur J Pediatr. Dec; 158 Suppl 2:S89-94.
7. Strauss KA, Robinson DL, Vreman HJ., et al (2006) *Management of hyperbilirubinemia and prevention of kernicterus in 20 patients with Crigler-Najjar disease. Eur* J Pediatr 165:306–319
8. Sellier AL, Labrune P, Kwon., et al (2012) *Successful plasmapheresis for acute and severe unconjugated hyperbilirubinemia in a child with cirgler Najjar type I syndrome.* JIMD Rep 2:33-36
9. Muiesan P, Vergani D, Mieli-Vergani G (2007) *Liver transplantation in children.* J Hepatol 46:340–348
10. Arias IM, Gartner LM, Cohen M., et al (1969) *Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologicand genetic evidence for heterogeneity*. Am J Med 47: 395–409
11. Fox IJ, Chowdhury JR, Kaufman SS., et al (1998) *Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation.* N Engl J Med 338:1422–1426
12. Ambrosino G, Varotto S, Strom SC., et al (2005) *Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1.* Cell Transplant 14:151–157
13. Bessis N, Garcia-Cozar FJ and Boissier MC (2004) *Immune responses to gene therapy vectors: influence on vector function and effector mechanisms*. Gene Therapy 11, S10–S17.
14. Cavazza A, Moiani A, Mavilio F (2013) *Mechanisms of retroviral integration and mutagenesis.* Hum Gene Ther 24:119–131
15. Samulski RD and Muzyczka N (2014) *AAV-mediated gene therapy for research and therapeutic purposes.* Annual review of virology 1:427-451
16. Nathwani AC, Reiss UM, Tuddenham EGD et al. (2014) *Longterm safety and efficacy of factor IX gene therapy in hemophilia B.* N Engl J Med 371: 1994-2004
17. Buning H (2013*) Gene therapy enters the pharma market: the short story of a long journey.* EMBO Mol Med 5:1–3
18. Seppen J, Bakker C, de Jong B., et al (2006) *Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats.* Molecular Ther. 13(6):1085-1092
19. Nathwani AC, Gray JT, Ng CY, et al (2006) *Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver*. Blood 107:2653-2661

20. Wu Z, Sun J, Zhang T, et al (2008) *Optimization of Self-complementary AAV Vectors for Liver-directed Expression Results in SustainedCorrection of Hemophilia B at Low Vector Dose.* Molecular Ther. 16(2):280-289
21. Ward NJ, Buckley SM, Waddington SN, et al (2011) *Codon optimization of human factor VIII cDNAs leads to high-level expression.* Blood 117: 798–807.
22. Foster H, Athanasopoulos T et al (2008) *Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer.* Mol. Ther. Nov;16(11): 1825-32
23. Bullock TN and Eisenlohr LC (1996) *Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames.* J Exp med 184: 1319-1329
24. Sharp PM and Li WH (1987) *The codon adaptation index – a measure of directional synonymous codon usage bias, and its potential applications.* Nucleic Acids Res 15: 1281-1295
25. Murray EL and Schoenberg DR (2007) *A+U-rich instability elements differentially activate 5′-3′ and 3′-5′ mRNA decay.* Mol Cell Biol 27: 2791–2799
26. Rouleau M, Collin P, Bellemare J, et al (2013) *Protein-protein interactions between the bilirubin-conjugating UDPglucuronosyltransferase UGT1A1 and its shorter isoform 2 regulatory partner derived from alternative splicing.* Biochemical Journal 15: 107-114
27. Nakai H, Montini E, Fuess S, et al (2003) *AAV serotype 2 vectors preferentially integrate into active genes in mice.* Nat. Genet. 34: 297-302
28. Penaud-Budloo M, Le Guiner C, Nowrouzi A, et al (2008) *Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle.* J Virol 82: 7875-7885
29. Bortolussi G, Zentillin L, Vanikova J, et al (2014) *Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crilger-Najjar syndrome.* Hum Gene Ther 25: 844-855
30. Wang Z, Zhu T, Qiao C, et al (2005) *Adeno-associated virus serotype 8 efficiently delivers genest o muscle and heart.* Nat Biotechnol 23: 321-328
31. Cunningham SC, Dane AP, Qiao C, et al (2008) *Gene delivery to the juvenile mouse liver using AAV2/8 vectors.* Mol Ther 16: 1081-1088
32. Vandendriessche T, THorrez L, Acosta-Sanchez A, et al (2007) *Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for haemophilia B gene therapy.* J Thromb Haemost 5(1): 16-24
33. Mingozzi F and High KA (2013) *Immune responses to AAV vectors: overcoming barriers to successful gene therapy.* Blood 122(1): 23-26
34. Zaiss, AK, and Muruve, DA (2008*) Immunity to adenoassociated virus vectors in animals and humans: a continued challenge.* Gene Ther. 15: 808–816
35. Zaiss AK, Liu Q, Bowen GP, et al (200) *Differential activation of innate immune responses by adenovirus and adenoassociated virus vectors.* J. Virol. 76: 4580–4590
36. Haisma HJ, Kamps JA, Kamps GK, et al (2008) *Polyinosinic acid enhances delivery of adenovirus vectors in vivo by preventing sequestration in liver macrophages.* J. Gen. Virol. 89: 1097–1105
37. Goh JW, Tan YS, Dodds AW, et al (2010) *The class A macrophage scavenger receptor type I (SR-AI) recognizes complement iC3b and mediates NF-kappaB activation*. Protein Cell 1: 174–187
38. Manno CS, Pierce GF, Arruda VR, et al (2006*) Successful transduction of liver in hemophilia by*

AAV-factor IX and limitations imposed by the host immune response. Nat Med 12: 342–347

39. Scallen CD, Jiang H, Liu T et al (2006) *Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titres in SCID mice.* Blood 107(5) 1810-1817
40. Gao GP, Alvira MR, Wang L et al (2002) *Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy.* Proc Natl Acad Sci USA 99(18): 11854-11859
41. Boutin S, Monteilhet V, Veron P et al (2010) *Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, , 6, 8 and 9 in the healthy population: implications for gene therapy using AAV vectors.* Human Gene Ther (21(6): 704:-712
42. Li C, Narkbunnam N, Samulski RJ et al (2012) *Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia.* Gene Ther 19(3): 288-294
43. Calcedo R and Wilson JM (2013) *Humoral immune response to AAV.* Fron in Immun 4: 1-7
44. Monteilhet V, Saheb S, Boutin S et al (2011) *A 10 patient case report on the impact of plasmapheresis upon neutralizing factors against adeno-associated virus (AAV) types 1, 2, 6 and 8.* Mol Ther 19(11): 2084-2091
45. Mingozzi F, Chen Y, Edmonson SC et al (2013) *Prevalence and pharmacological modulation of humoral immunity to AAV vectors in gene transfer to synovial tissue.* Gene Ther (20)4: 417-424
46. Bartel M, Schaffer D and Buning H (2011) *Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity.* Front Microbiol 2: 204
47. Brunetti-Pierri N, Ng T, Iannitti DA et al (2006) *Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates.* Hum Gene Ther 17(4): 391-404
48. Fields PA, Arruda VR, Armstrong E et al (2001) *Risk and prevention of anti-factor IX formation in AAV-mediated gene transfer in the context of a large deletion of F9.* Mol Ther 4: 201-210
49. Mendell RJ, Cambell K, Rodino-Klapac L et al (2010) *Dystrophin immunity in Duchenne's muscular dystrophy.* NEJM 363: 1429-1437
50. Jooss K, Yang Y, Fisher KJ et al (1998) *Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers*. J Virol 72: 4212–4223
51. Veron P et al (2007) *Major subsets of human dendritic cells are efficiently transduced by self-complementary adeno-associated virus vectors 1 and 2.* J Virol 81:5385–5394
52. Vandenberghe LH, et al (2006) *Heparin binding directs activation of T cells against adeno-*associated virus serotype 2 capsid. Nat Med 12:967–971
53. Yuanqing Lu and Sihong Song(2009) *Distinct immune responses to transgene products from rAAV1 and rAAV8 vectors*. PNAS 106(40): 17158–17162
54. Mingozzi F, Liu YL, Dobrzynski E et al (2003) *Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer.* J Clin Invest 111(9):1347–1356
55. Martino AT, Nayak S, Hoffman BE et al (2009) Tolerance induction to cytoplasmic beta-galactosidase by hepatic AAV gene transfer: implications for antigen presentation and immunotoxicity. PLoS ONE 4(8): e6376
56. Cao O, Dobrzynski E, Wang L et al (2007) *Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer.* Blood 110: 1132- 1140
57. Arruda VR et al. (2004) *Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1.* Blood 103: 85–92

58. Kappas A and Drummond GS (1985) *Synthetic metalloporphyrins: a class of compounds of pharmacological interest.* Bioessays 3(6):256-9
59. Baranano DE, Rao M, Ferris CD et al (2002) *Biliverdin reductase: a major physiologic cytoprotectant.* PNAS 99(25): 16093-16098
60. Nytofte NS, Serrano MA, Monte MJ et al (2011*) A homozygous nonsense mutation (c.214C->A) in the biliverdin reductase alpha gene (BLVRA) results in accumulation of biliverdin during episodes of cholestasis.* J Med Genet 48(4): 219-25
61. Bosma PJ, Chowdhury JR, Bakker C et al (1995*) The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome.* N Engl J Med 1995(33): 1171–1175
62. Beuers U (2006) *Drug insight: Mechanisms and sites of action of ursodeoxycholic acid in cholestasis.* Nat Clin Pract Gastroenterol Hepatol 3(6): 318-28
63. Bachs L, Pares A, Elena M et al (1992) *Effects of longterm rifampicin administration in primary biliary cirrhosis.* Gastroenterology 102(6): 2077-80
64. Marschall HU, Wagner M, Zollner G et al (2005) *Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans.* Gastroenterology 129(2): 476-85
65. Boyer JL, Trauner M, Mennone A et al (2006) *Upregulation of a basolateral FXR-dependent bile efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents.* Am J Physiol Gastointest Liver Physiol 290(6):G1124-G1130
66. Srivastava A (2014) *Progressive familial intrahepatic cholestasis.* J of Clin and Ep Hepatol 4(1): 25-36
67. Meier Y, Zodan T, Lange C et al (2008) *Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump.* World J Gastroenterol 14(1): 38-45
68. Lang C, Meier Y, Stieger B et al (2007) *Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury.* Pharmacogenet Genomics 17(1): 47-60
69. Stapelbroek JM, van Erpecum KJ, Klomp LW (2010) *Liver disease associated with canalicular transport defects: current and future therapies.* J hepatol 52(2): 258-71
70. Kremer AE, Martens JJ, Kulik W et al (2010) *Lysophosphatidic acid is a potential mediator of cholestatic pruritus.* Gastroenterology 139: 1008-1018

ADDENDUM

Nederlandse samenvatting

Crigler-Najjar syndroom

Crigler-Najjar syndroom is een zeldzame genetische aandoening die wordt gekenmerkt door geelzucht vanaf de geboorte vanwege een ophoping van ongeconjugeerd bilirubine. Bilirubine is het afbraakproduct van heme, een ijzerhoudend molecuul dat voornamelijk in rode bloedcellen voorkomt en belangrijk is voor zuurstoftransport in het bloed. Bij de afbraak van rode bloedcellen ontstaat eerst heme waarna het in twee stappen wordt omgezet naar ongeconjugeerd bilirubine. Hoge concentraties ongeconjugeerd bilirubine is potentieel giftig voor de hersenen (neuro-toxisch). Ongeconjugeerd bilirubine wordt in de lever omgezet in een niet schadelijke vorm door er één of twee suiker (glucuronyl) moleculen er aan vast te maken. Dit wordt verricht door het enzym UDP-glucuronosyltransferase 1A1 (UGT1A1). De aanwezigheid van suikergroepen maakt het ongeconjugeerd bilirubine wateroplosbaar en kan daardoor via de gal worden uitgescheiden uit het lichaam.

Bij patiënten met het Crigler-Najjar syndroom ontbreekt de UGT1A1 activiteit, hierdoor kan het schadelijke ongeconjugeerde bilirubine niet uit het lichaam worden verwijderd. Bij hoge concentraties kan ongeconjugeerd bilirubine zich opstapelen in de hersenen. Dit kan in eerste instantie een ontwikkelingsachterstand bij het kind veroorzaken, maar kan uiteindelijk overlijden tot gevolg hebben als de patiënt onbehandeld blijft.

Er zijn twee vormen van Crigler-Najjar bekend. De zwaarste vorm is type 1, deze patiënten hebben helemaal geen UGT1A1 activiteit. In de milde type 2 variant is er wel UGT1A1 eiwit in de lever, maar heeft dit een verminderde activiteit.

Huidige therapieën voor Crigler-Najjar syndroom

Op dit moment is fototherapie de meest gebruikte vorm van therapie bij het Crigler-Najjar syndroom. Het blauwe licht dat gebruikt wordt bij fototherapie veranderd de structuur van het ongeconjugeerde bilirubine, waarop het in de vorm van foto-isomeren via de gal kan worden uitgescheiden. Fototherapie is echter niet heel effectief, en patiënten met Crigler-Najjar type 1 moeten dagelijks tussen de 8 en 14 uur onder de blauwe lamp liggen. Dit heeft een grote impact voor het dagelijks functioneren en welbevinden van de patiënt.

De enige curatieve optie op dit moment is levertransplantatie. Een levertransplantatie is echter een grote operatie met daarbij risico's en mogelijke complicaties. Ook moet na de transplantatie levenslang medicijnen worden ingenomen om het immuunsysteem te onderdrukken. Deze medicijnen en het onderdrukken van het immuunsysteem vergroten de risico's op bijwerkingen zoals infecties en het ontwikkelen van suikerziekte. Met daarbij ook een te kort aan leverdonoren, is levertransplantatie niet de meest ideale therapie en daarom in Nederland zelden uitgevoerd bij patiënten met Crigler-Najjar syndroom.

Voor Crigler-Najjar type 2 patiënten is er ook nog de mogelijkheid om dagelijks het medicijn fenobarbital te nemen, wat de UGT1A1 expressie in de lever verhoogd. Echter, patiënten hebben vaak last van bijwerkingen zoals moeheid, duizeligheid en vertraagd denken, daarom wordt in de praktijk fenobarbital niet door alle type 2 patiënten gebruikt.

Vanwege de tekortkomingen van de huidige therapieën voor Crigler-Najjar syndroom hebben wij in het eerste deel van dit proefschrift gekeken naar nieuwe therapeutische opties voor patiënten met het Crigler-Najjar syndroom.

Virale gentherapie voor Crigler-Najjar syndroom

Recente ontwikkelingen laten zien dat gentherapie effectief kan zijn bij de behandeling van genetische afwijkingen in de lever. Een Engelse groep onder leiding van Prof. Natwhani gebruikte het adeno-assocaited virus (AAV) om een werkende versie van het factor IX gen, het gemuteerde gen bij hemofilie B, in de lever tot expressie te brengen. Dit resulteerde in langdurige correctie van de ziekte bij de behandelde patiënten zonder dat daarbij ernstige bijwerkingen werden gezien.

In **hoofdstuk 2** laten wij in twee diermodellen voor Crigler-Najjar syndroom, de Gunn rat en de UGT1A1 knock-out muis, zien dat middels een eenmalige toediening van AAV met het UGT1A1 gen langdurige expressie van het UGT1A1 eiwit in de lever kan worden bereikt. Daarbij daalde de concentratie van het ongeconjugeerde bilirubine naar normale waarden. Tevens hebben we gekeken naar het verbeteren van UGT1A1 expressie in de lever, door het gebruikte UGT1A1 gen te optimaliseren. Dit leidde tot meer expressie in cellijnen, maar helaas niet tot een betere uitkomst in de diermodellen. Ook hebben we laten zien dat het gebruik van AAV gentherapie in een nog groeiende lever kan leiden tot verlies van

therapeutische effectiviteit, en dat daarom AAV gentherapie gericht op de lever op dit moment alleen moet worden uitgevoerd op een volgroeide lever.

We hebben in **hoofdstuk 3** gekeken naar het effect van het immuunsysteem op de effectiviteit van de gentherapie met AAV. We hebben laten zien dat een receptor op immuun cellen in de lever in staat is om AAV partikels weg te vangen. Daarnaast hebben we aangetoond dat poly[i] in staat is om deze receptor te blokkeren. Gebruik van poly[i] in de Gunn rat vergrootte de effectiviteit van AAV gentherapie, voornamelijk bij ratten behandeld met de laagste dosis AAV. Tevens had het blokkeren van de receptor middels poly[i] meer effect op vrouwelijke ratten, dan mannelijk ratten. Blokkeren van de receptor, het inactief maken van Kuppfer cellen of op een andere manieren interfereren in het immuunsystem kan daarom een strategie zijn om de effectiviteit van AAV gentherapie bij Crigler-Najjar patiënten te verhogen.

Naast virale gentherapie middels AAV hebben we in **hoofdstuk 4** gekeken of het verminderen van de aanmaak van ongeconjugeerd bilirubine een mogelijke therapeutische optie is voor patiënten met Crigler-Najjar. Het enzym biliverdine reductase A (BVRA) is verantwoordelijk van de omzetting van biliverdine in ongeconjugeerd bilirubine. Biliverdine is een niet toxisch wateroplosbaar molecuul dat zonder omzettingen in de lever via de gal kan worden uitgescheiden. Na het opzetten en valideren van een assay hebben we 1200 medicijnen getest op het remmen van biliverdine reductase activiteit. Uiteindelijk zijn twee medicijen, disulfiram en montelukast, geselecteerd om in de Gunn rat te worden getest voor hun capaciteit om het serum bilirubine concentratie te verlagen. Toediening van disulfiram bleek echter toxisch te zijn voor Gunn ratten. Alhoewel de Gunn ratten montelukast goed verdroegen, resulteerde dit niet tot verlaging van het ongeconjugeerd bilirubine in het bloed. Op dit moment lijkt het verminderen van de biliverdine reductase activiteit geen haalbare therapie voor Crigler-Najjar patiënten. Meer onderzoek naar meer potente biliverdine reductase remmers, met een hoge beschikbaarheid in alle weefsels is echter wenselijk.

Een promoter is een stukje DNA voor een gen dat geactiveerd moet worden voordat een gen kan worden geactiveerd. Crigler-Najjar patiënten hebben een mutatie in het UGT1A1 gen. Echter zijn er ook patiënten bekend met de kenmerken van een Crigler-Najjar patiënt (verhoogd ongeconjugeerd bilirubine in het bloed) zonder een mutatie in het UGT1A1 gen. Wij hebben in **hoofdstuk 5** de promoter

geanalyseerd van een Crigler-Najjar patiënt die geen mutatie heeft in het UGT1A1 gen. De belangrijkste ontdekking was een mutatie in de promoter regio ter plaatse waar de nucleaire receptor HNF1α bindt. Het was al reeds bekent dat binding van HNF1α belangrijk is voor UGT1A1 gen activatie. Wij hebben aangetoond dat een mutatie ter plaatse van de plek waar HNF1α bindt aan de promoter leidt tot een reductie van 95% van de UGT1A1 expressie. Dit verklaart dus waarom onze patiënt zonder een mutatie in het UGT1A1 gen, wel dezelfde ziekte kenmerken heeft als een Crigler-Najjar patiënt.

In het tweede deel van het proefschrift hebben we gekeken naar patiënten met een belemmering van de gal afvloed, zogenaamde cholestase (chole= gal, stase= stilstand). Cholestase leidt tot een ophoping van (schadelijke) moleculen, zoals bilirubine en galzouten, in het bloed. Cholestase kan worden veroorzaakt door ziekte in de lever of door obstructie van de galwegen. **Hoofdstuk 6** dient als inleiding van het begrip cholestase, de mogelijk oorzaken, complicaties en behandel opties bij cholestatische ziektes.

In **hoofdtuk 7** beschrijven we dertien patiënten met 'persistent hepatocellular secretory failure' (PHSF). Deze aandoening wordt kenmerkt door cholestase met zeer hoge bilirubin concentraties in het bloed (> 255µmol/L, normaal is < 17µmol/L). PHSF kan worden veroorzaakt door medicatie (bijvoorbeeld antibiotica), hormonen (oestrogenen en steroïden) en kortdurende galwegobstructie (door bijvoorbeeld een tumor of galstenen). Waar normaal gesproken bilirubine waardes normaliseren bij het stoppen van de schadelijke medicatie of het opheffen van de galwegobstructie, is dit bij PHSF niet het geval.

Rifampicine is een antibiotica dat onder andere wordt gebruikt voor de behandeling tuberculose. Echter, bekend is ook dat rifampicine genen kan activeren in de lever die belangrijk zijn voor het verwerken van toxische moleculen, zoals bilirubine, die zich opstapelen bij cholestase.

Wij laten zien dat rifampicine een effectief en veilig middel is voor de behandeling van PHSF. Na de start van rifampicine normaliseerde de bilirubine waardes binnen 10 dagen tot enkele weken bij alle patiënten. Waarschijnlijk heeft rifampicine zijn effect door het activeren van de nucleair receptor pregnane-x-receptor in de lever, een receptor verantwoordelijk voor de inductie van detoxificatie eiwitten zoals CYP3A4, UGT1A1, MRP2 en OSTβ.

Een veel voorkomende complicatie van cholestase is jeuk. Ondanks decennia lang onderzoek is de oorzaak hiervan nog onbekend. In h**oofdstuk 8** laten we zien dat verhoogde autotaxine activiteit correspondeert aan de jeukbeleving van cholestatische patiënten en dat verhoogde autotaxine activiteit wordt gezien bij alle cholestatische aandoeningen. Daarnaast tonen we aan de effectiviteit van de behandeling van cholestatische jeuk (met colesevelam, rifampicine, albumine dialyse en nasobiliaire galdrainage) correspondeert met vermindering van de serum autotaxine. Autotaxine activiteit en daarbij behorende productie van LPA spelen daarom meest waarschijnlijk een essentiële rol in de etiologie van cholestatsiche jeuk.

Concluderend laat dit proefschrift zien dat gentherapie middels AAV effectief en veilig is in dieren modellen voor Crigler-Najjar en dat rifampicine effectief is voor de behandeling van 'persistent hepatocellular secretory failure' en cholestatische jeuk.

List of abbreviations

6-ECDC	6-ethyl-chenodeoxycholate
aAbs	Autoantibodies
AAP	Assembly activating protein
AAV	Adeno-associated virus
AAV8	Adeno-associated virus serotype 8
ADA-SCID	Adenosine deaminase deficiency
AIH	Autoimmune hepatitis
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
ASBT/IBAT	Apical sodium dependent bile acid transporter
AST	Aspartate transaminase
ATX	Autotaxin
BACS	Bile acid: CoA synthase
BAT	Bile acid: amino acid transferase
BRIC	Benign recurrent intrahepatic cholestasis
BSA	Bovine serum albumin
BSEP/*ABCC11*	Bile salt export pump
BVRA	Biliverdin reductase
CA	Cholic acid
CAI	Codon adaptation index
CAR	Constitutive androstane receptor
CCC	Cholangiocarcinoma
CDCA	Chenodeoxycholic acid
CHO	Chinese hamster ovary
ChO	Choline oxidase
CNS	Crigler-Najjar syndrome
CO	Carbon monoxide
CT	Computedtomography
CYP3A4	Cytochrome P450 3A4
CYP7A1	Cytochrome P450 cholesterol 7 alpha-hydroxylase
DCs	Dendritic cells
DCB	Diglucuronidated bilirubin
DILI	Drug-induced liver injury
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
ERC	Endoscopic retrograde cholangiography

FGF19	Fibroblast growth factor 19
FBS	Fetal bovine serum
FXR/*NR1H4*	Farnesoid X receptor
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GS	Gilbert syndrome
gtPBREM	Phenobarbital-responsive enhancer module
γGT	Gamma glutamyl tranferase
hAAT	Human apolipoprotein E enhancer
HBB2	Hemoglobin subunit beta
HBSS	Hanks' Balanced Salt solution
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HNF1α	Hepatic nuclear factor 1α
HO	Heme oxygenase
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cells
HVA	Homovanillinic acid
ICP	Intrahepatic cholestasis of pregnancy
IL	Interleukin
ITR	Inverted terminal repeat
ISS	Itch severity score interleukin
LPA	Lysophosphatidic acid
LPC	Lysophosphatdiylcholine
LKM3	Liver-kidney microsomal antibodies 3
MARS	Molecular absorbance recirculating system
MCB	Mono glucuronidated bilirubin
MF2	Macrophage type 2
MRC	Magnetic resonance cholangiopancreatography
mRNA	Messenger RNA
MRP2/*ABCC2*	Multidrug resistance-associated protein 2
Nab	Neutralizing antibodies
norUDCA	24-norursodeoxycholic acid
NTCP/*SLC10A1*	Na^+-taurocholate co-transporting polypeptide
NT	Nucleotide
OATP	Organic anion-transporting polypeptide
OCA	Obeticholic acid
OLT	Orthotopic liver transplantation
OSTα	Organic solute transporter alpha

OSTβ	Organic solute transporter beta
OTC	Ornithine transcarbamylase
P3MS	5β-pregnan-3α,-20α-diol-3-sulfate
PBC	Primary biliary cholangitis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PFIC	Progressive familial intrahepatic cholestasis
PHSF	Persistent hepatocellular secretory failure
PMA	Phorbol-12-myristate-13-acetate
Poly[i]	Polyinosinic acid
PPV	Positive predictive value
PXR	Pregnane X Receptor
PSC	Primary sclerosing cholangitis
PUO	Pruritus of unknown origin
qPCR	quantitative real-time polymerase chain reaction
RMP	Rifampicin
RS	Rotor syndrome
RXR	Retinoid x receptor
ROC	Receiver operator characteristic
SCID	Severe combined immune deficiency disease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresi
SHP	Short heterodimer partner
shRNA	Short hairpin RNA
SR-A	Scavenger receptor A
TB	Total bilirubin
TBS	Total serum bile salt
TNFα	Tumour necrosis factor alpha
UCB	Unconjugated bilirubin
UDCA	Ursodeoxycholic acid
UGT1A1	Uridine diphospho-glucuronosyl transferase 1A1
ULN	Upper limit of normal
US	Ultrasound
VAS	Visual analog scale
VGCN	Vector genome copy number
WT	Wild-type

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PhD PORTFOLIO

PhD Student: R. van Dijk
Period: September 2010 – November 2014
Supervisors: Prof. Dr. U.W.H. Beuers, Prof. Dr. R.P.J. Oude Elferink , dr. P.J. Bosma

1. PhD training	**Year**	**ECTS**
General courses		
AMC World of Science	2010	0.7
Crash course	2010	0.4
Project management	2010	0.6
Basic lab safety	2010	0.4
Basic course in legalization and organization for clinical researchers (BROK)	2010	0.8
Reference manager basic and SPSS	2010	0.6
Specific courses		
DNA technology	2010	3.9
Clinical data management	2010	1.1
Laboratory animals (article 9 WOD)	2011	2.1
Macroscopic, microscopic and pathological anatomy of the house mouse	2010	0.3
Seminars, workshops and master classes		
Weekly MDL seminar, quarterly Ruysch lecture	2010-2014	2.1
EASL school of Hepatology, course 6: cell biology of the liver	2010	0.6
(Inter)national conferences		
ACM MDL PHD retreat, Lunteren *(oral presentation)*	2011	1.0
Falk symposium, Vienna, Austria	2012	0.6
NHV voorjaarsmeeting, Veldhoven *(oral presentation)*	2012	1.0
AASLD Liver Meeting, Boston, USA *(poster)*	2012	1.0
Dutch liver retreat, Spier *(oral presentation)*	2012	1.0
NVH najaarsmeeting, Veldhoven *(oral presentation)*	2012	1.0
EASL international liver congress, Amsterdam	2013	0.7

ESGCT international congress, Madrid, Spain *(poster)*	2013	1.0
NVGCT meeting, Lunteren *(oral presentation)*	2013	1.0
Dutch liver retreat, Spier *(oral presentation)*	2013	1.0
NVH najaarsmeeting, Veldhoven	2013	1.0
ACM MDL PHD retreat, Lunteren *(oral presentation)*	2013	1.0
ESGCT international congress, Den Haag *(poster)*	2014	1.0
NVGCT meeting, Lunteren *(oral presentation)*	2014	1.0
NVH najaarsmeeting, Veldhoven	2014	0.5
AASLD Liver Meeting, San Francisco, USA *(poster)*	2015	0.5
NVH najaarsmeeting, Veldhoven *(oral presentation)*	2015	0.5
AASLD Liver Meeting, Boston, USA *(poster)*	2016	1.0

2. Teaching

Supervising

Wouter Smit *(Bsc thesis medicine)*	2013
Isabella Mayayo-Peralta (Msc thesis Biomedical science)	2014

3. Parameters of esteem

Grants

Dutch Crigler-Najjar society - €32.500 (project bilverdine reductase inhibitor)	2014

Award and prizes

Presidential Poster of Distinction AASLD	2015
Nominated as one of three candidates for young hepatologist award (Dutch Liver Society)	2016

4. Publications

Peer reviewed

van Dijk R*, Aronson SJ*, de Waart DR, van de Graaf SF, Duijst S, Seppen J, Elferink RO, Beuers U, Bosma PJ. *Biliverdin Reductase inhibitors did not improve severe unconjugated hyperbilirubinemia in vivo*. Sci Rep. 2017 May 10;7(1):1646

(*shared first authorship)

Ronzitti G*, Bortolussi G*, **van Dijk R***, Collaud F, Charles S, Leborgne C, Vidal P, Martin S, Gjata B, Sola MS, van Wittenberghe L, Vignaud A, Veron P, Bosma PJ, Muro AF, Mingozzi F. *A translationally optimized AAV-UGT1A1 vector drives safe and long-lasting correction of Crigler-Najjar syndrome.* Mol Ther Methods Clin Dev. 2016 Jul 20;3:16049

(*shared first authorship)

van der Mark VA, Ghiboub M, Marsman C, Zhao J, **van Dijk R**, Hiralall JK, Ho-Mok KS, Castricum Z, de Jonge WJ, Oude Elferink RP, Paulusma CC. *Phospholipid flippases attenuate LPS-induced TLR4 signaling by mediating endocytic retrieval of Toll-like receptor 4.* Cell Mol Life Sci. 2017 Feb;74(4):715-730

van Dijk R, Mayayo-Peralta I, Aronson SJ, Kattentidt-Mouravieva AA, van der Mark VA, de Knegt R, Oruc N, Beuers U, Bosma PJ . *Disruption of HNF1α binding site causes inherited severe unconjugated hyperbilirubinemia.* J Hepatol. 2015 Dec;63(6):1525-9

van Dijk R, Beuers U, Bosma PJ. *Gene replacement therapy for genetic hepatocellular jaundice.* Clin Rev Allergy Immunol. 2015 Jun;48(2-3):243-53

Kremer AE, Bolier R, **van Dijk R**, Oude Elferink RP, Beuers U. *Advances in pathogenesis and management of pruritus in cholestasis.* Dig Dis. 2014;32(5):637-45

van Dijk R, Kremer AE, Smit W, van den Elzen B, van Gulik T, Gouma D, Lameris JS, Bikker H, Enemuo V, Stokkers PC, Feist M, Bosma P, Jansen PL, Beuers U.

Characterization and treatment of persistent hepatocellular secretory failure. Liver Int. 2015 Apr;35(4):1478-88

van Dijk R, Montenegro-Miranda PS, Riviere C, Schilderink R, ten Bloemendaal L, van Gorp J, Duijst S, de Waart DR, Beuers U, Haisma HJ, Bosma PJ. *Polyinosinic acid blocks adeno-associated virus macrophage endocytosis in vitro and enhances adeno-associated virus liver-directed gene therapy in vivo.* Hum Gene Ther. 2013 Sep;24(9):807-13

Kremer AE, **van Dijk R**, Leckie P, Schaap FG, Kuiper EM, Mettang T, Reiners KS, Raap U, van Buuren HR, van Erpecum KJ, Davies NA, Rust C, Engert A, Jalan R, Oude Elferink RP, Beuers U. *Serum autotaxin is increased in pruritus of cholestasis, but not of other origin, and responds to therapeutic interventions.* Hepatology. 2012 Oct;56(4):1391-400

van Maldegem F, Jibodh RA, **van Dijk R**, Bende RJ, van Noesel CJ. *Activation-induced cytidine deaminase splice variants are defective because of the lack of structural support for the catalytic site*. J Immunol. 2010 Mar 1;184(5):2487-9

van Maldegem F, **van Dijk R**, Wormhoudt TA, Kluin PM, Willemze R, Cerroni L, van Noesel CJ, Bende RJ. *The majority of cutaneous marginal zone B-cell lymphomas expresses class-switched immunoglobulins and develops in a T-helper type 2 inflammatory environment.* Blood. 2008

Curriculum vitae

Remco van Dijk was born on 27 February 1985 in Leidschendam, The Netherlands. Upon graduating from Dalton-Vatel Voorburg, he started his medical training at the University of Amsterdam in 2003. During his first years of study he developed an interest in fundamental research and was given the opportunity to work at the lab of prof. dr. C van Noesel at the pathology department of the Academic Medical Centre in Amsterdam. This extracurricular work resulted in his first two co-authorships while he was still a medical student. After travelling through Australia for 4 months he started his rotations in 2008. During his rotations his interest in working abroad was established by an internship of two months at the department of pediatrics at the University Hospital Bruxelles, Belgium and a three months internship at the rural St Joseph's hospital in Nguludi, Malawi.

After obtaining his medical degree in August 2010, Remco started working as a PhD-student in the department of Gastroenterology and Hepatology of the Academic Medical Centre in Amsterdam. He was under the supervision of prof. dr. U. Beuers, prof. dr. R. Oude Elferink and dr. P.J. Bosma and focused on advanced treatment of severe hyperbilirubinemia and cholestasis. His work resulted in several publications and he presented his results at various (inter)national conferences.

To obtain more clinical experience, after spending four-and-a-half-year in the laboratory, Remco worked for a year as a resident (SHO/ANIOS) at the world-renowned department of Hepatology at King's College Hospital London, and subsequently as a resident in General Medicine at the same hospital for another six months.

In January 2017 he started his training as a resident in Gastroenterology and Hepatology at the Leiden University Medical Centre and is currently working at the Haaglanden Medical Centre in The Hague.

Dankwoord

Voor de totstandkoming van dit proefschrift ben ik talloze mensen dank verschuldigd. Ten eerste gaat mijn dank uit naar de Crigler-Najjar stichting en patiënten die al jaren ons het vertrouwen geven voor de ontwikkeling van gentherapie voor het Crigler-Najjar syndroom.

Ik had het geluk om al vroeg tijdens mijn coschappen op de afdeling maag-, darm- en leverziekten van het AMC werkzaam te zijn, waar ik voor het eerst kennis maakte met prof. dr. Beuers. Klaarblijkelijk had ik geen slechte indruk achter gelaten, want toen ik aan het einde van mijn coschappen informeerde naar de mogelijkheid tot een promotieplek, het liefst of het gebied van basale hepatologie, was prof. dr. Beuers bereidt om mij onder zijn vleugels te nemen.

Beste Ulrich, ik had mij geen betere promoter kunnen wensen. De liefde die je hebt voor zowel de kliniek als de wetenschap is ongekend, en het enthousiasme waarmee je dit uitdraagt is erg inspirerend. Een van de vele factoren wat promoveren bij Ulrich zo leuk en succesvol maakt is de samenwerking met prof. dr. R. Oude Elferink. Beste Ronald, hartelijk dank je mijn promoter bent en dat ik op je lab van het Tytgat Instituut heb mogen werken. Heel belangrijk voor dit proefschrift is dr. P.J. Bosma. Piter, dank dat je met hebt opgenomen in je onderzoek naar virale gentherapie voor het Crigler-Najjar syndroom en mijn co-promoter bent geworden.

Prof. dr. A.K. Groen, prof. dr. E.S.G. Stroes, prof. dr. P.L.M. Jansen, prof. dr. V. Keitel, prof. dr. H.J. Verkade en dr. R.J. de Knegt, dank voor het plaatsnemen in de commissie, het lezen en beoordelen van mijn proefschrift.

Op het Tytgat Instituut werd ik onderdeel van de cholestase groep. Andy, jij begeleide mij met mijn eerste experimenten op het lab. Het is alom bekend hoe kundig en vernieuwend jouw experimenten waren op het gebied van de cholestatische jeuk. Het was inspirerend om dat van dichtbij mee te mogen maken. Simon, Lucas, Serge en later ook Ruth en Luca maakten de groep compleet, het waren mooie tijden zowel op als buiten het lab.

Sem, ik had me geen beter opvolger kunnen wensen. Het is het mooi om te zien dat je met heel veel enthousiasme je hebt gestoord om het Crigler-Najjar project.

Mijn experimenten waren nooit geslaagd zonder de hulp van Liesbeth, Suzanne, Dagmar, Rudi en Johan. Bedankt voor jullie geduld en de hulp bij het maken van virus, uitvoeren van dierenexperimenten, bilirubine bepalingen met HPLC, de ELISA's.

Ik hoop mij na mijn verdediging te mogen voegen bij de exclusieve groep van 'Yellow giants', onderzoekers op het gebied van bilirubine. Jurgen en Coen behoren evident tot deze groep, altijd kritisch, maar ook met een hoop humor en goede smaak voor muziek en eten.

Ook had ik het privilege om twee studenten te mogen begeleiden. Wouter en Isabel, het is misschien een cliché, maar ik denk dat ik misschien nog wel meer heb geleerd van jullie, dan jullie van mij.

Verder wil ik iedereen van het Tytgat instituut bedanken, wat een leuk en gezellig lab om te werken. Op het Tytgat instituut wordt hoogstaand onderzoek verricht en altijd kritisch meegedacht.

Graag wil ik dr. Alexandra Langers en dr. Roelof Veenendaal danken dat ik mijn opleiding tot MDL-arts aan het LUMC heb mogen beginnen. Tevens ook dr. Lars Perk en de rest van de MDL-artsen van het HMC die mij begeleiden in mijn eerste stappen tijdens opleiding tot maag-, darm en lever dokter.

Ook ben ik de groep van Federico Mingozzi bij Genethon in Frankrijk dank verschuldigd, als onderdeel van een vruchtbare samenwerking mocht ik een week meelopen op hun lab in Evry. Het is altijd goed voor je ontwikkeling om een kijkje te nemen in andermans keuken.

In het bijzonder wil ik mijn paranimfen bedanken. Tom, met jou begon deze reis toen wij op 18 jarige leeftijd samen vanuit Leidschendam-Voorburg naar de Jan van Galenstraat vertrokken om te studeren. In de tussentijd hebben we veel meegemaakt, mooie avonturen beleefd door heel Europa waar jij en Roger een van je vele optredens hadden. Met als hoogte punt natuurlijk de legendarische Amstereo feesten in de Flexbar waar ik Janey heb ontmoet.

Paula de Sousa Montenegro Miranda, het was oprecht een eer om het gentherapie project van jou over te nemen. Hoewel de Hollandse nuchterheid soms kan botsen

met het Portugese temperament is dit bij ons niet het geval, en hebben we samen veel gepraat en gelachen.

Natuurlijk wil ik mijn familie bedanken. Lieve papa en mama, zonder jullie onvoorwaardelijke steun en vertrouwen was dit nooit gelukt. Ook wil ik mij twee grote broers bedanken, Erik en Peter die toen ik klein was alle paden voor mij hebben bewandeld zodat ik als 'kleine broertje van' nooit ergens zorgen hoefde te maken.

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