Efficacy of AAV8-hUGT1A1 with Rapamycin in neonatal, suckling, and juvenile rats to model treatment in pediatric CNs patients

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A clinical trial using adeno-associated virus serotype 8 (AAV8)-human uridine diphosphate glucuronosyltransferase 1A1 (hUGT1A1) to treat inherited severe unconjugated hyperbilirubinemia (Crigler-Najjar syndrome) is ongoing, but preclinical data suggest that long-term efficacy in children is impaired due to loss of transgene expression upon hepatocyte proliferation in a growing liver. This study aims to determine at what age long-term efficacy can be obtained in the relevant animal model and whether immune modulation allows re-treatment using the same AAV vector. Neonatal, suckling, and juvenile Ugt1a1-deficient rats received a clinically relevant dose of AAV8-hUGT1A1, and serum bilirubin levels and anti-AAV neutralizing antibodies (NAbs) in serum were monitored. The possibility of preventing the immune response toward the vector was investigated using a rapamycin-based regimen with daily intraperitoneal (i.p.) injections starting 2 days before and ending 21 days after vector administration. In rats treated at postnatal day 1 (P1) or P14, the correction was (partially) lost after 12 weeks, whereas the correction was stable in rats injected at P28. Combining initial vector administration with the immune-suppressive regimen prevented induction of NAbs in female rats, allowing at least partially effective re-administration. Induction of NAbs upon re-injection could not be prevented, suggesting that this strategy will be ineffective in patients with low levels of preexisting anti-AAV NAbs.

INTRODUCTION

Crigler-Najjar syndrome (CNs) type 1, the most severe form of inherited unconjugated hyperbilirubinemia, is lethal during infancy or early childhood.1 This autosomal-recessive liver disorder is caused by the deficiency of a hepatic enzyme, uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1).2 UGT1A1 converts the toxic and lipophilic unconjugated bilirubin (UCB) to water-soluble conjugated bilirubin (CB) that can be excreted into bile by active transport across the hepatocyte canalicular membrane.2,3 Deficiency of UGT1A1 leads to UCB accumulation in all tissues, causing irreversible and lethal brain damage, characterized by kernicterus, the yellow pigmentation of the globus pallidus in the basal ganglia. Current treatments consist of intensive phototherapy, a cumbersome treatment that becomes less effective over time.4,5 For the most severely affected patients, a liver transplantation is inevitable at some point in their lifetime.4–6 Important shortcomings of liver transplantation, such as donor availability, procedure-associated complications and mortality, graft survival, and increased cancer and infection risks due to life-long need for immune suppression, indicate that alternative treatments are urgently needed.7

A promising alternative curative treatment for inherited severe liver disorders, such as CNs, is in vivo gene therapy using recombinant adeno-associated virus (AAV) vectors. Liver-directed gene therapy trials for hemophilia B, a bleeding disorder caused by factor IX deficiency, have achieved a sustained reduction of bleeding episodes after a single systemic injection of an AAV vector containing cDNA encoding the human factor IX protein.8,9 The safety and efficacy of this treatment strategy is currently being investigated for several monogenic inherited severe liver disorders. Clinical trials are ongoing for ornithine transcarbamylase deficiency (ClinicalTrials.gov: NCT02991144), familial hypercholesterolemia (ClinicalTrials.gov: NCT02651675), and glycogen storage disease type 1a (ClinicalTrials.gov: NCT03517085), and also the feasibility of liver-directed gene therapy to treat severe CNs is currently being investigated in clinical trials (ClinicalTrials.gov: NCT03466463 and NCT03223194).

Recombinant AAV vectors do not actively integrate into the host genome and are lost upon cell division.10 Studies in neonatal animals, modeling AAV gene therapy to treat CNs early after birth, showed loss of correction over time due to hyper-proliferation of hepatocytes in a developing liver. This study aims to determine at what age long-term efficacy of a hepatic enzyme, uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1), to treat inherited severe unconjugated hyperbilirubinemia (Crigler-Najjar syndrome) is ongoing, but preclinical data suggest that long-term efficacy in children is impaired due to loss of transgene expression upon hepatocyte proliferation in a growing liver. This study aims to determine at what age long-term efficacy can be obtained in the relevant animal model and whether immune modulation allows re-treatment using the same AAV vector. Neonatal, suckling, and juvenile Ugt1a1-deficient rats received a clinically relevant dose of AAV8-hUGT1A1, and serum bilirubin levels and anti-AAV neutralizing antibodies (NAbs) in serum were monitored. The possibility of preventing the immune response toward the vector was investigated using a rapamycin-based regimen with daily intraperitoneal (i.p.) injections starting 2 days before and ending 21 days after vector administration. In rats treated at postnatal day 1 (P1) or P14, the correction was (partially) lost after 12 weeks, whereas the correction was stable in rats injected at P28. Combining initial vector administration with the immune-suppressive regimen prevented induction of NAbs in female rats, allowing at least partially effective re-administration. Induction of NAbs upon re-injection could not be prevented, suggesting that this strategy will be ineffective in patients with low levels of preexisting anti-AAV NAbs.
increased presence of regulatory T cells,16 was studied in sucking regimen of rapamycin, based on shifting the T cells toward an liver maturation feasible. The ef
would render initial treatment earlier after birth and re-treatment af-
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dependent of age and sex, treatment with AAV8-h
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titer of NAbs toward the vector induced by the 
at neonatal or juvenile age. In case of loss of correction over time, re-
dose as part of the clinical trial. Furthermore, liver damage due to, for
AAV8 has relevance beyond application in young patients, since it

To model long-term efficacy in children of a clinically relevant dose of AAV8-hUGT1A1, the vector similar to the one used in the ongoing clinical trial for CNs, patients (CureCN), Ugt1a1 deficient rats 1, 14, and 28 days of age received 5 \times 10^{12} \text{ vector genomes (vg)/kg by intravenous injection (Figure 1A). To monitor the effect on serum bilirubin, blood was sampled every 2 or 4 weeks after vector administration. Independent of age and sex, treatment with AAV8-hUGT1A1 resulted in complete normalization of total serum bilirubin levels at 2 weeks after injection. In rats treated at 28 days after birth, this complete normalization persisted in males, while in females after 12 weeks a low level of serum bilirubin was detectable (Figure 1B). Treatment at postnatal day 1 (P1) or P14 resulted in complete correction followed by a gradual increase of serum bilirubin levels over time (Figures 1C and 1D). After

To determine at what age gene therapy would result in long-term ef-
ficacy when using the vector that is also evaluated in an ongoing clin-
cical trial, Ugt1a1 deficient rats received a clinically relevant dose either at neonatal or juvenile age. In case of loss of correction over time, re-
treatment will be necessary to retain therapeutic efficacy. The high titer of NAbs toward the vector induced by the first administration will impair hepatocyte transduction efficiency of a second administration unless we are able to reduce the initial formation of NAbs. Immune suppression to reduce vector capsid-mediated B and T cell activation could prevent or reduce NAb formation, and, if effective, would render initial treatment earlier after birth and re-treatment after liver maturation feasible. The efficacy of an immune-suppressive regimen of rapamycin, based on shifting the T cells toward an increased presence of regulatory T cells,16 was studied in sucking Ugt1a1 deficient rats to model its suitability in children suffering from CNs type 1. An effective strategy to block NAbs toward AAV8 has relevance beyond application in young patients, since it will also allow re-treatment of patients who may receive a sub-optimal dose as part of the clinical trial. Furthermore, liver damage due to, for instance, a viral infection or use of alcohol may result in loss of correction, rendering a re-treatment needed later in life.

RESULTS

Long-term efficacy of AAV8-hUGT1A1 depends on the age of treatment
To model long-term efficacy in children of a clinically relevant dose of AAV8-hUGT1A1, the vector similar to the one used in the ongoing clinical trial for CNs, patients (CureCN), Ugt1a1 deficient rats 1, 14, and 28 days of age received 5 \times 10^{12} \text{ vector genomes (vg)/kg by intra-}

To determine the presence of NAbs, a reporter vector AAV8-luciferase was preincubated with increasing dilutions of sera from treated or control animals. Subsequently, the presence of NAbs was determined as the highest dilution at which 50% or greater inhibition of the luciferase expression was measured. As shown in Figure 2B, in rapamycin-treated rats the titer was similar to that in serum from

Rapamycin blocks humoral response toward AAV8 capsid
The loss of therapeutic efficacy when treating suckling rats implies that AAV vector re-administration is needed to obtain life-long correction when aiming to treat these young animals. The presence of a high titer of antibodies toward the vector induced by the first treatment blocks hepatocyte transduction, rendering re-administration ineffective.17 We hypothesized that immune suppression covering the exposure of the vector to the immune system using rapamycin and prednisolone may prevent this. In addition, rapamycin seems a promising option because it increases autophagy, resulting in an increased liver transduction in non-human primates (NHPs).18 Considering the heterogeneous anti-AAV response in rats treated at P1, the rats treated at P14 were used to study the efficacy of rapa-

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cicacy associated with

To ensure
a transient correction pattern in male rats, a 2-fold lower dose of AAV8-hUGT1A1 was used (5 × 10^{12} vg/kg), and because no additive effect of prednisolone was seen in females, the combination of rapamycin and prednisolone was omitted. This dose did not induce a detectable titer of anti-AAV IgG but did induce NAbs toward AAV8, albeit with a low titer not reaching a statistically significant difference with the serum from naive rats (Figures 2C and 2D). This reduced response could be due to the 50% lower dose or a less mature immune system in males or a combination of both.

Absence of NAbs toward AAV8 allows effective re-treatment with AAV8-hUGT1A1 but is transient in adult female rats
Administration of 1 × 10^{12} vg/kg AAV8-hUGT1A1 at P14 in female rats resulted in a transient correction, with the serum bilirubin level returning to that of untreated rats at 12 weeks after vector injection (Figure 3A). Rapamycin with or without prednisolone resulted in some delay in the growth of juvenile rats that did not reach statistical significance (Figure S3). After having shown that rapamycin blocked the formation of antibodies directed against the AAV8 vector.
irrespective of the presence of prednisolone (Figures 2A and 2B), the efficacy of re-dosing AAV8-hUGT1A1 was investigated by injecting the animals at P14 with a sub-therapeutic dose of $1 \times 10^{12}$ vg/kg, and re-injecting the animals at week 14 (W14) with a dose previously shown to provide long-term correction in female rats injected at P28 ($5 \times 10^{11}$ vg/kg, Figure 1B). The rats received an immune-suppressive regimen identical to the first time to investigate whether this could prevent a humoral response in primed animals. In rats receiving the immune suppression the second injection did result in a significant reduction of serum bilirubin, whereas in vehicle-treated rats no effect was seen (Figure 3A; Table 1). This response, however, appeared transient. At 4 weeks after vector re-dosing a 50% reduction of serum bilirubin in rapamycin with and without prednisolone-treated rats was seen, while the effect in vehicle-treated rats did not result in a significant reduction of serum total bilirubin. At later time points the difference between both groups did not reach statistical significance, although the levels in rats receiving rapamycin remained below that of the vehicle-treated groups. Excretion of bilirubin conjugates into bile and the presence of vector genomes in these naive animals were also very comparable to those in the re-injected rats (Figures 3C and 3D). Overall, this indicates that the reduced efficacy seems due to age of these female rats at the time of (re)dosing. In males, re-treatment upon loss of correction did result in a persistent therapeutic reduction of serum bilirubin levels (Figure 3E; Table 2). Different from the transient correction in females, at 4 weeks after vector re-dosing a more than 75% reduction of serum bilirubin was found compared to rats receiving a single dose at P14 in males. This increased efficacy is also reflected by the excretion of bilirubin conjugates into bile and vector genomes present in liver at the time of sacrifice (Figures 3F and 3G).

Rapamycin does not prevent a humoral response toward AAV in primed rats

A rapamycin base regimen did prevent the induction of a humoral response in suckling female rats. To investigate whether this treatment would also effectively prevent a response upon re-administration of the vector, all animals received the same immune-suppressive regimen covering the second AAV dosing. NAbs and IgG levels toward AAV8 were determined at week 10 after the second injection. As shown in Figures 4A–4D, immune suppression did not block the induction of NAbs and IgG upon re-injection in both sexes,
Figure 3. In the absence of NAbs the efficacy of re-administered AAV8-hUGT1A1 is comparable to that in naive rats
At 12 weeks after the first AAV8-hUGT1A1 administration half of the vehicle-treated rats were re-injected with 5 \times 10^{12} \text{vg/kg} of vector. All rats treated the first time with vector in combination with immune suppression were re-dosed with 5 \times 10^{12} \text{vg/kg} of AAV8-hUGT1A1 in combination with the same immune-suppressive regimen. (A and E)
although it did reduce the titer compared to vehicle-treated rats. In contrast to rats re-injected with AAV, the immune-suppressive regimen did completely block the immune response in the naive rats (Figures S4B–S4E). Also, in the 14-week-old animals the immune-suppressive regimen resulted in a small effect of weight, but the difference did not reach statistical significance (Figure S3) and induced a mild leukopenia (Figure S5).

Rapamycin affects the biodistribution of AAV
To investigate whether the rapamycin treatment modified the biodistribution upon systemic administration of AAV8-hUGT1A1, the amount of viral genomes in liver, lung, kidney, and spleen was determined. In all four organs, viral genomes were detectable (Figure S6). In the rats receiving a single injection at P14 the vector genomes were undetectable. In re-injected female rats, in none of the vehicle rats could vector be detected in the liver (Figure 3D). In immune suppression-treated rats and in naive rats, vector genomes were detectable in part of the animals, albeit at a low level. Five- to 10-fold higher levels of vector genomes were present in male livers, but the results were also heterogeneous, with two rapamycin-treated rats having very low levels (Figure 3G). At the time of sacrifice high numbers of vector genomes were detected in the spleen of naive rats injected at week 14 (Figure 5). Presence of vector copies in the spleen and not in the liver could play a role in the loss of efficacy seen in older animals. These increased levels were not seen in the spleen of animals receiving rapamycin. To investigate whether rapamycin could increase the efficacy by preventing vector loss to the spleen, naive 14-week-old males and females were treated with rapamycin as before and received a dose of $5 \times 10^{12}$ vg/kg AAV8-hUGT1A1. Although this indeed reduced the presence of vector genomes in the spleen, it did not improve the treatment efficacy (Figure 5). In males the serum bilirubin levels were reduced with 75%, which is less than that in rats not receiving rapamycin. The 10%–20% reduction in females is comparable to that seen in the retreated animals receiving rapamycin (Figure S4A).

DISCUSSION
Administration of an AAV8-hUGT1A1 vector is currently being evaluated as a potential treatment of severe unconjugated hyperbilirubinemia, CNs (ClinicalTrials.gov: NCT03466463). Although effective phototherapy does allow treatment of adults, the risk for irreversible brain damage in severely affected patients persists and the disease burden during all of these years is significant. In addition, presence of liver fibrosis was seen in livers from CNs type 1 patients, indicating that this severe syndrome can result in liver damage. Gene therapy at a younger age therefore seems preferable. Furthermore, in view of the gradual increase in the prevalence of pre-existing immunity toward AAV vectors during aging, treating at a younger age would be preferable. Screening adult CNs type 1 patients showed that 30.6% were not eligible for AAV-mediated gene therapy because of preexisting NAbs toward AAV8.

The episomal persistence of AAV vector genomes, however, compromises long-term efficacy due to dilution of the vector in growing livers. To investigate whether the vector used in the ongoing clinical trial can provide long-term correction at a younger age, a clinically relevant dose was administered to neonatal, sucking, and juvenile rats to model its use in young children suffering from CNs. When treating neonatal rats the correction was lost completely, and only sporadic clusters of UGT1A1-expressing hepatocytes were present, most likely resulting from random integration of the vector in the host genome (Figure 1; Figure S1). Since the vector dose administered (vg/kg) was based on the weight of the animals at the time of treatment, this loss of efficacy can, at least partially, be explained by the lower number of vector genomes given to the neonatal and sucking rats. At the time of sacrifice the presence of vector genomes in the liver was >10-fold lower than that seen with the same vector administered to adult rats. Since the difference in weight between week 4 (P28) and week 8 is less than a factor 2, the difference in absolute vector dose between these two groups is <2-fold. The 10-fold difference of vector in the liver at the time of sacrifice indicates that in addition to vector dilution, hepatocyte proliferation enhances vector loss. A comparable loss of efficacy was reported in this model and in a Ugt1a1 deficient mouse model also reporting presence of some integrations. These data indicate that treatment very early after birth compromises efficacy, with only minimal expression from a few integrated gene copies. Re-treatment using virus serotype switching is an option to overcome the hurdle imposed by the induction of NAbs due to the first treatment, but it is limited by cross-reactivity between serotypes. Moreover, a development process for the production of two different vectors complicates this approach. Gene therapy treatment at P1 limited the induction of NAbs in several rats (data not shown) and in mice allowed effective re-treatment. Treatment very early after birth will be complicated in patients for several reasons. Since physiological jaundice occurs in almost all newborns, and pathological jaundice can be due to several much more frequent causes, jaundice will only be recognized first a few days after delivery and treated with phototherapy. Only upon reappearance of severe jaundice after stopping phototherapy will additional diagnostic tests be performed to diagnose UGT1A1 deficiency and to establish the severity. Only the severe form of CNs should be treated with gene therapy. Thus, treatment at a juvenile age seems more representative to model gene therapy treatment for CN patients. In sucking rats treated at P14, the loss of correction is less compared to P1, although already at 12 weeks after treatment a significant increase in serum bilirubin is seen in females. This more prominent loss in females may be due to the lower efficacy of AAV vectors to transduce females reported for murine animals. In contrast to treatment at P1, the
in the naive dog model lifelong correction was established. In patients seen in adults was demonstrated by the immune-mediated loss of life, and in most adults the immune system may be primed toward humoral response toward the AAV capsid in patients. Since AAV were primed and not naive toward this vector anymore. This observation of a detectable humoral response upon the first trial to treat hemophilia B, while in children the increase is <20% during the first month, but this is an unwanted side effect. In this respect a single injection of a targeted rapamycin also seems preferable. Other effects of the rapamycin treatment, such as a mild leukopenia, are expected and the effect is only transient and does not appear to render this approach less feasible.

This targeted approach may also reduce the adverse effects of a 3-week course of rapamycin. Our data indicate that this had a minor non-significant delay effect in the growth of the suckling rats (Figures S3A and S3B). The growth retardation of a 3-week course in children may be smaller due to a slower growth, as rats double their weight from week 2 to 5 after birth, while in children the increase is <20% during the first month, but this is an unwanted side effect. In this respect a single injection of a targeted rapamycin also seems preferable. Other effects of the rapamycin treatment, such as a mild leukopenia, are expected and the effect is only transient and does not appear to render this approach less feasible.

Table 1. Reduction in serum bilirubin in female rats in different groups

| Group | Serum bilirubin, p value versus AAV P14 |
|-------|------------------------------------------|
| AAV P14 | 170 ± 27 μM, p = 0.2281 204 ± 43 μM |
| Vehicle/(AAV P14 + AAV W14) | 151 ± 33 μM, p = 0.8458 |
| Rapamycin/(AAV P14 + AAV W14) | 108 ± 23 μM, p = 0.0008 172 ± 49 μM, p = 0.3173 |
| Rapamycin/prednisolone/(AAV P14 + AAV W14) | 92 ± 33 μM, p = 0.0297 143 ± 48 μM, p = 0.1245 |

Average serum bilirubin was calculated during periods of 13–16 and 18–26 weeks after injection. Mean ± SD. A mixed effects analysis of variance was used to calculate p values.

Table 2. Reduction in serum bilirubin in male rats in different groups

| Group | Serum bilirubin, p value versus AAV P14 |
|-------|------------------------------------------|
| AAV P14 | 185 ± 9 μM |
| Vehicle/(AAV P14 + AAV W14) | 53 ± 53 μM, p = 0.0588 12 ± 5 μM, p = 0.0002 |
| Rapamycin/(AAV P14 + AAV W14) | 76 ± 48 μM, p = 0.0151 |
| Rapamycin/prednisolone/(AAV P14 + AAV W14) | 46 ± 31 μM, p = 0.004 |

Average serum bilirubin was calculated during periods of 13–16 and 18–26 weeks after injection. Mean ± SD. A mixed effects analysis of variance was used to calculate p values.

intravenous administration of a clinical dose at P14 resulted in the presence of anti-AAV antibodies in all animals, indicating that this is a good time for injection to study the efficacy of immune suppression to allow effective re-administration.

Our results showed that a 3-week course of rapamycin prevents the humoral response against AAV in naive suckling and adult female rats (Figures 2 and 4). In NHPs an immune-suppressive regimen containing rapamycin combined with AAV may provide some protection. AAV1 is not efficient murine models to investigate whether this approach can be applicable to CNs patients.

The transient correction in 14-week-old female rats (Figure 3) using a dose of 5 × 1012 vg/kg is comparable to the correction of serum bilirubin levels previously obtained with a 10-fold lower dose of this vector. As shown in Figure 1, in 4-week-old juvenile rats this dose did provide sustained therapeutic correction. Also, in female rats 6–8 weeks old, this dose proved to be effective. This indicates that in the 14-week-old female rats AAV8 liver transduction appears less efficient. In male rats the difference in efficacy between the different ages was much smaller, but when administered at week 14, a low level of bilirubin was detectable while at earlier time points after treatment no bilirubin was detectable. In this respect a single injection of a targeted rapamycin also seems preferable. Other effects of the rapamycin treatment, such as a mild leukopenia, are expected and the effect is only transient and does not appear to render this approach less feasible.
reduced efficacy in older animals (Figures 5A and 5B). The presence of NABs does cause increased uptake of AAV vectors by the spleen in rats and mice.\textsuperscript{17,31} Treating the animals with rapamycin for 3 weeks reduced the presence of vector in the spleen, most likely by delaying the induction of NABs until most of the vector had disappeared from the circulation (Figure S9). This prolonged rapamycin treatment did not prevent the induction of NABs at later time points and did not improve the correction, indicating that it appears not to be a promising approach to improve AAV efficacy. Several studies have shown macrophages do phagocytose AAV vectors and, as such, are involved in the innate immune response toward this vector.\textsuperscript{35,36} Previously we have shown that preventing the uptake of a scAAVLP1-\textit{UGT1A1} by these cells does improve correction of serum bilirubin levels and liver transduction, especially in female Gunn rats.\textsuperscript{37} The latter suggests that in addition to the reported less efficient single- to double-stranded conversion of AAV vector genomes in females, the higher number of Kupffer cells and their higher phagocytosis activity may be responsible for the reduced efficacy.\textsuperscript{38–40} This sexual dimorphism also exists in humans and may affect the vector efficacies, especially in adults.

Overall, our studies showed that AAV-mediated \textit{in vivo} gene therapy to provide long-term correction of inherited hyperbilirubinemia in Gunn rats is feasible at P28. Earlier treatment results in loss of efficacy. How this translates to human age requires studies comparing liver growth rates in children and juvenile rats. Rapamycin could prevent the induction of NABs in naive animals. However, its potential effect on growth and its poor effectivity after prior exposure to AAV, which is endemic in the human population, indicates that other treatments are needed to prevent the NABs. Depletion of AAV NABs using immune adsorption does allow efficient liver transduction with AAV but seems only sufficiently effective when antibody titers are low.\textsuperscript{41} Other options such as removal of IgGs using endopeptidase therefore seem more promising.\textsuperscript{42,43}

**MATERIALS AND METHODS**

**Animal study**

\textit{Ugt1a1} deficient Gunn rats from our own breeding colony received AAV8-\textit{hUGT1A1} (produced by Genethon, France; \( n = 6–8 \) of equal sex) treatment by facial vein injection at P1, or by tail vein administration at P14 or P28. Rats were housed in a temperature-controlled environment with a 12-h light/12-h dark cycle and permitted \textit{ad libitum} consumption of C1000 control diet (Altromin, Triple A Trading, Germany) and water. Blood sampling was performed by tail vein puncture under isoflurane anesthesia in heparin tubes. At the time of sacrifice, bile was sampled as previously described,\textsuperscript{44} and blood was collected by cardiac puncture and plasma (heparin) was separated by centrifugation at 1,000 \( \times \) \( g \) for 5 min. Organs were fixed overnight in 4% paraformaldehyde or snap-frozen in liquid \( \text{N}_2 \) and stored at \(-80^\circ \text{C}\) for further analysis. All animal experiments were performed in accordance with the European Directive 2010/63/EU and with
approval of the Institutional Animal Care and Use Committee of the Amsterdam UMC.

Rapamycin (Sanbio; 15 mg/mL) and prednisolone (Sigma-Aldrich; 50 mg/mL) stock solutions were made in DMSO and stored at −20°C. Stocks were diluted 10 times in 5% Tween 80 (Sigma-Aldrich) and 5% polyethylene glycol 400 (PEG 400) (Hampton Research), as previously described.35 Rats received 1.5 mg/kg rapamycin or 1.5 mg/kg rapamycin + 5 mg/kg prednisolone or vehicle + DMSO control by a daily i.p. injection, starting 2 days prior to vector administration and ending 21 days after.

**Bilirubin quantification**

Bilirubin metabolites in bile were quantified by high-performance liquid chromatography (HPLC) as described44 with the modification that a Pursuit column (Agilent Technologies, the Netherlands) was used. Bilirubin in serum was quantified on a Roche Cobas c502/702 analyzer (Roche Diagnostics, USA) by the hospital Routine Clinical Chemistry Department.

**Determination of AAV8 NAb s**

The AAV8 NAb titer in plasma was determined using the protocol reported previously.19 Briefly, plasma samples were preincubated at 56°C for 30 min to inactivate complement. AAV8-luciferase was preincubated for 1 h at 37°C with plasma samples diluted in fetal calf serum (FCS) and added to HEK293T cells cultured at 37°C, 5% CO2. One day later luciferase expression was determined using the ONE-Glo luciferase assay system (Promega), according to the manufacturer’s protocol.

**ELISA for AAV8 and hUGT1A1 antibody**

An indirect ELISA approach was used to detect the anti-AAV8 or anti-UGT1A1 IgG in rat serum as previously described.46 An ELISA plate was coated overnight (O/N) at 4°C with 50 μL/well of lysate from HEK293T cells, HEK293T cells expressing AAV8 capsid, or 1 μg/mL human rUGT1A1 protein (Bio Connect) in 50 mM carbonate coating buffer. The next day the wells were incubated with 200 μL of blocking buffer (1% gelatin in phosphate-buffered saline (PBS)). After 2 h, the blocking buffer was removed and 50 μL/well of heparin plasma samples diluted in washing buffer with 0.05% Tween 20 in PBS was added and incubated for 1.5 hours at room temperature. After three washings with washing buffer pDP8/UGT1A1, binding rat immunoglobulins were detected with horseradish peroxidase (HRP) conjugated anti-rat IgG at 1:1,000 dilution in conjugation buffer containing four-fifths blocking buffer and one-fifth washing buffer followed by o-phenylenediamine (Sigma) conversion.

**Biodistribution and hUGT1A1 mRNA levels**

Genomic DNA was isolated as previously described47 from the tissues. hUGT1A1 copy number was determined by qPCR in a Bio-Rad CFX96 system (Roche Diagnostics, USA) using the TaqMan assay, iQ Supermix (Bio-Rad, Germany), and the primers mentioned in Table S1. Results were processed and analyzed using LinRegPCR software and normalized to β-actin. qRT-PCR was performed on a Bio-Rad CFX96 system (Roche Diagnostics, USA) using the Sensi-FAST SYBR No-ROX kit (Bioline) and the primers listed (Table S1). Data were normalized to β-actin.

**Histology**

For conventional bright-field light microscopy, tissues were fixed in 4% formaldehyde solution and embedded in paraﬃn. Hematoxylin (Sigma, 51275) and eosin (Sigma, E4382) (H&E) staining was performed on tissue sections as previously described.48

**RNA in situ hybridization**

Detection of UGT1A1 mRNA in liver tissue was done using RNA-scope technology according to the manufacturer’s protocol (Advanced Cell Diagnostics, Hayward, CA, USA). Target probes for hUGT1A1, based on the sequences listed in Table S1, were hybridized on liver tissue and visualized after signal amplification with diaminobenzidine (DAB), and counterstained using hematoxylin. UBC and DapB probes served as positive and negative controls, respectively.

**Statistical analysis**

Serum bilirubin data are presented as mean values ± standard error of the mean (SEM) and were analyzed for significance using a mixed-effects analysis. Other data are presented as mean values ± standard deviation (SD) and were analyzed for significance using the independent t test for the comparison of parametric variables between two groups, unless stated otherwise. For nonparametric variables, we performed a Mann-Whitney test, and for the comparison of three or more groups a one-way analysis of variance (ANOVA) was performed using GraphPad Prism 8 software (GraphPad, CA, USA). *p <0.05, **p <0.01, and ***p <.001 were considered significant.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.11.016.

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AUTHOR CONTRIBUTIONS

Study Design, X.S., and P.J.B.; Data Collection, Analysis, and Interpretation, X.S., S.J.A., L.t.B., S.D., R.S.B., and D.R.d.W.; Manuscript Preparation, X.S., and P.J.B.; Critical Appraisal and Manuscript Review and Editing, P.J.B., G.R., A.F.M., F.M., G.B., F.C., and R.P.O.E.

DECLARATION OF INTERESTS

F.C., G.B., G.R., A.F.M., and F.M. are inventors of patents describing liver gene transfer approaches for metabolic diseases and/or treatment of hyperbilirubinemia. F.M. is an employee of Spark Therapeutics, a Roche company. The remaining authors declare no competing interests.

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