

# Determining the Minimally Effective Dose of a Clinical Candidate AAV Vector in a Mouse Model of Crigler-Najjar Syndrome

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**Liver metabolism disorders are attractive targets for gene therapy, because low vector doses can reverse the buildup of toxic metabolites in the blood. Crigler-Najjar syndrome is an inherited disorder of bilirubin metabolism that is caused by the absence of uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1) activity. This syndrome is characterized by hyperbilirubinemia and jaundice. Unfortunately, current phototherapy treatment is not effective long term. We intravenously injected phototherapy-rescued adult UGT1 knockout mice with  $2.5 \times 10^{10}$ – $2.5 \times 10^{13}$  genome copies (GC)/kg of a clinical candidate vector, AAV8.TBG.hUGT1A1co, to study the treatment of disease compared to vehicle-only control mice. There were no apparent vector-related laboratory or clinical sequelae; the only abnormalities in clinical pathology were elevations in liver transaminases, primarily in male mice at the highest vector dose. Minimal to mild histopathological findings were present in control and vector-administered male mice. At vector doses greater than  $2.5 \times 10^{11}$  GC/kg, we observed a reversal of total bilirubin levels to wild-type levels. Based on a significant reduction in serum total bilirubin levels, we determined the minimally effective dose in this mouse model of Crigler-Najjar syndrome to be  $2.5 \times 10^{11}$  GC/kg.**

## INTRODUCTION

Monogenic disorders of liver metabolism are attractive targets for gene therapy applications because the buildup of toxic metabolites in the blood and other tissues potentially can be reversed at low vector doses by the transduction of a few hepatocytes. Crigler-Najjar syndrome is an ultra-rare autosomal recessive disorder of bilirubin metabolism, characterized by hyperbilirubinemia and jaundice. Patients with Crigler-Najjar syndrome type 1 lack the enzyme uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1), which is responsible for the conjugation of bilirubin for excretion. Without intervention, the resulting elevated blood bilirubin levels can lead to irreversible neurological damage. Current treatment for Crigler-Najjar syndrome includes an average of 12 hr per day of phototherapy. However, most patients eventually require a liver transplant because phototherapy becomes less effective with age.

A potential approach to treating Crigler-Najjar syndrome is systemic delivery of a gene therapy vector expressing UGT1A1, which would enable the liver to continuously synthesize UGT1A1. The aim of the current study was to determine the minimally effective dose (MED) of a clinical candidate vector, AAV8.TBG.hUGT1A1co, before clinical trials. We have successfully used mouse models of specific human liver diseases to determine the MED and toxicity of vectors.<sup>1,2</sup> Two UGT1 knockout (KO) mouse strains are phenotypically similar to patients with Crigler-Najjar syndrome.<sup>3,4</sup> One strain contains the same point mutation as the Gunn rat, and the other has a neomycin cassette inserted into the *Ugt1* gene locus.<sup>3–6</sup> Both UGT1 KO mouse models display lethal hyperbilirubinemia in the immediate postnatal period, and mice do not survive past day 11 of life without intervention. Phototherapy via exposure to blue fluorescent light for 12 hr per day for the first 21 days of life has been shown by us and others to allow the UGT1 KO mice to survive until adulthood and exhibit serum total bilirubin levels of  $9.1 \pm 3.0$  mg/dL following weaning from phototherapy.<sup>4,7–10</sup> Thus, pretreatment with phototherapy allows the administration of gene therapy vectors to be delayed until adulthood, which is after the most proliferative phase of liver development, increasing the likelihood of stable, long-term gene expression. Therefore, we intravenously (i.v.) injected phototherapy-rescued adult UGT1 KO mice with a clinical candidate vector, AAV8.TBG.hUGT1A1co. We evaluated levels of serum total bilirubin and liver transaminases and performed a comprehensive histopathological examination to determine vector efficacy and the MED to support a future clinical trial.

## RESULTS

### Rationale and Design

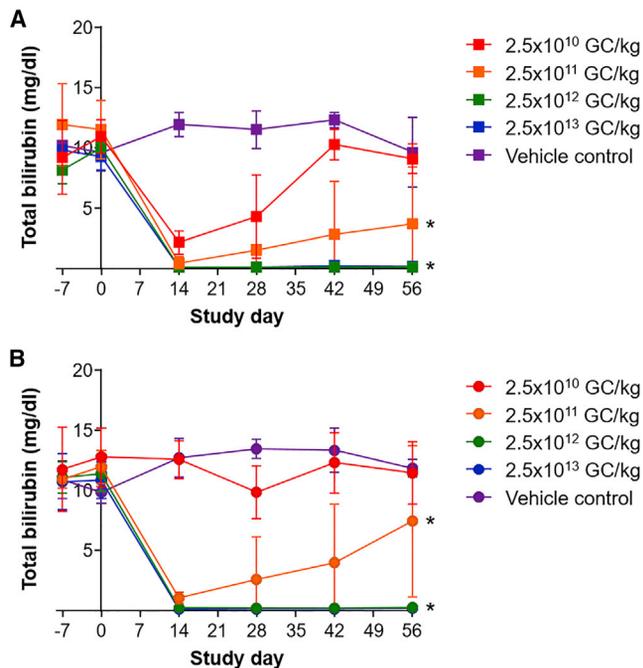
The UGT1 KO mouse is a disease model for Crigler-Najjar syndrome. As this disease can affect both sexes in the human population, we used

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**Figure 1. Serum Total Bilirubin Levels in Vector- or Vehicle Control-Injected UGT1 KO Mice**

Male (A) and female (B) UGT1 KO mice received an i.v. injection of  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  GC/kg of AAV8.TBG.hUGT1A1co or vehicle control. Total bilirubin levels were measured in serum samples. Values are expressed as mean  $\pm$  SD. \* $p < 0.05$ .

both male and female UGT1 KO mice to determine the MED. We exposed all UGT1 KO mice to phototherapy immediately after birth for 12 hr per day for up to 21 days (blue fluorescent light,  $\lambda = 450$  nm; 10–30  $\mu\text{W}/\text{cm}^2/\text{nm}$ ). Male and female UGT1 KO mice aged 6–20 weeks with a body weight of more than 15 g received an i.v. tail vein injection of vehicle control or AAV8.TBG.hUGT1A1co (AAV8 viral capsid with thyroxine binding globulin [TBG] promoter and a codon-optimized version of human UGT1A1 [hUGT1A1co]) at one of four doses:  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  genome copies (GC)/kg ( $n = 5/\text{sex}/\text{group}$ ). The composition of the groups is shown in Table S1. We performed necropsies on all mice 56 days after administration to capture hUGT1A1 expression profiles and detect any potential toxicity against the transgene.

### Clinical Findings

During the course of the study, we euthanized one mouse for humane reasons. The mouse had received a dose of  $2.5 \times 10^{11}$  GC/kg and showed a moribund condition on day 8 after administration. Following a full necropsy, we collected tissue samples and performed histopathological analyses. The microscopic findings for this mouse were moderate extramedullary hematopoiesis in the spleen and marked apoptosis of thymic lymphocytes, which is a background finding related to stress. As this mouse was euthanized at such an early stage in the study, we enrolled an additional male mouse into the same cohort to provide a complete dataset.

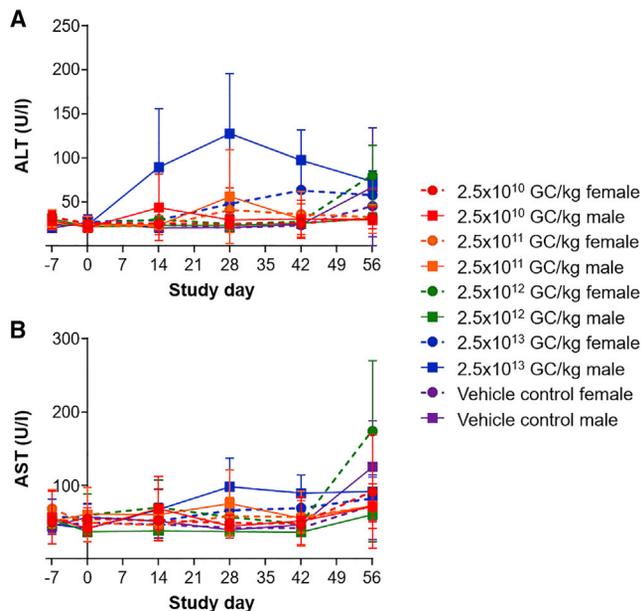
### Reversal to Wild-Type Total Bilirubin Levels

The contract facility Antech Good Laboratory Practice (GLP) measured the serum total bilirubin levels of all animals before vector administration and throughout the study. UGT1 KO mice injected with vehicle control showed similar total bilirubin levels across all time points with no sex-specific variation (Figure 1A for males; Figure 1B for females). Elevated total bilirubin levels rapidly reversed to normal wild-type baseline levels (0.1–0.3 mg/dL)<sup>10</sup> by day 14 after vector administration at two doses ( $2.5 \times 10^{12}$  and  $2.5 \times 10^{13}$  GC/kg) in both males and females. Male mice administered with  $2.5 \times 10^{11}$  GC/kg showed significantly reduced serum total bilirubin levels, reaching  $0.5 \pm 0.5$  mg/dL at day 14 (mean  $\pm$  SD, Wilcoxon rank-sum test,  $p < 0.05$ ). By contrast, female mice administered with the same dose of vector ( $2.5 \times 10^{11}$  GC/kg) had serum total bilirubin levels of  $1.0 \pm 0.5$  mg/dL at day 14 (mean  $\pm$  SD). This reduction in bilirubin was transient in some mice administered with  $2.5 \times 10^{11}$  GC/kg, with two males and three females showing rising levels after day 14 after vector administration. In male mice, administration of the  $2.5 \times 10^{10}$  GC/kg dose reduced serum total bilirubin levels by 79% at day 14 after vector administration; these levels gradually increased to 57% at day 28 and returned to baseline hyperbilirubinemia by day 42 (Figure 1A). Administration of the  $2.5 \times 10^{10}$  GC/kg dose in female UGT1 KO mice did not change serum total bilirubin levels from baseline values. We compared total bilirubin levels across the groups using linear mixed-effect modeling and stratified the analysis by sex. We observed a significant reduction in total bilirubin compared to the respective control group for all dose groups with the exception of female mice administered with  $2.5 \times 10^{10}$  GC/kg ( $p < 0.05$ ).

### Dose-Dependent Elevations in Transaminases

Antech GLP also analyzed blood chemistries throughout the in-life phase of the study and at the time of necropsy. We evaluated alanine transaminase (ALT) (Figure 2A) and aspartate transaminase (AST) (Figure 2B) values for male and female mice after vector administration and compared them to control mice (e.g., the average value for day 28 vector-administered mice was compared to the average value for day 28 control male mice). Transaminase abnormalities were primarily present in a dose-dependent manner on day 28 after vector administration in males that received the highest vector dose. Only small changes in ALT or AST levels were present among mice that received lower vector doses. Elevations in ALT greater than 4-fold above baseline levels were restricted to the highest dose group ( $2.5 \times 10^{13}$  GC/kg), with the exception of one male mouse in the lowest dose group ( $2.5 \times 10^{10}$  GC/kg) at day 14 and one male mouse that received a dose of  $2.5 \times 10^{11}$  GC/kg at day 28 (Figure 2A). Elevations in AST showed a similar pattern but were less than 4-fold above baseline levels, with the exception of one female that received a dose of  $2.5 \times 10^{12}$  GC/kg at day 56 (Figure 2B).

We compared ALT and AST levels across the cohorts using linear mixed-effect modeling, stratified by sex. ALT was significantly elevated compared to the control group in both male and female mice administered with the highest vector dose ( $p = 0.015$  for males;



**Figure 2. ALT and AST Levels in Vector- or Vehicle Control-Injected UGT1 KO Mice**

Male and female UGT1 KO mice received an i.v. injection of  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  GC/kg of AAV8.hUGT1A1co or vehicle control. (A) ALT and (B) AST levels were measured in serum samples. Values are expressed as mean  $\pm$  SD.

$p = 0.049$  for females). By contrast, AST was significantly elevated compared to the control group only in female mice administered with the highest vector dose ( $p = 0.042$ ). However, there was no correlation between the age of mice at the time of vector administration and transaminase elevation ( $R^2 = 0.026$  for ALT and  $R^2 = 0.0005$  for AST at day 28).

### Histopathology Findings

We harvested tissues from all animals at the time of necropsy and stained them with H&E for a full histopathology analysis. An experienced board-certified veterinary pathologist microscopically examined sections from all harvested tissues in a blinded manner for the highest vector dose group and the vehicle control group. Liver sections were evaluated for all dose groups (Table 1). Gross observations were noted in a few animals, including an enlarged mandibular lymph node in a male administered with  $2.5 \times 10^{12}$  GC/kg of vector, a dilated uterus in a female administered with  $2.5 \times 10^{13}$  GC/kg, and a dilated uterus and slight icterus in a female administered with vehicle control.

Microscopic findings were predominantly found in the liver and characterized by centrilobular single-cell hepatocellular necrosis and/or degeneration and mononuclear cell infiltration (Table 1). Observations of centrilobular single-cell hepatocellular necrosis and/or degeneration were minimal in all female mice and most male mice. However, we observed mild centrilobular single-cell hepatocellular

necrosis and/or degeneration in two male mice administered with  $2.5 \times 10^{13}$  GC/kg. Three male mice that received the highest vector dose displayed minimal mononuclear cell infiltration, which was more prevalent in female mice; one female mouse administered with  $2.5 \times 10^{10}$  GC/kg showed mild mononuclear cell infiltration. Other findings in the liver included minimal hepatocellular mitotic figures in one female dosed with  $2.5 \times 10^{11}$  GC/kg, and minimal hepatocellular mitotic figures and minimal bile stasis in one female dosed with  $2.5 \times 10^{12}$  GC/kg. Among male mice that received the highest vector dose, one had minimal hepatocellular mitotic figures and two had bile stasis. We did not detect liver abnormalities in female mice administered with the vehicle control; in males from the control group, we found only minimal centrilobular single-cell hepatocellular necrosis and/or degeneration and one mouse with intracanalicular bile stasis (Table 1).

We only reviewed non-liver tissues for the highest dose ( $2.5 \times 10^{13}$  GC/kg) and the vehicle control groups (Table S2). In the lung, we observed minimal and mild accumulation of acidophilic material in alveolar macrophages in two males and one female administered with the vehicle control. Findings in the uterus consisted of minimal focal subacute inflammation in one female administered with the vehicle control. The skin on the injection site showed mild dermal fibrosis in one female administered with the highest vector dose.

### Liver Vector Genome Copy and Transgene RNA Analysis

At the time of necropsy, we snap froze the liver and extracted DNA and RNA to quantify the vector GC and hUGT1A1 transcript levels, respectively. Both vector GC (Figure 3A) and RNA levels (Figure 3B) increased in a dose-dependent manner, with no detectable levels in the vehicle control mice. The presence or absence of a statistical difference between the sexes administered with the same vector dose was consistent across vector GC and RNA levels, with the exception of mice dosed with  $2.5 \times 10^{10}$  or  $2.5 \times 10^{12}$  GC/kg. Male mice dosed at  $2.5 \times 10^{10}$  GC/kg showed significantly higher hUGT1A1 RNA levels than females that received the same dose ( $p < 0.01$ , Mann-Whitney test). We observed a correlation between hUGT1A1 RNA levels and serum total bilirubin levels among mice administered with the  $2.5 \times 10^{11}$  GC/kg dose. More specifically, we observed higher bilirubin levels on day 56 occurring in mice with lower levels of hUGT1A1 RNA.

### hUGT1A1 Expression in the UGT1 KO Mouse Liver

We collected liver samples at necropsy to measure hUGT1A1 expression at both mRNA and protein levels. We performed ViewRNA *in situ* hybridization (ISH) to detect hUGT1A1co mRNA expression and immunohistochemistry to evaluate protein expression (Figure 4). To avoid cross-hybridization with any potential endogenous mouse RNA from the gene locus, we used an ISH probe that is specific to the codon-optimized hUGT1A1 sequence. Whereas ISH and immunohistochemistry staining increased with vector dose in female mice, male mice that were given the highest vector dose showed a slight reduction in staining. Although quantification of the ISH images confirmed this sex-specific observation, the difference was not

**Table 1. Liver Histopathology Scoring**

Dose (GC/kg)	$2.5 \times 10^{10}$		$2.5 \times 10^{11}$		$2.5 \times 10^{12}$		$2.5 \times 10^{13}$		Vehicle Control	
Sex	M	F	M	F	M	F	M	F	M	F
Single-cell hepatocellular necrosis and/or degeneration, centrilobular										
Minimal	2/5	2/5	1/5	4/5	3/5	2/5	1/5	1/5	1/5	–
Mild	–	–	–	–	–	–	2/5	–	–	–
Mononuclear cell infiltration										
Minimal	–	1/5	1/5	5/5	–	2/5	3/5	1/5	–	–
Mild	–	1/5	–	–	–	–	–	–	–	–
Mitotic figures, hepatocellular										
Minimal	–	–	–	1/5	–	1/5	1/5	–	–	–
Bile stasis										
Minimal	–	–	–	–	–	1/5	2/5	–	1/5	–

Male and female UGT1 KO mice received an i.v. injection of  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  GC/kg of AAV8.TBG.hUGT1A1co or vehicle control (n = 5/sex/group). Liver sections positive for histopathology were scored as minimal, mild, moderate, marked, or severe. M, male; F, female.

statistically significant (Figure 4B). A stepwise increase of the dose by 10-fold did not result in significantly increased transgene expression, with the exception of male mice administered with  $2.5 \times 10^{11}$  and  $2.5 \times 10^{12}$  GC/kg that showed a statistically significant increase (Figure 4B).

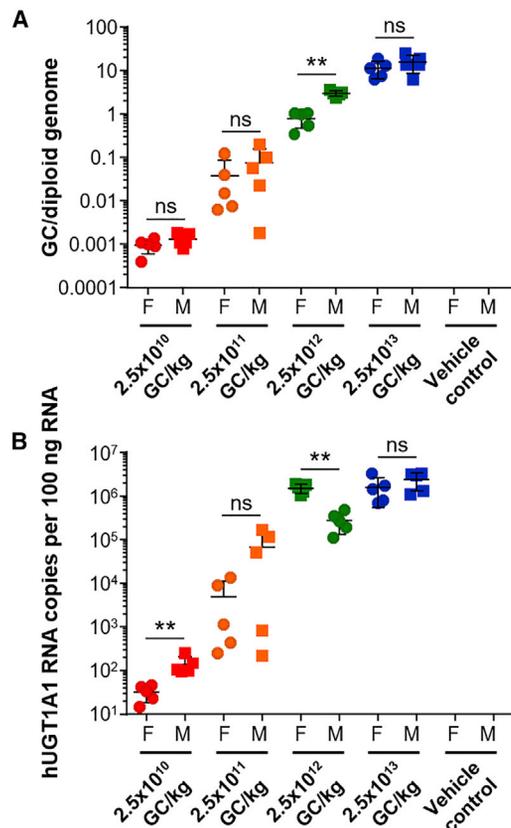
## DISCUSSION

We took several factors into consideration while designing this study. We chose to conduct the experiments in UGT1 KO mice, rather than wild-type mice or Gunn rats, for two reasons. First, we wanted to evaluate vector-associated toxicity within the setting of any pathology associated with the defect in UGT1A1, the associated hyperbilirubinemia, and its sequelae. Although we did not expect any liver pathology in the model, we were concerned that some level of chronic severe hyperbilirubinemia could influence the response of the host liver to the vector. Second, although others have previously evaluated adeno-associated viral (AAV) gene therapy for Crigler-Najjar syndrome in the classical animal model, the Gunn rat, this model does not require phototherapy treatment to survive to adulthood.<sup>5,11–13</sup> In a short-term study, i.v. administration with  $5 \times 10^{11}$  vector genomes (vg)/kg corrected total bilirubin levels in Gunn rats, and in a long-term study, seven of ten Gunn rats showed improvement following a dose of  $5 \times 10^{12}$  vg/kg.<sup>13</sup> The transient affect seen in three male rats was attributed to growth of the animal following vector administration at 6–8 weeks of age. Therefore, for the determination of the MED, we chose to use the more severe mouse model of Crigler-Najjar syndrome, the UGT1 KO mouse. Similar vector doses have been evaluated previously by others in *Ugt1*<sup>–/–</sup> mice, with correction of total bilirubin levels seen following intraperitoneal injection of  $8 \times 10^9$  vg/mouse at post-natal day (P) 11<sup>13</sup> (which equates to a dose of  $\sim 1.6 \times 10^{12}$  vg/kg, assuming a P11 animal weighs  $\sim 5$  g<sup>14</sup>). Although this dose is approximately 10-fold higher than the MED reported here, the route of administration and age at dosing have to be taken into consideration when comparing efficacy.

In this study, male and female UGT1 KO mice aged 6–20 weeks received an i.v. injection of our clinical candidate vector, AAV8.TBG.hUGT1A1co, at one of four doses:  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  GC/kg. We chose these doses to reflect the span of the proposed dosing regimen in a clinical trial. An additional cohort of animals received vehicle control. We collected blood samples from animals during the in-life phase to capture serum total bilirubin levels. On day 56 after administration, we necropsied the mice and harvested tissues for a comprehensive histopathological examination. We did not observe any clinical sequelae during the study.

Although we observed histopathological findings in male mice administered with the vehicle control, most findings were in male mice administered with the highest vector dose. All findings were minimal to mild, and microscopic findings were predominantly in the liver, characterized by centrilobular single-cell hepatocellular necrosis and mononuclear cell infiltration. In addition, we observed hepatocellular mitotic figures and minimal bile stasis. However, the liver findings occurred at all dose levels and in one vehicle control mouse. As the severity of the findings in the liver was consistent between the control- and the vector-administered groups, these can be considered background findings in the UGT1 KO mouse. Therefore, we observed no dose-limiting toxicities, and we can conclude that the maximally tolerated dose based on this mouse model study was greater than or equal to the highest dose tested ( $2.5 \times 10^{13}$  GC/kg).

We observed a dose-dependent increase in liver transaminases; however, there was only a significant difference in ALT and AST following administration of the highest dose of vector ( $2.5 \times 10^{13}$  GC/kg). The peak ALT and AST values occurred at day 28 and resolved by the end of the study. Although we did not evaluate immune responses to the AAV8 capsid, the hUGT1A1 transgene, or antibodies to hUGT1A1, there was no evidence of loss of transgene expression indicative of an adaptive immune response in these mice. In mice administered



**Figure 3. Liver Vector Biodistribution and hUGT1A1 RNA Transcript Levels in Vector- or Vehicle Control-Injected UGT1 KO Mice**

Male and female UGT1 KO mice received an i.v. injection of  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  GC/kg of AAV8.TBG.hUGT1A1co or vehicle control. At necropsy, livers were harvested and snap frozen. (A) DNA was extracted for quantification of vector GC. (B) RNA was extracted for quantification of hUGT1A1 transcript levels. Values are plotted as mean  $\pm$  SD. NS, not significant. \*\* $p < 0.01$ .

with  $2.5 \times 10^{13}$  GC/kg of vector, we did not observe a rebound in serum total bilirubin levels. Furthermore, liver vector genomes and hUGT1A1 RNA levels determined after necropsy on day 56 increased in a dose-dependent manner; we observed a slight reduction in ISH staining in male mice administered with the highest vector dose, but this difference was not statistically significant. In addition, the finding of minimal or mild mononuclear cell infiltration in this group by histopathological analysis was the same as in several vector-administered groups.

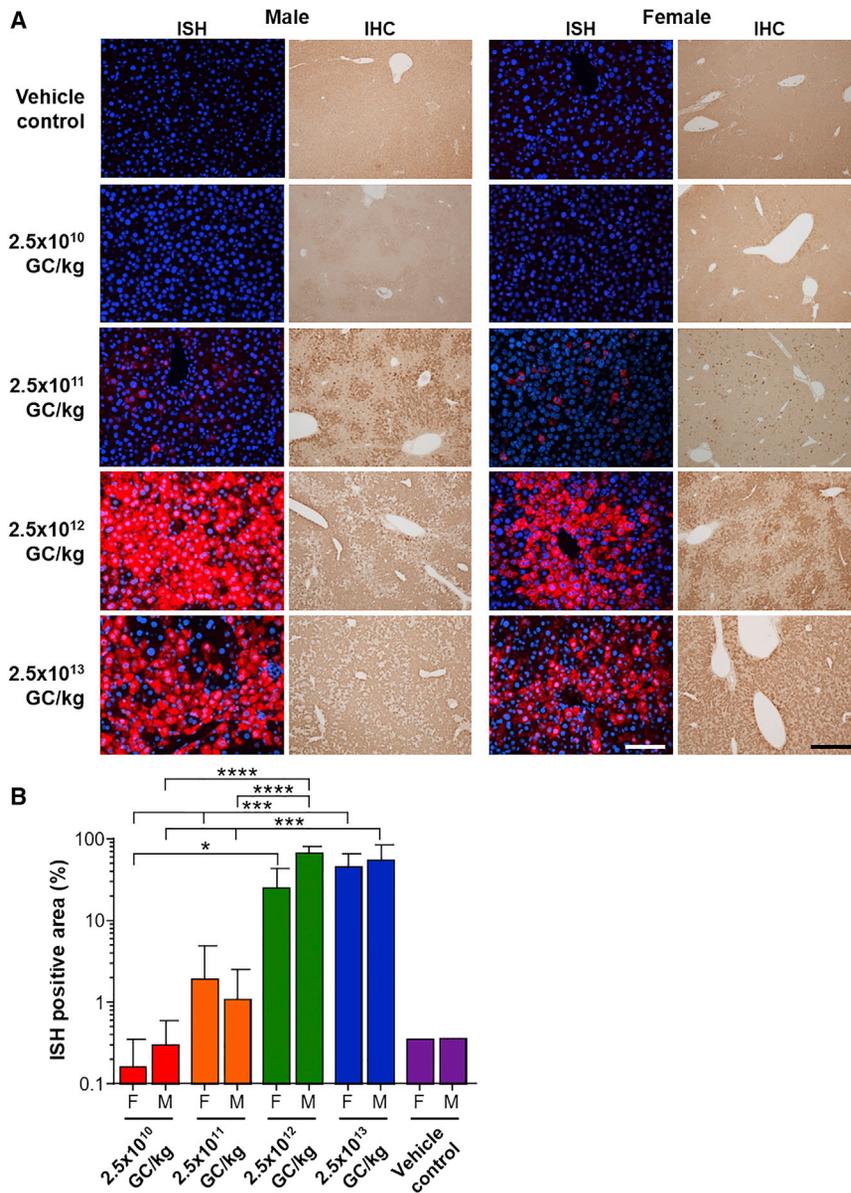
Following vector administration at doses of  $2.5 \times 10^{12}$  and  $2.5 \times 10^{13}$  GC/kg, serum total bilirubin levels rapidly and completely reversed to baseline levels of 0.1–0.3 mg/dL by day 14 after vector administration. At vector doses greater than  $2.5 \times 10^{10}$  GC/kg, total bilirubin levels were significantly reduced ( $p < 0.05$ ). However, at the lowest dose ( $2.5 \times 10^{10}$  GC/kg), the reduction in total bilirubin levels was temporary in male mice, and serum total bilirubin levels returned to baseline hyperbilirubinemia by day 42. At a dose of  $2.5 \times 10^{11}$  GC/kg, we noticed some

variation in the response across five mice of each sex. Two males and three females showed a transient decrease in total bilirubin, with levels rising after day 14 after vector administration.

The transient serum total bilirubin levels following administration of  $2.5 \times 10^{11}$  GC/kg in both males and females could potentially allude to a threshold effect at this vector dose, such that a dose of  $2.5 \times 10^{11}$  GC/kg is effective in some animals, but not others. Again, because four of the five mice with transient expression also showed a concurrent rise in ALT, peaking at day 28, and reduced vector GC and RNA levels compared to other mice in the group, this effect could be due to an immune response to the vector or human transgene. We did not harvest splenocytes at the time of necropsy to determine the presence of a cytotoxic T lymphocyte response. Although these mice had minimal histopathology findings, the findings were not enhanced compared to other mice in the group or in other vector dose groups. Transient effects of gene therapy are commonly seen following newborn administration due to rapid growth of the animal.<sup>4,8,10,14</sup> However, it is unlikely that the transient effect seen here was due to growth of the mice during the study, because the average body weight only increased from  $18.8 \pm 2.5$  to  $22.0 \pm 2.2$  g (mean  $\pm$  SD) over the two-month study.

Administration of the lowest vector dose ( $2.5 \times 10^{10}$  GC/kg) in female UGT1 KO mice did not result in deviation from baseline total bilirubin values; however, there was a 79% reduction in total bilirubin in male mice at the same dose. In addition, although there was no significant difference in liver vector GCs between male and female mice administered with  $2.5 \times 10^{10}$  GC/kg of vector, male mice showed 4-fold higher hUGT1A1 RNA levels compared to females. This indicates that gene transfer to hepatocytes was similar but that the higher transgene RNA levels in males was sufficient to be transiently therapeutic. Other researchers have reported a sex difference in both transgene expression and vector GC following vector dosing in mice.<sup>9,15</sup> Following i.v. administration of  $3.3 \times 10^{11}$  vg/mouse (calculated by the authors to be  $\sim 1.1 \times 10^{13}$  vg/kg) at P60 in Ugt1<sup>-/-</sup> mice, male mice showed a correction of total bilirubin to wild-type levels. However, female mice showed a transient reduction in total bilirubin levels with a significant reduction in vector genomes.<sup>9</sup> This sex difference does not appear to translate to non-human primates.

Based on the significant reduction in serum total bilirubin levels in mice following vector administration, the MED in this mouse model of Crigler-Najjar syndrome is  $2.5 \times 10^{11}$  GC/kg. The dose required for sustained and complete normalization of total bilirubin levels was  $2.5 \times 10^{12}$  GC/kg; however, because the doses evaluated here increased by one log, it is possible that a dose between  $2.5 \times 10^{11}$  and  $2.5 \times 10^{12}$  GC/kg will also achieve this. The MED corresponds to approximately 1.9% and 1.2% transduction of hepatocytes in female and male mice, respectively, based on ISH data (Figure 4B). This is the same percentage of liver transduction in adult mice that is retained following newborn systemic vector administration and was sufficient to rescue UGT1 KO mice from lethal hyperbilirubinemia.<sup>10</sup>



**Figure 4. hUGT1A1 Expression in Liver of Vector-Injected UGT1 KO Mice**

Male and female UGT1 KO mice received an i.v. injection of  $2.5 \times 10^{10}$ ,  $2.5 \times 10^{11}$ ,  $2.5 \times 10^{12}$ , or  $2.5 \times 10^{13}$  GC/kg of AAV8.TBG.hUGT1A1co or vehicle control. (A) ISH and immunohistochemistry (IHC) staining were performed on liver sections for detection of hUGT1A1. In ISH sections, hUGT1A1co RNA is shown with red staining, and sections were counterstained with the nuclear stain DAPI (blue). Representative images are from mice with stable serum total bilirubin levels. Scale bars: 100  $\mu$ m for ISH, 500  $\mu$ m for IHC. (B) ISH staining was quantified, and the percentage of positively stained area was calculated. Values are expressed as mean  $\pm$  SD. \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

used for this study were derived from this colony. All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Mice were individually housed in disposable micro-isolator mouse caging with corncob bedding and Nestlets provided for enrichment (Innovive, San Diego, CA). Certified irradiated Laboratory Rodent Diet 5002 (LabDiet, St. Louis, MO) was provided *ad libitum*. All interventions were performed during the light cycle, and mice were not fasted before blood collection.

Male and female UGT1 KO mice ( $n = 51$ , 26 male and 25 female) 6 to 20 weeks in age and greater than 15 g in body weight were used in this study and necropsied at day 56 after administration (Table S1). Group dosing dates were staggered based on availability of UGT1 KO mice. At the initiation of the study, available mice within the dosing age range were randomly assigned first to the high-dose group ( $2.5 \times 10^{13}$  GC/kg) or to the control group by an online program (Research Randomizer, <http://www.randomizer.org/>). Subsequently, mice were assigned to dose groups in the following order:  $2.5 \times 10^{12}$ ,  $2.5 \times 10^{10}$ , and  $2.5 \times 10^{11}$  GC/kg.

#### Serum Analyses

Blood was collected every other week and allowed to clot. Serum was isolated and sent to Antech GLP (Morrisville, NC) for analysis of ALT, AST, and total bilirubin levels.

#### Histopathology

At day 56 after administration, mice were euthanized. Blood was collected by cardiac puncture, and a full necropsy was performed. Tissues were harvested for comprehensive histopathological examination (see Table S3 for the list of harvested tissues). Tissues were fixed

## MATERIALS AND METHODS

### AAV Vector Production

All AAV vectors were produced by the Penn Vector Core at the University of Pennsylvania, as described previously.<sup>16</sup> Plasmids expressing a codon-optimized version of hUGT1A1 from the TBG promoter were packaged with the AAV8 viral capsid to create the single-stranded vector, AAV8.TBG.hUGT1A1co.

### Mice

Breeding pairs of heterozygous Ugt1<sup>+/-</sup> mice (mixed B6 and 129 background strains) were obtained from The Jackson Laboratory (Bar Harbor, ME), and a colony was maintained at the University of Pennsylvania under specific pathogen-free conditions. All mice

using 10% neutral buffered formalin, paraffin embedded, sectioned, and stained for histopathology using H&E stain. An experienced board-certified veterinary pathologist microscopically examined all tissues from the highest vector dose group and the vehicle control group in a blinded manner. Liver sections were evaluated for histopathology from all dose groups in the same manner.

### Immunohistochemistry

Liver sections were deparaffinized through an ethanol and xylene series, boiled for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval, and sequentially treated with 2% H<sub>2</sub>O<sub>2</sub> (15 min), avidin and biotin blocking reagents (15 min each; Vector Laboratories, Burlingame, CA), and blocking buffer (1% donkey serum in PBS with 0.2% Triton for 10 min). Sections were incubated with 5 µg/mL of primary antibody against UGT1A1 (R&D Systems, Minneapolis, MN) for 1 hr and then with a biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted in blocking buffer for 45 min. A Vectastain Elite ABC Kit (Vector Laboratories) was used according to the manufacturer's instructions with 3,3'-diaminobenzidine as the substrate to stain bound antibodies as a brown precipitate.

### In Situ Hybridization

ISH was performed on formalin-fixed, paraffin-embedded liver sections using the ViewRNA ISH Tissue Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Z-shaped probe pairs specific for hUGT1A1co were synthesized by the kit manufacturer. The deposition of Fast Red Substrate (Thermo Fisher Scientific) precipitates indicating positive signals was imaged by fluorescence microscopy using a rhodamine filter set. Sections were counterstained with DAPI to visualize nuclei.

To quantify RNA expression, five random pictures were taken from each liver section. Using ImageJ software (<https://imagej.nih.gov/ij/>), a threshold was determined to select UGT1A1-ISH-positive areas, and the percentage of positive area per image area was established. Likewise, an empty liver area (i.e., veins) was quantified in a second measurement. The final percentage of ISH-positive liver tissue (i.e., the percentage of positive hepatocytes) was calculated as the adjusted area (i.e., total area minus empty area), and the values were averaged for each liver.

### Vector Genome Copy and Transgene RNA Analysis

Liver was snap frozen at the time of necropsy, and DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). Detection and quantification of vector GC in extracted DNA and relative hUGT1A1 transcript expression in extracted RNA were performed by real-time PCR, as described previously.<sup>17,18</sup> Vector GC and RNA levels were quantified using primers and probe designed against the poly(A) sequence of the vector and a transgene-specific sequence, respectively.

### Statistical Analysis

We calculated and reported the cohort average and SD for ALT, AST, total bilirubin, vector GC, and hUGT1A1 RNA transcript data. Differ-

ences in ALT, AST, and total bilirubin levels compared to baseline were analyzed statistically by the Wilcoxon rank-sum test, and overall differences in ALT, AST, and total bilirubin values across all time points were analyzed using a linear mixed-effect model. Analyses were stratified by sex. Comparisons between two groups were performed using a non-parametric Mann-Whitney test, and comparisons among multiple groups were performed using a one-way ANOVA (Tukey's multiple comparison post-test). A p value < 0.05 was considered significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables and can be found with this article online at <https://doi.org/10.1016/j.omtm.2018.07.008>.

### AUTHOR CONTRIBUTIONS

Conceptualization, J.A.G., J.T.G., and J.M.W.; Methodology, J.A.G. and J.M.W.; Validation, E.A.C.; Investigation, J.A.G., J.M.L.N., C.D., D.M., P.B., and L.K.R.; Writing – Original Draft, J.A.G.; Writing – Review & Editing, J.A.G., J.T.G., and J.M.W.; Visualization, J.A.G. and J.M.W.; Supervision, J.A.G., J.T.G., and J.M.W.; Funding Acquisition, J.M.W.

### CONFLICTS OF INTEREST

J.M.W. is an advisor to, holds equity in, and has a sponsored research agreement with REGENXBIO and Scout Bio; he also has sponsored research agreements with Ultragenyx, Biogen, and Janssen, which are licensees of Penn technology. In addition, he has sponsored research agreements with Precision Biosciences and Moderna Therapeutics. J.M.W. holds equity in Solid Bio, and he is an inventor on patents that have been licensed to various biopharmaceutical companies.

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