Chapter 2

Characterization of the hyperbilirubinemic and hypocholesterolemic phenotype of the UGT1A1\textsuperscript{j/BluHsdRrrc} Gunn rat strain

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Abstract

Unconjugated hyperbilirubinemia occurs in physiological jaundice in neonates and in patients with Crigler-Najjar syndrome. A widely used animal model for unconjugated hyperbilirubinemia is the Gunn rat, which has a deficiency in the bilirubin-conjugating enzyme uridine diphosphoglucuronosyl transferase (UGT1A1). Various Gunn rat strains are available, which exhibit differences in phenotype and response to treatment. For studies testing the efficacy of bilirubin-lowering treatment strategies, homozygous Gunn rats have been used, such as the commonly used RHA/jj strain. However, using only homozygously Ugt1a1-deficient Gunn rats doesn’t allow analysis of other clinically relevant phenotypes such as reduced plasma levels of low-density lipoprotein cholesterol (LDL-C). To obtain a better insight into the role of Ugt1a1 in the bilirubin and LDLc phenotype, we analyzed neonatal and adult rats of all three genotypes of the Gunn-Ugt1a1\textsuperscript{J/BluHsdRrrc} strain. We first determined the effect of a high-fat diet (HFD) on plasma levels of unconjugated bilirubin (UCB). In neonatal Gunn rats, plasma and brain levels of UCB were increased at all time points as compared to those of heterozygous and wild type littermates and this was most prominent before postnatal day 21. Furthermore, plasma and brain UCB levels of heterozygous pups displayed a small but significant increase as compared to wild type littermates. On chow, adult homozygous female Gunn rats displayed decreased levels of LDLc in plasma, as compared to heterozygous and wild type rats. In line with earlier findings in the RHA/jj strain, a HFD significantly decreased plasma and biliary UCB in Gunn rats. We conclude that the hypobilirubinemic response to dietary fat treatment is independent of the Gunn rat strain. Since heterozygous Gunn rats have slightly increased plasma levels of UCB, the use of wild type littermates as normobilirubinemic controls for Gunn rat studies is preferred in studies aimed to mimic human unconjugated hyperbilirubinemia.
Introduction

Severe unconjugated hyperbilirubinemia or jaundice can occur in preterm neonates and patients with Crigler-Najjar syndrome and, when left untreated, can cause kernicterus which eventually can be lethal. UCB is the breakdown product of heme, derived from heme-containing molecules such as haemoglobin in erythrocytes. In plasma, UCB is bound to albumin and transported to the liver where it can be conjugated by UGT1A1 into less toxic and more water-soluble bilirubin monoglucuronide and bilirubin diglucuronide. Conjugated bilirubin (CB) is transported across the bile canalicular membrane and secreted into bile by the ATP-binding cassette transporter 2 (ABCC2, MRP2). UGT1A1 is also expressed in the human intestine where it contributes to conjugation of UCB, especially in neonates as they do not yet fully express UGT1A1 in the liver. Under physiological conditions beyond neonatal age, around 98% of the bilirubin secreted into the bile is CB and less than 2% is UCB. Under conditions of UCB accumulation in the body (unconjugated hyperbilirubinemia), UCB can be excreted, both into the bile and across the intestinal epithelium (see for review). Alternatively, it can be oxidized by cytochrome P450 family 1A1 (CYP1A1) and 1A2 (CYP1A2) after which its oxidation products are secreted into the bile, functioning as an alternative pathway to ameliorate unconjugated hyperbilirubinemia. In the intestinal lumen, CB is deconjugated into UCB by the hydrolysing enzyme β-glucuronidase, which is present in the intestinal microbiota but also in breast milk. After hydrolysis, UCB can be taken up by enterocytes and transported back to the liver, thereby completing its enterohpatic circulation (EHC). UCB that has not been taken up by the enterocytes is excreted via the stool, either as UCB or as non-toxic urobinoids or other products of microbial catabolism. Since an enhanced EHC of bilirubin contributes to unconjugated hyperbilirubinemia, interrupting the EHC is a strategy to lower plasma bilirubin levels. Previously, we demonstrated that plasma bilirubin can be lowered by capturing luminal UCB in unabsorbed fat, for example by increasing fecal fat excretion through dietary strategies such as a HFD or administration of the lipase inhibitor orlistat. In these studies, we used the Gunn rat, a well-established animal model for studying severe unconjugated hyperbilirubinemia, due to the complete absence of the conjugating activity by UGT1A1. The specific Gunn rat strain we used, RHA/jj, has been homozygously bred for many generations, precluding the comparison to heterozygous or wild type littermates. Furthermore, using only homozygously Ugt1a1-deficient Gunn rats doesn’t allow analysis of other clinically relevant phenotypes such as its possible effect on plasma lipids. We therefore chose to establish a new colony of Gunn rats in our facility, which could be used to compare and obtain a better insight into the effect of Ugt1a1 on the bilirubin and plasma lipid phenotype between homozygous Gunn rats and heterozygous and wild type littermates.

Several Gunn rat strains with different genetic backgrounds exist, with varying levels of plasma bilirubin levels. For example, Leyten et al. reported mean serum total bilirubin levels of ~150 µmol/L in the Gunn rat strain R/APfd-j/j. In 1997, Kotal et al. reported serum
bilirubin levels of ~80 µmol/L in the RA/jj Gunn rat strain, and of ~120 µmol/L in the RHA/jj Gunn rat strain. Finally, Kordes et al. reported a mean serum UCB concentration of ~177 µmol/L in the Gunn-Ugt1a1/Jr/BluHsdRrrc strain, whereas Bakrania et al. observed lower blood UCB levels (~46 µmol/L) in rats of the same strain. These differences in serum bilirubin levels indicate variations in bilirubin metabolism that could be caused by strain or environmental differences such as housing conditions, microbiome or diet.

We established a colony of Gunn rats from the Gunn-Ugt1a1/Jr/BluHsdRrrc strain, that we purchased from RRRC. This strain has been used in several other studies but their bilirubin metabolism has never been extensively characterized. Moreover, it is not known to what extent heterozygous Gunn rats exhibit differences from wild type rats in terms of bilirubin and lipid metabolism, and thus could be used as normobilirubinemic controls. Literature indicated a link between plasma bilirubin levels and a plasma lipid phenotype under mild hyperbilirubinemic conditions.

Individuals with Gilbert Syndrome (GS) have mildly elevated plasma levels of UCB due to an increased number of TA repeats in the UGT1A1 promoter. Recently, an association between a mildly increased bilirubin profile and an effect on circulating LDL-C and triglyceride (TG) levels, and a higher protection against cardiovascular and metabolic diseases was demonstrated in GS individuals. However, the underlying mechanism of this protective beneficial association of bilirubin has not been fully elucidated. Here we assessed the bilirubin and lipid phenotype in wild type, heterozygous and homozygous Gunn-Ugt1a1/Jr/BluHsdRrrc rat littermates in neonatal and adult conditions and determined to what extent these rats can serve as a reliable model to study human normo- and hyperbilirubinemia.

**Methods**

**Animals**

Heterozygous Gunn rats (Gunn-UGT1A1/Jr/BluHsdRrrc) were obtained from the Rat Resource & Research Centre (Columbia, Missouri, USA) and bred in the animal facility of the University Medical Centre Groningen (Groningen, the Netherlands). Animals were housed in a light- and temperature-controlled facility (12h dark/light rhythm, 21°C) with ad libitum access to normal drinking water and control semi-synthetic low-fat diet (LFD) (code 4063.02, Hope Farms B.V., Woerden, The Netherlands). Animal experiments were performed with the approval of the local Ethics Committee for Animal Experiments of the University of Groningen. All experiments were performed in accordance with relevant guidelines and regulations (including laboratory and biosafety regulations).
Experimental design

Neonatal characterization

Litters from breeding pairs formed of a heterozygous female and a homozygous male Gunn rat were used to acquire heterozygous and homozygous Gunn rat pups. From breeding pairs consisting of a heterozygous male and heterozygous female rat, heterozygous pups and wild type littermates were obtained. Neonatal characterization of this strain was performed in wild type (n=11), heterozygous (n=8) and homozygous (n=11) Gunn rat pups of both gender with the age of 4 – 42 days. The rat pups were housed per litter with 9 – 11 animals per cage. The body weight of the pups was determined daily and transcutaneous bilirubin (TcB) was measured at postnatal days 4, 7, 14, 21 and 42. In order to determine plasma UCB in homozygous pups, 5 animals per time point were terminated. Blood was collected by decapitation in EDTA-coated collecting tubes (MiniCollect, Greiner Bio-One, Kremsmünster, Austria) on ice under light-protecting conditions and processed immediately. Brains were snap-frozen upon harvesting. Plasma and brains were stored in amber-coloured tubes (Eppendorf, Hamburg, Germany) under argon gas at -80°C upon analysis.

Adult characterization under different dietary conditions

We aimed to study the effect of a HFD on plasma UCB in the Gunn-UGT1A1/J/HsdRrc strain, similar to our earlier report in the RHA/jj Gunn rat strain. The effect of dietary fat intake on bilirubin metabolism in this strain was performed using adult (age 14–18 weeks) individually housed homozygous wild type, heterozygous and homozygous Gunn rats of either gender. A run-in period of 4 weeks on a semi-synthetic LFD (code 4063.02, Hope Farms B.V., Woerden, The Netherlands) was introduced for all rats (n=8 per gender and genotype). After this run-in period, half of the animals per genotype randomly received a semi-synthetic HFD (code 4141.07, Hope Farms B.V., Woerden, The Netherlands) whilst the other half of the group remained on the LFD for a consecutive period of 4 weeks (n=4 per gender and genotype). Determination of the body weight was performed weekly for a total period of 8 weeks. Feces were collected for 24 hours after 4 weeks of LFD (t=4, n=8) and 4 weeks of HFD (t=8, n=4). Blood was sampled from the tail under isoflurane anaesthesia before the start of the run-in LFD (t=0 weeks), after 2 and 4 weeks on run-in LFD (t=2 and 4) and experimental diet (t=6 and 8). Blood was collected in EDTA-coated capillaries and kept in EDTA-coated collecting tubes on ice under light-protecting conditions upon analysis. At t=8 weeks, bile cannulation was performed under isoflurane anaesthesia for 20 minutes and the bile flow was determined gravimetrically (assuming 1g = 1mL bile secretion). Subsequently, blood was collected through cardiac puncture and animals were terminated by decapitation. The liver and brain were harvested and snap-frozen in liquid nitrogen. Plasma, bile and brains were protected from light and stored in amber-coloured tubes under argon at -80°C.
Analytical methods

Transcutaneous bilirubin measurements

Transcutaneous measurement of bilirubin is a commonly used non-invasive method in neonates, and has also been shown to strongly correlate with serum total bilirubin in neonatal Gunn rats. TcB levels were determined using a Minolta Airshield Jaundice Meter (JM-103, Dräger Medical, Lübeck, Germany) and the bilirubinometer was calibrated daily according to the instructions of the manufacturer. The heads of the animals were shaved before three consecutive measurements were performed of which the mean was used (µmol/L).

Plasma and biliary bilirubin measurements

Bilirubin monoglucuronide and bilirubin diglucuronide levels in plasma and bile were determined using ultra high-performance liquid chromatography (UPLC) analysis linked with UV/VIS (Shimadzu, Kyoto, Japan). The protocol was adapted from the method described by Spivak and Carey with adaptations as described below. For precipitation of proteins in plasma, 80 µL of methanol (Merck, Burlington, Massachusetts, USA) with 2 mM dithiothreitol (DTT; Sigma Aldrich, St. Louis, MO) was added to 20 µL plasma from wild type and heterozygous Gunn rats, and 98 µL to 2 µL plasma from homozygous Gunn rats, giving a total volume of 100 µL. To determine bilirubin fractions in bile, an amount of 90 µL methanol/DTT mix was added to 10 µL of bile for wild type, heterozygous and homozygous Gunn rats. Mesobilirubin (MSB, Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal standard and added to plasma of wild type and heterozygous Gunn rats and to bile samples of all three genotypes in a concentration of 1.8 ng/µL. To plasma samples of homozygous Gunn rats, a concentration of 3.6 ng/µL MSB was added. MSB was stored at -80°C in amber-coloured Eppendorf tubes under argon for maximally 2 months till analysis. Subsequently, samples were spun down for 10 minutes (14000g, 4°C) and protected against light by using dark containers. The supernatant was transferred to a centrifugal filter (VWR, Radnor, Pennsylvania, USA) and again spun down for 10 minutes (3000g, 4°C). Finally, the samples were loaded into an amber-coloured vial (2 mL, VWR) and treated with argon gas in order to prevent oxidation. A volume of 20 µL of the sample solution was injected into the UPLC/UV-VIS instrument and chromatography was performed with an Acquity UPLC BEH C18 column (2.1x100 mm, 1.7 µM, Waters, Milford, MA, USA). An Acquity UPLC BEH C18 guard column (2.1x5 mm, 1.7 µM, Waters) and a pre-column filter (Waters, Milford, MA, USA) were fitted to the analytical column. Plasma and bile bilirubin fractions were separated by eluting the column with a rate of 0.4 mL/min and a starting eluent of 30% (v/v) dissolvent A (0.1% formic acid in Millipore water) and 70% dissolvent B (0.1% formic acid in acetonitrile) for 16 minutes, followed by a linear gradient to 100% dissolvent A in 17.5 minutes, continuing to 21.5 minutes, and from 100% eluent B to 30% eluent B in 1 minute. The column temperature was 30°C and the bilirubin fractions were detected at 450 nm. Analysis and interpretation
of the chromatograms were done with Analyst MD Software (version 1.6.2, ScieX). Biliary UCB secretion rates were calculated using the bile flow (μL/h) and corrected for body weight (grams).

**Brain bilirubin measurements**

Bilirubin levels in whole brains were analysed using high-performance liquid chromatography (HPLC) with diode array detector (Agilent, Santa Clara, CA, USA) as described earlier.

**Plasma lipid levels & lipoprotein profile**

Plasma total cholesterol (TC), triglycerides (TG) and non-esterified fatty acids (NEFA) were analyzed using commercially available kits (Roche Diagnostic, Basel, Switzerland and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma lipoproteins of individual rats were fractionally separated and determined by using fast-performance liquid chromatography (FPLC) on a Jasco FPLC system (Easton, MD, USA) as described earlier. The plots of the individual FPLC profiles were generated with R, version 3.6.1 (2019-07-05) using RColorBrewer_#fff7bc, #fec44f and #d95f0e.

**Fecal neutral sterols (FNS) determination**

Feces were collected of individually housed adult rats over a period of 24h. Fecal total neutral sterols were extracted from 50 mg of dried, homogenized feces and measured by gas-liquid chromatography as described earlier.

**Gene expression analysis**

Total RNA isolation from liver was performed using TRI- Reagent (Sigma, St. Louis, USA). Quantification of RNA was done by NanoDrop (NanoDrop Technologies, Wilmington, DE) and subsequently 1 μg was used to produce cDNA using a mix of the appropriate primers and probes. For amplification of cDNA for quantitative PCR (qPCR) either Hi-ROX SensiMix SYBR Green (Bioline, London, UK) or Taqman Fast Mix (Applied Biosystems, Foster City, CA) was used and analysed on a QuantStudio 7 Flex machine (Applied Biosystems, Thermo Fisher, Darmstadt, Germany). Gene expression levels were normalized using the housekeeping gene 36B4. An overview of the Taqman and SYBR primer sequences can be found in respectively Supplemental Table 1 and 2.

**Statistics**

For statistical analysis, GraphPad Prism 8.01 (GraphPad Software, La Jolla, CA, USA) was used. Differences in body weight between three different genotypes over days of age in pups and between genotypes over weeks in adult rats were tested using a two-way ANOVA repeated measurement followed by a Tukey’s multiple comparisons test. Statistical significance of transcutaneous, plasma and brain bilirubin were tested with an
one-way ANOVA Friedman test, followed by Dunn’s multiple comparisons test. The effect of diet on plasma and biliary bilirubin between adult wild type and heterozygous Gunn rats was tested using an one-way ANOVA Kruskal-Wallis test followed by Dunn’s multiple comparisons test. Differences in plasma and biliary bilirubin in homozygous Gunn rats were assessed using a non-parametric Mann-Whitney U-test. Differences in plasma lipid profile and hepatic gene expression were tested using a Kruskal-Wallis test followed by Dunn’s multiple comparisons test. All values are given as means ± standard deviation (SD) unless stated otherwise. Significance is indicated as * P < 0.05, ** P < 0.01, *** P < 0.001.

Results

Natural course of plasma and brain UCB levels in neonatal Gunn rat pups

A lower body weight has been reported for homozygous Gunn rats of both the RHA/jj strain and Gunn-UGT1A1j/BluHsdRrrc as compared to a combined group of wild type and heterozygous control rats. Because these studies did not distinguish between wild type and heterozygous Gunn rats, the question remained if wild type and heterozygous animals differ in body weight. Interestingly, in our current study, heterozygous pups of the Gunn-Ugt1a1j/BluHsdRrrc strain had a significantly higher body weight as compared to wild type and homozygous Gunn rat pups, starting from postnatal day (PD) 18 (Figure 1A). To assess their bilirubin phenotype, TcB levels were measured over time in wild type, heterozygous and homozygous Gunn rat pups (Figure 1B). In wild type and heterozygous pups, TcB was below the limit of detection at all time points. In homozygous Gunn rat pups, TcB levels peaked at PD13, and significantly decreased thereafter until the end of follow up, at PD42 (P=0.03). To compare TcB with plasma UCB levels (biochemically assessment using UPLC), homozygous Gunn rat pups were terminated at different ages. Plasma levels of UCB were similar between PD4 and PD21, after which a significant decrease in UCB was observed between PD21 and PD42 (-80%, p=0.0014) (Figure 1C).

During unconjugated hyperbilirubinemia, UCB can accumulate in the brain, eventually causing kernicterus. Levels of UCB in whole-brain homogenates of homozygous Gunn rat pups displayed a similar pattern as plasma UCB levels (Figure 1D). Dependent on the method of bilirubin analysis (TcB or UPLC), the peak in the natural UCB course in this Gunn-Ugt1a1j/BluHsdRrrc strain in hyperbilirubinemic rat pups occurred between PD13 and 21.
Effect of a HFD on plasma levels of UCB

As indicated above, previous studies have reported varying plasma UCB levels between different strains of Gunn rats. However, little is known about the levels of plasma UCB between wild type and heterozygous Gunn rats of the same strain. Therefore, plasma UCB levels of adult wild type, heterozygous and homozygous rats were assessed in response to a HFD, a condition known to affect bilirubin levels in RHA/j Gunn rats. Increasing dietary fat content has been an effective manner to decrease plasma UCB in homozygous RHA/j Gunn rats, but its effects in the Gunn-Ugt1a1/BlahsdRrrc strain were unknown. We first characterized the effect of dietary fat on body weight and bilirubin levels in adult wild type,
heterozygous and homozygous male and female Gunn-Ugt1a1/JmuHsdRrrc rats. In both males and females, no difference in initial body weight was found between wild type and heterozygous rats. However, female homozygous Gunn rats showed a lower initial body weight compared to normobilirubinemic littermates (Figure 2A, B). After 2 to 4 weeks on a LFD, male heterozygous rats were significantly heavier compared to wild type and homozygous male Gunn rats (Figure 2A). Administration of a HFD for a period of 4 weeks increased the body weights of all genotypes and obliterated the significant difference between heterozygous and homozygous male Gunn rats. No difference in body weight between wild type and heterozygous animals was observed after 4 weeks of HFD. The body weight of female heterozygous rats was not significantly different from wild type females but higher compared to female homozygous Gunn rats under LFD and HFD conditions (Figure 2B). Plasma UCB was significantly higher in heterozygous rats compared to wild type rats under LFD conditions, but this difference disappeared upon feeding the HFD (Figure 2C). In line with earlier studies in the RHA/jj strain, a high dietary fat intake significantly decreased plasma UCB in homozygous Gunn rats (from 80 to 46 µmol/L, -43%, P<0.001; Figure 2D). Under unconjugated hyperbilirubinemia, oxidation of UCB by CYP1A1 and CYP1A2 can function as an alternative means to metabolize UCB \(^6,32\). No significant differences, however, were found in the hepatic expression of Cyp1a1, Cyp1a2 and the bilirubin transporter Abcc2 between the three genotypes (Supplemental Figure 1A). The difference in dietary fat content between the LFD and HFD had no effect on the hepatic expression of Cyp1a1, Cyp1a2 and Abcc2. Administration of a HFD decreased plasma UCB levels in mild and severe unconjugated hyperbilirubinemic conditions, and this was independent of mRNA expression of major elements of the bilirubin-oxidizing pathway.

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)
Effect of a HFD on biliary UCB secretion

During unconjugated hyperbilirubinemia, UCB can be excreted via the bile and via a transintestinal pathway\textsuperscript{11,14,16}. Homozygous Gunn rats had significantly higher biliary UCB excretion compared to wild type and heterozygous animals under LFD and HFD conditions (Figure 3). Feeding the Gunn-Ugt1a1\textsuperscript{J/BluHsdRrrc} strain a HFD did not affect biliary UCB secretion rates in wild type animals compared to LFD. However, administration of the HFD significantly decreased biliary UCB secretion both in heterozygous and in homozygous Gunn rats as compared to LFD (Figure 3). These data demonstrate that a higher dietary fat intake decreases biliary UCB secretion both under mild and severe unconjugated hyperbilirubinemia.

Plasma lipid levels in wild type, heterozygous and homozygous Gunn rats

Recently, an association between lipid-lowering effects of bilirubin and interactions between cholesterol and bilirubin have been described in humans with GS, who have mildly elevated plasma levels of UCB. This effect was also reported in RHA/jj and Gunn-Ugt1a1\textsuperscript{J/BluHsdRrrc} Gunn rats and in humanized Ugt1a1 mice (HuUGT*28)\textsuperscript{20,22,23,33,34}. Plasma TC and TG levels were lower in homozygous male and female Gunn rats compared to normobilirubinemic rats, as well as in individuals with GS compared to control subjects\textsuperscript{20,22,25}. It has remained unclear, however, to what extent this hypolipidemic observation is an intrinsic feature of unconjugated hyperbilirubinemia and is related to Ugt1a1 effects, because studies reporting plasma lipid levels in wild type and heterozygous rats of the
Gunn-Ugt1a1/j/BluHsdRrrc strain have been lacking. Therefore, we assessed plasma lipid levels and fecal neutral sterol (FNS) excretion in all three genotypes of this specific strain after 4 weeks of LFD or HFD (Figure 4). No significant differences between wild type, heterozygous and homozygous Gunn rats were observed in plasma TG, free fatty acids (FFA), TC or FNS (Figure 4A-D) under either LFD or HFD conditions. Figure 4B shows that administration of HFD significantly increased plasma FFA levels in heterozygous animals. Plasma TC levels were significantly increased in all genotypes in response to a HFD (Figure 4C). In mice and rats, administration of a HFD has been shown to increase FNS excretion \(^{35,36}\). Additionally, a HFD was also effective in the RHA/jj strain to lower plasma UCB levels, and this was associated with a higher FNS output. The FNS was significantly increased after administration of HFD in wild type and homozygous, but not in heterozygous Gunn rats compared to rats on LFD (Figure 4D). Taken together, these hypobilirubinemic findings of a HFD in homozygous Gunn rats of the Gunn-Ugt1a1/j/BluHsdRrrc strain are in line with our earlier findings in the RHA/jj strain. However, the hypobilirubinemic effects in heterozygous Gunn rats did not completely align with increased FNS.

Additionally, we determined whether the differences in plasma FAs and TC under LFD and HFD conditions were accompanied by changes in hepatic expression levels of genes related to lipid metabolism. Bilirubin has been reported to affect FA and cholesterol metabolism through regulation of the nuclear receptors PPAR\(\alpha\), PPAR\(\gamma\) and other genes involved in lipid metabolism \(^{37,38}\). The hepatic expression of the PPAR\(\alpha\), pyruvate dehydrogenase lipoamide kinase isozyme 4 (Pdk4), 3-hydroxy-3-methylglutaryl-CoA (Hmgcr) and the cholesterol transporters low-density lipoprotein receptor (Ldlr) and ATP-binding cassette transporter A1 (Abca1) was not altered between genotypes and dietary fat intake (Supplemental Figure 1B). However, the PPAR\(\alpha\) target gene carnitine palmitoyltransferase 1a (Cpt1a) was significantly increased in heterozygous and homozygous rats compared to wild type rats under LFD conditions. Hepatic expression of the lipogenic gene sterol regulatory element-binding protein 1c (Srebp1c) was significantly decreased under LFD conditions in heterozygous rats, but this did not reach significance in homozygous rats (P=0.08) compared to wild types. These results show that the lower Cpt1a and higher Srebp1c mRNA expression in wild type animals compared to heterozygous Gunn rats were not accompanied by differences in lipid- or cholesterol levels in either plasma or feces.
The relationship between plasma bilirubin and cholesterol levels

Longitudinal and cross-sectional studies in healthy human individuals as well as in subjects with GS, type 2 diabetes and kidney disease, showed a negative correlation between bilirubin and LDL-C, TG and TC. Bilirubin was positively correlated with high-density lipoprotein (HDL) cholesterol in these studies. In female Gunn-Ugt1a1/BluHsdRrrc homozygous Gunn rats, plasma HDL levels were significantly lower compared to normobilirubinemic rats.

After administration of semi-synthetic LFD or HFD, we found no differences in plasma TC between male and female wild type, heterozygous and homozygous Gunn rats (Figure 4C; Supplemenal Figure 2). To determine the relation between hyperbilirubinemia and cholesterol under non-semi-synthetic dietary conditions, we performed an additional experiment: we assessed plasma TC and lipoprotein levels after feeding a standard chow diet (Figure 5).

Under chow conditions, plasma TC levels tended to be lower (p=0.06) in female homozygous Gunn rats compared to normobilirubinemic wild type and heterozygous rats (Figure 5A), which is in line with literature. A similar decrease in plasma TC was found for male hyperbilirubinemic Gunn rats compared to normobilirubinemic rats (data not shown). Interestingly, the difference seems to be diet-dependent, because this phenomenon was not
observed when female rats were fed the semi-synthetic LFD or HFD (Supplemental Figure 2). In female rats, the fraction in LDL and to a smaller extent in HDL, was lower in homozygous Gunn rats compared to heterozygous and wild type female rats (Figure 5B). Our results show that only under chow conditions, plasma TC levels were decreased in female homozygous rats of the Gunn-Ugt1a1\textsuperscript{j/BluHsdRrrc} strain, and that female homozygous Gunn rats displayed lower LDL-cholesterol levels compared to normobilirubinemic animals.

![Figure 5](image.png)

**Figure 5.** Plasma cholesterol levels and lipoprotein profile after control chow diet in female wild type, heterozygous and homozygous Gunn rats. (A) Plasma total cholesterol in female rats. (B) Fast-performance liquid chromatography profiles of plasma cholesterol of female Gunn rats. The lines represent the mean intensity analyzed (uV). An n=3 – 4 was used.

**Discussion**

We aimed to investigate the effect of Ugt1a1 deficiency on bilirubin metabolism and its interaction with lipoprotein metabolism. Since not much is known about bilirubin levels in plasma and brain during the neonatal period of rat models of unconjugated hyperbilirubinemia, we characterized the bilirubin metabolism both in the neonatal period and in adulthood. We also tested the efficacy of a previously identified hypobilirubinemic treatment strategy (high-fat diet, HFD) in adult rats of another Gunn strain as well as its effect on cholesterol metabolism.

During the early days of life, increased metabolism of fetal haemoglobin together with a low expression of Ugt1a1 results in elevated plasma UCB levels. The intestinal microbiota that normally convert UCB into urobilinoids, are not yet fully established in neonates and therefore more UCB is available for EHC, further contributing to increased plasma UCB levels \textsuperscript{41-43}. We characterized the course of bilirubin levels in early life, the equivalent of neonatal jaundice, of wild type, heterozygous and homozygous Gunn rat pups of the Gunn-Ugt1a1\textsuperscript{BluHsdRrrc} strain. We analysed bilirubin using two independent methods, transcutaneous bilirubin (TcB) and biochemical plasma analysis, which showed some discrepancies. We found that at PD4 and PD7, the estimated bilirubin values were higher in the biochemical plasma analyses than those obtained by the transcutaneous method, for
which we do not have a clear explanation. One possible explanation is that bilirubin accumulation is delayed in tissues compared to plasma UCB accumulation, although it is not completely understood how. Furthermore, we cannot exclude that transcutaneous measurements in rat pups is less reliable due to their small size at the earliest time points. The peak in plasma UCB in homozygous Gunn-Ugt1a1/JBluHsdRrrc rat pups (at ~PD21) in our study appeared somewhat later than described in studies on the RHA/jj strain pups, namely between PD9 and 19 \[44-47\]. The age of the peak bilirubin level observed in our present study, however, did correspond with that reported by Zarattini et al. in homozygous Gunn rats of the RHA/jj strain (PD17) \[19\], although the UCB peak concentration was lower in our present study (162 vs. 233 μmol/L) \[19,44\]. A possible explanation for this discrepancy involves differences in the genetic background of the two strains (RHA/jj and Gunn-Ugt1a1/JBluHsdRrrc), which may affect the plasma UCB levels as indicated above. Also, environmental differences such as housing conditions or microbiota can affect plasma UCB levels within one strain. For future studies investigating new therapies for (neonatal) unconjugated hyperbilirubinemia, it should thus be kept in mind that Gunn rat strains display differences in plasma UCB levels and it is unclear if this could affect outcomes of therapeutic interventions.

To investigate the effect of Ugt1a1 on bilirubin and lipid phenotype in the three genotypes of the Gunn-Ugt1a1/JBluHsdRrrc strain, we applied a dietary treatment that was previously shown hypobilirubinemic in RHA/jj Gunn rats and, more importantly, correlated with a hypobilirubinemic effect in hyperbilirubinemic Crigler Najjar patients \[49,50\]. In accordance with these previous observations, feeding the homozygous Gunn-Ugt1a1/JBluHsdRrrc rats a HFD decreased plasma UCB levels.

The results of this study support the link between plasma bilirubin levels and hypolipidemic phenotype in homozygous Gunn rats. It has remained unclear, however, to what extent heterozygous rats could be used as normobilirubinemic controls. Our present results clearly demonstrate that, despite a non-jaundiced appearance, heterozygous Gunn rats have significantly increased plasma UCB levels under LFD conditions compared to wild type rats, which could be lowered by a HFD. During the neonatal period, no differences in body weight were found between normobilirubinemic wild type and hyperbilirubinemic rats, a finding also reported in other studies investigating neonatal hyperbilirubinemia in Gunn rat pups \[21,51,52\]. In adults, a higher body weight of normobilirubinemic Gunn rats compared to jaundiced Gunn rats has also been reported in earlier studies, but it is important to note that most of these studies did not distinguish normobilirubinemic wild type from heterozygous Gunn rats, or did not use wild type littermates at all \[20,22,51,53,54\]. An exception is Zarratini et al. who showed that wild type (RHA/JJ) Gunn rats were heavier compared to heterozygous (RHA/Jj) and homozygous (RHA/jj) Gunn rats \[19\]. In contrast, our heterozygous Gunn rats were significantly heavier compared to both their wild type and homozygous Gunn rat littermates during their neonatal and adult period. We do not have a clear explanation for these differences in body weight but it is most likely not related to levels of UCB, because normobilirubinemic wild
type rats had similar body weight as hyperbilirubinemic homozygous Gunn rats. Although
the increase in plasma bilirubin concentrations in heterozygous Gunn rats are limited
compared to wild type Gunn rats, the use of wild type littermates as normobilirubinemic
controls for Gunn rat studies would still be preferred, aimed to serve as a better control for
hyperbilirubinemia. An unexplained difference in body weight of heterozygous Gunn rats
further strengthens this preference to use wild type littermate controls rather than
heterozygotes.

Recently, there has been considerable interest in the relationship between (unconjugated)
bilirubin and serum cholesterol levels. Several studies reported a negative
association between bilirubin and plasma TC levels: a decreased HDL-cholesterol, LDL-
cholesterol or both, has been shown in individuals with GS and in hyperbilirubinemic Gunn
rats. In line with these studies, we found a decreased plasma LDL-cholesterol concentration in our homozygous female Gunn rats compared to wild type and
heterozygous Gunn rats, upon feeding a control chow diet. The LDL receptor (LDLR) is
involved in the uptake of LDL into the hepatocytes, but hepatic Ldlr mRNA did not differ
between homozygous Gunn rats and wild types or heterozygous Gunn rats. Although
the mechanism underlying the lipid phenotype in hyperbilirubinemia is still unresolved (and
beyond the scope of the present studies), our data support the usefulness of this strain
as model to obtain further mechanistic insights into this epidemiological observation in
humans.

In conclusion, the bilirubin metabolism in neonatal and adult homozygous Gunn rats of
the Ugt1a1/J/BluHsdRrrc strain is rather similar to that observed in Gunn rats of the RHA/jj
strain. Our earlier reported therapeutic findings of dietary fat appeared reproducible in
heterozygous and homozygous Gunn-Ugt1a1/J/BluHsdRrrc rats, indicating that the bilirubin-
lowering effect of HFD is independent of the genetic background of a Gunn rat strain. Discrete phenotypical differences between wild type and heterozygous Gunn rats were
observed, what makes heterozygous rats less suitable for their use as controls in studies on
intestinal bilirubin and fat metabolism. In line with previous reports, plasma TC and LDL-
C were decreased under severe hyperbilirubinemic conditions in female Gunn rats, but was
not affected by diminished Ugt1a1 expression as seen in heterozygous animals compared
to wild type animals. For future studies, also wild type animals are important to include for
analysis in order to exclude UGT1A1-dependent effects on lipid phenotype.

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Eilers, Natalia Loaiza Velasquez and the colleagues from the animal facility.

Contributions: MB and LWEvdS designed and performed the experiments, analysed and
interpreted data. MB wrote the manuscript. JJ analysed data and reviewed the manuscript. LV
interpreted data and reviewed the manuscript. JWJ and HJV designed the experiments, interpreted
data and wrote the manuscript.
**Supplemental Information**

**Supplementary Table 1. Taqman primer sequences.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (3’ to 5’)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>36b4</td>
<td>acidic ribosomal phosphoprotein</td>
<td>CCC AGA GCA AAA AGC GAC TC</td>
<td>GGT CAT CAT CAT TGG TCT TG</td>
<td>AGA CTA CTC TGT TGT TCA TCA GAC AAC ACT TGA CCA AG</td>
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<tr>
<td>Abca1</td>
<td>ATP-binding cassette, subfamily A (ABC1), member</td>
<td>CCC AGA GCA AAA AGC GAC TC</td>
<td>GGT CAT CAT CAT TGG TCT TG</td>
<td>AGA CTA CTC TGT TGT TCA TCA GAC AAC ACT TGA CCA AG</td>
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<td>Cpt1a</td>
<td>carnitine palmitoyltransferase 1a</td>
<td>CAG TGG GAG CGA CTC TCT AAT</td>
<td>GCC CTC TGT GGT ACA CAA CAA</td>
<td>CTT GGG GAA GAG ACA GAC ACC ATC CAA C</td>
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<tr>
<td>Cyp1a2</td>
<td>cytochrome P450, family 1, subfamily A, polypeptide 2</td>
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<td>CTG GGC GGA ACA CAA AGG</td>
<td>TGT TCC ACT GCT. TCT CAT GGT TGA CCT</td>
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<tr>
<td>Hmgr</td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A reductase</td>
<td>CCG GCA ACA ACA AGA TGT CG</td>
<td>ATG TAC AGG ATG GCG ATG CA</td>
<td>TGT GGC TGC TCA GCA CGT CTT C</td>
</tr>
<tr>
<td>Ldrl</td>
<td>low density lipoprotein receptor</td>
<td>GCA TCA GCT TGG ACA AGG TGT</td>
<td>GGG AAC AGC CAC CAC CAT TGT TG</td>
<td>CAC TCC TTG ATG GCC TCA TCC GAC C</td>
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<tr>
<td>Ppara</td>
<td>peroxisome proliferator activated receptor alpha</td>
<td>CAC CCT CTC TCC AGC TCT CA</td>
<td>GCC TTG TCC CCA CAT ATT CG</td>
<td>TCC CCA CCA GTA CAG ATG AGT CCC CTG</td>
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<tr>
<td>Pdk4</td>
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<td>CAG CAC ATC CTC ATA TTC AGT GAC TCA AAG AC</td>
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<tr>
<td>Srebp1c</td>
<td>sterol regulatory element binding protein 1c</td>
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<td>CCT GTC TCA CCC CCA GCA TA</td>
<td>CAG CTC ATC AAC AAC CAA GAC AGT GAC TCC C</td>
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**Supplementary Table 2. SYBR Green primer sequences.**

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<th>Gene</th>
<th>Description</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (3’ to 5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36b4</td>
<td>acidic ribosomal phosphoprotein</td>
<td>GCT CCA AGC AGA TGC AGC A</td>
<td>CCG GAT GTG AGG CAG CAG</td>
</tr>
<tr>
<td>Abca2</td>
<td>ATP-binding cassette, subfamily C (CFTR/MRP), member 2</td>
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<td>ATACGCCGCATAAGACCGAG</td>
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<tr>
<td>Cyp1a2</td>
<td>cytochrome P450, family 1, subfamily A, polypeptide 1</td>
<td>TCCTATCTCCGGTTACCTCCC</td>
<td>CCGATGTGGCCCTTCTCAAAT</td>
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</tbody>
</table>
Supplemental Figure 1. Hepatic gene expression under LFD and HFD conditions for wild type, heterozygous and homozygous Gunn rats. Expression of genes involved in (A) metabolism and transport of bilirubin and (B) lipid metabolism (n=6 – 9). Data is represented as bar graphs with mean values ± standard deviation.

Supplemental Figure 2. Plasma total cholesterol in female rats after 4 weeks of LFD or HFD. Data is represented as individual animals with the median ± interquartile range. N=3-5.
References


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46. Cannon C, Daood MJ, O'day TL, Watchko JF. Sex-Specific Regional Brain Bilirubin Content in Hyperbilirubinemic Gunn Pups. 2006. doi:10.1159/000091843


