Chapter 6

General discussion and conclusion
1. Summary

The increasing worldwide prevalence of obesity is a major contributor to the development of metabolic syndrome and increases the risk of CVD, T2DM and NAFLD. Recently, the feasibility of NRs as new therapeutic targets for these metabolic diseases has been investigated based on the role of NRs in a plethora of metabolic pathways. Currently, around 13% of the FDA-approved drugs target NR and examples are PPAR-ligands (fibrates and thiazolidinediones) that have been used to treat T2DM and dyslipidemia. Recently, cholesterol and UCB metabolism has been linked as individuals with GS have a lower risk of CVD and peripheral arterial disease accompanied by a leaner phenotype characterized by mildly elevated plasma UCB levels (>17.1 µM). The underlying mechanisms of this interaction, however, are not known yet.

The aim of the research conducted in this thesis was to improve our understanding of disorders of bilirubin and lipid metabolism and find new targets of intervention. We therefore developed and/or characterized new relevant model systems and addressed the potential role of NRs as therapeutic target.

Severe unconjugated hyperbilirubinemia can occur in preterm neonates and patients with Crigler-Najjar disease type 1 and 2 and when left untreated, can cause neurotoxicity, including kernicterus, which can be lethal. The most used animal model to study mechanisms of disease and novel treatments is the Gunn rat, which genetically lacks the enzyme activity essential for bilirubin glucuronidation. Various Gunn rat strains have been developed over time, including the RHA/jj and Gunn-Ugt1a1/J/BluHsdRrrc strains, which differ their bilirubin phenotype.

Recently, several studies demonstrated a lipid-lowering effect of bilirubin in hyperbilirubinemic animal models as well as in individuals with GS. This was associated with a reduced risk of cardiovascular disease (CVD). In chapter 2, we investigated the role of UGT1A1 in lipid metabolism in the three genotypes of the Gunn-Ugt1a1/J/BluHsdRrrc rat strain. We assessed the bilirubin and lipid phenotype in neonates and adults of this Gunn-Ugt1a1/J/BluHsdRrrc strain and determined to what extent these rats can serve as a reliable model to study human normo- and hyperbilirubinemia. Adult female homozygous Gunn rats had a decreased levels of low-density lipoprotein cholesterol (LDL-C) in plasma, as compared to heterozygous and wild type rats. Furthermore, 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.

A transintestinal secretion pathway has been described for both UCB and cholesterol, but it is not known if these pathways interact and whether they are regulated in the same manner. In chapter 3 we therefore explored if stimulation of TICE by increasing fecal neutral sterol (FNS) secretion by LXR and FXR activation could affect hyperbilirubinemia.
in Gunn rats. We found no effect on unconjugated hyperbilirubinemia in Gunn rats after stimulation of either LXR or FXR, suggesting that transintestinal excretion of cholesterol and unconjugated bilirubin are not quantitatively linked.

Several mouse studies have been performed in our lab to obtain a better understanding of the transintestinal cholesterol excretion (TICE) pathway and its underlying molecular mechanisms. Transcriptome analysis of experiments in which TICE was stimulated revealed an associated upregulation of the gene \(Pxmp4\), encoding the peroxisomal membrane protein 4, suggesting that this gene could be involved in TICE. In chapter 4, we investigated the metabolic function of PXMP4 using a \(Pxmp4\) knockout (\(Pxmp4^{-/-}\)) mouse model using CRISPR/Cas9-mediated gene editing. Preliminary results showed no change in fecal neutral sterol excretion nor in plasma cholesterol levels between wild type and \(Pxmp4^{-/-}\) mice, suggesting no important quantitative role for PXMP4 in the TICE pathway. Under standard chow conditions and after stimulation of PPARx, the function of PXMP4 is redundant, possibly due to compensation by other peroxisomal proteins.

Outside the main scope of this thesis, it has been shown that dietary protein restriction improves metabolic health and can decrease the risk of CVD, T2DM and different types of cancer. Ageing itself is a risk factor for (chronic) metabolic disorders and whether a short-term protein restriction, starting at an advanced age, has beneficial effects on metabolic health is not known. In chapter 5 we determined whether a short-term dietary intervention with reduced protein content would be able to improve metabolic health at an advanced age. A short-term protein restriction increased energy expenditure and thermogenesis in aged mice, without affecting muscle function. We believe that the underlying mechanism for the improved metabolic health is an increased fibroblast growth-factor 21 (FGF21) signaling, mediating changes in subcutaneous white adipose tissue (scWAT).

2. Bilirubin and lipid phenotype

Since recently, there has been growing interest in the association between (unconjugated) bilirubin and cholesterol metabolism based on the epidemiologically lower risk of CVD and peripheral arterial disease in individuals with Gilbert Syndrome (GS). A meta-analysis of ~15,000 men showed that the risk of CVD decreased with 6.5% with every increase of 1 \(\mu\)mol/L in serum bilirubin levels. Aiming to further elucidate the underlying mechanisms, several studies reported decreased plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in individuals with GS as well as in hyperbilirubinemic homozygous Gunn rats of the RHA/jj strain. Also, individuals with T2DM and kidney disease showed a negative association between plasma bilirubin levels and LDL-C, triglycerides (TG) and total cholesterol (TC). However, a relationship between plasma UCB and plasma lipids has not been confirmed in...
some other studies. The study of Wang et al. stated that humans with plasma bilirubin levels between 12 and 86 µmol/L have a risk of CVD similar as individuals with bilirubin levels lower than 7 µmol/L. In addition, Lippi et al. reported no significant differences in plasma TC, LDL-C and TGs in a large population of GS individuals with normal plasma bilirubin levels (<1.10 mg/dL (equally ~97.2 µmol/L) and increased plasma bilirubin levels (>97.2 µmol/L). This retrospective epidemiological study did not find an association between plasma bilirubin levels and lipid profile. In line with this, Maruhashi et al. compared adult male GS individuals (mean plasma bilirubin 29.2 ± 11.6 µmol/L) with normobilirubinemic controls, and reported no significantly lower plasma TC or LDL-C levels in the former group. The mechanisms underlying the association between mildly elevated plasma bilirubin levels and a decreased risk of CVD remain to be elucidated. Therefore, no definite conclusions can be made for the use of bilirubin as a therapeutic strategy to lower plasma lipid profile.

In chapter 2 we assessed plasma TC and lipoprotein levels under physiological conditions in wild type, heterozygous and homozygous UGT1A1-deficient Gunn rats of the Gunn-Ugt1a1/J/HsdRrc strain. In line with literature, our present data showed that male homozygous Gunn rats had a significant lower plasma TC levels compared to normobilirubinemic wild type and heterozygous Gunn rats (data not shown). A trend towards lower plasma TC levels was also observed in female homozygous rats, although this did not reach statistical significance (P=0.06) compared to wild type and heterozygous female rats. The studies of Boon et al. and Wallner et al. reported that female hyperbilirubinemic Gunn rats had significantly lower plasma TC levels compared to female Wistar rats, although they did not compare this to wild type and heterozygous littermates.

Regarding plasma lipoprotein profile, decreased plasma LDL-C levels were observed in female homozygous rats compared to normobilirubinemic rats. We did not find major differences in plasma high-density lipoprotein cholesterol (HDL-C) levels between female homozygous Gunn rats and normobilirubinemic rats. This is in contrast with the findings of Boon et al. and Wallner et al., where lower plasma HDL-C levels in female Gunn rats compared to Wistar rats were reported but an effect was absent on plasma LDL-C in female hyperbilirubinemic Gunn rats. Although we did not confirm the previous reported significantly lowered TC in female Gunn rats, our results do suggest that hyperbilirubinemia is linked to hypocholesterolemia in female, as well as in male, hyperbilirubinemic Gunn rats. Different hypotheses regarding the lipid-lowering effect of (unconjugated) bilirubin will be discussed below.

2.1. Estrogen and bilirubin

Both existing literature and our present findings suggest sexual dimorphism in the relationship between UCB and cholesterol. One hypothesis is that this sexual dimorphism could be explained by endogenous levels of the steroid hormone estrogen, which is naturally higher in female rats. A link between estrogen and lipid levels has been
demonstrated in studies investigating the risk of CVD in premenopausal and postmenopausal women \(^{31,32}\). Estrogens can lower hepatic de novo lipogenesis and increase the fatty acid oxidation rate in the liver \(^{32}\). Estrogen deficiency, as is seen in (post)menopausal women, is associated with liver lipid accumulation, increased plasma TC, TG and LDL-C levels \(^{33}\). Also, women who have surgically-induced menopause showed a higher incidence of non-alcoholic fatty liver disease (NAFLD) \(^{34}\). It has been shown that estrogens can be glucuronidated by UGT1A1 \(^{35}\) and that UGT1A1 even has a high specificity for estradiol \(^{36}\), a precursor of estrogens. A possibility is that higher estrogen levels can give competition between estrogen and UCB for glucuronidation by UGT1A1, resulting in less glucuronidation of UCB and causing increased plasma UCB levels \(^{37}\). This concept was demonstrated in female Wistar rats where administration of the synthetic estrogen 17α-ethinylestradiol (EE) resulted in an increase of total serum bilirubin levels \(^{38}\).

Also in the study of Wallner et al., female Gunn rats exhibited higher plasma UCB levels (112 µM) compared to their male littermates (98 µM) \(^{15}\). However, conflicting data regarding sexual dimorphism and the possible role of estrogen in plasma bilirubin levels exist. In the study of Van der Veere et al., female UGT1A1-deficient Gunn rats exhibited plasma UCB levels of ~150 µmol/L whilst male Gunn rats had higher plasma UCB levels (~190 µmol/L) under standard dietary chow conditions \(^{39}\), levels which are in line with our findings (data not shown). Furthermore, Seppen et al. did not find a link between estrogenic activity and plasma UCB levels \(^{40}\). The phytoestrogen genistein binds to the estrogen receptor α and performs estrogenic effects, and administration of this compound for three weeks did not increase, but rather decreased (~32%) plasma bilirubin levels in Gunn rats compared to control diet \(^{40}\).

There is scarce information about estrogen levels in the presence of UGT1A1 mutations as found in individuals with GS or Crigler-Najjar Type 2, nor on estrogen levels in female normobilirubinemic and hyperbilirubinemic Gunn rats. Taken together, we cannot conclude if the hypocholesterolemic effects in female Gunn rats can be explained by naturally higher estrogen levels, or by competition between UCB and estrogen for glucuronidation by UGT1A1. Therefore, future studies could investigate the relation between estrogen, UCB and lipid levels in female normobilirubinemic and hyperbilirubinemic Gunn rats. Additionally, exogenous administration of synthetic estrogens such as EE to heterozygous female Gunn rats could provide more information about the effect on (plasma) UCB and lipid levels.

### 2.2. Regulation of cholesterol metabolism by Aryl Hydrocarbon Receptor

The association between (mildly) unconjugated hyperbilirubinemia and lower TC can potentially also be explained by a common regulation by the Aryl hydrocarbon Receptor (AhR). AhR is a ligand-activated cytosolic transcription factor and belongs to the family of basic helix-loop-helix/Per-Arnt-sim (bHLH/PAS) transcription factors \(^{41}\). AhR is expressed in many organs including the liver, kidney, lung, gut and lymphocytes in the
Exogenous ligands for AhR include small dietary compounds, environmental toxins, as well as polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibeno-p-dioxin (TCDD). Endogenous compounds functioning as a ligand for AhR are kynurenic acid, biliverdin and bilirubin. Ligand-binding to AhR causes translocation of AhR from the cytoplasm to the nucleus, where it dimerizes with ARNT. In order to execute transcriptional effects, the AhR/ARNT complex generally binds to specific sequences called dioxin-responsive elements (DRE) present in target genes, although DRE-independent target gene regulation has also been reported in humans and mice.

AhR is involved in bilirubin metabolism by inducing hepatic Ugt1a1 and Cyp1a1 expression and its activation has been reported to decrease plasma UCB in Gunn rats. Conflicting results, however, have been reported about the role of AhR in lipid and cholesterol metabolism as well as bilirubin metabolism, which partially could be explained by species-specific responses what has been reported in some, but not in all studies.

Regarding lipid metabolism, several studies reported that administration of the AhR ligand TCDD resulted in a different transcriptional response between rats and mice. Nault et al. reported that administration of TCDD decreased mRNA expression of lipoprotein lipase (LPL) (1.5-fold) in rats, opposed to a 3-fold increase of Lpl in mice. Harrill et al. also investigated species-specific AhR regulation of cholesterol metabolism using AhR-knockout (AhR-KO) rats and AhR-KO mice. In AhR-KO mice, plasma total cholesterol (TC) was significantly decreased as compared to wild type mice. This lipid-lowering effect was also reported by Biljes et al. where young (<5 months) AhR-KO mice had significantly lower postprandial serum triglycerides (TG), TC, HDL-C and VLDL levels compared to wild type littermates. Interestingly, no effect on plasma TC was observed in AhR-KO rats compared to their wild type littermates.

Harrill et al. studied the effect of AhR inactivation on bilirubin metabolism in both mice and rats and found species-specific differences as well. AhR-KO mice had increased plasma bilirubin levels, but in AhR-KO rats no effect on plasma total bilirubin was found compared to their wild type littermates.

In contrast, other studies did not find a species-specific effect of AhR. Activation of AhR through β-naphthoflavone (BNF) administration in human hepatocytes and intestinal Caco-2 cells, as well as in mice, all resulted in downregulation of cholesterol biosynthesis genes and the cholesterol absorption transporter NPC1L1 through a DRE-independent pathway. Inactivation of AhR in human Caco-2 cells by small-interference RNA (siRNA) resulted in the opposite effect and increased for example the rate-limiting enzyme in cholesterol biosynthesis HMCGR. In ApoE-deficient mice, administration of the phenolic acid ferulic acid (FA) increased the hepatic levels of AhR which was associated with a decreased hepatic expression of fatty acid synthase (Fasn) and Srebp1c, resulting in reduced deposition of TG and TC in the liver. Additionally, another gene involved in lipid metabolism such as Srebf1 showed an decreased expression in mice and rats after AhR activation.
Besides species-specific differences, also heterogeneity in studies about the effects of AhR activation or inactivation on lipid and bilirubin metabolism was reported. Three hypotheses can be suggested from this: 1) Lipid-lowering effects of bilirubin are regulated by AhR activation, and the effects of AhR are species-specific; illustrated by lower cholesterol levels found in mice with increased bilirubin but not in rats; 2) Bilirubin and cholesterol levels are both regulated by AhR activation and bilirubin can have a cholesterol-lowering effect; 3) Bilirubin and cholesterol are not directly regulated by AhR and lipid-lowering effects of UCB cannot be explained by altered AhR levels.

Not much is known about the expression of the AhR receptor in hyperbilirubinemic Gunn rats or in humanized UGT1A1 mice. The interaction between AhR activation by endogenous UCB, and the effects on cholesterol metabolism remain to be elucidated. For future experiments, double knockout of AhR and UGT1A1 in mouse or rat models can be used to investigate if the beneficial effect of UCB are executed by AhR or not. Also, exogenous administration of UCB to an AhR-knockout model can be used to elucidate if the beneficial metabolic effects of UCB are regulated through AhR activation. This will give more insight if targeting the UCB-AhR pathway could be a new therapeutic strategy for metabolic disorders.

2.3. Bilirubin and PPAR

Recently, the heme oxygenase (HO) system and its metabolites biliverdin and bilirubin have gained more interest due to their involvement in insulin sensitivity, oxidative stress, lipid metabolism and energy metabolism. One of the important regulators of these metabolic processes is the family of PPARs and a bidirectional interaction has been shown between the HO-pathway and PPAR regulatory pathway. Involvement of PPAR on the HO-pathway was demonstrated by the HO-1 gene promoter containing a PPAR response element (PPRE) as well as a phenobarbital-responsive enhancer module (grPBREM) containing a binding motif for transcription factor PPARα. Conversely, regulation of PPARα either directly or indirectly, has also been demonstrated by several studies. Biliverdin reductase A (BVRA) converts biliverdin into UCB, and BVRA can phosphorylate the serine 9 of glycogen synthase kinase 3β (GSK3β), thereby inhibiting GSK3β activity. GSK3β is an inhibitor of PPARα, hence, the upregulation of BVRA can result in activation of PPARα.

A more direct interaction between the HO-pathway and PPAR has been demonstrated by the discovery that bilirubin can act as a selective modulator of the PPAR family of nuclear receptors (SPPARM) and is able to directly bind to PPARα. This is attributed to the pyrrole-ring like structure of bilirubin, resembling the structure of other ligands for PPARα such as WY-14643 and fenofibrate. In vivo data showed beneficial effects of bilirubin administration on body weight and lipid homeostasis in mice. In the study of Liu et al., intraperitoneally (IP) administration of 20 µmol/kg bilirubin twice per day for a period of two weeks decreased body weight, hepatic lipid content and epididymal fat weights in diet-induced obese (DIO) mice.
Additionally, plasma TC levels were lower in bilirubin-treated DIO mice compared to vehicle-treated mice, but no significant effect was found on plasma LDL, HDL and TG levels. Bilirubin administration decreased hepatic mRNA expression of Sterol Regulatory Binding Protein 1 (Srebp1), acetyl-Coenzyme A carboxylase alpha (Acac) and fatty acid synthase (Fasn), genes involved in de novo lipogenesis and fatty acid synthesis. It has been suggested that these beneficial effects of bilirubin are regulated through activation of PPARα, that in its turn increases adiponectin levels. Adiponectin can increase fatty acid oxidation (FAO) and improve insulin sensitivity.

In wild type mice, IP injection of 30 mg/kg bilirubin lowered body weight and fat mass, an effect that was blunted in bilirubin-treated PPARα knockout (PPARα-/-) mice. Furthermore, PPARα-/- mice did not show mRNA upregulation of the PPARα target gene fibroblast growth factor 21 (Fgf21) or increased plasma FGF21 levels. FGF21 is an endocrine signal able to regulate endogenous glucose and fatty acids (FA) and is associated with metabolic control. Levels of FGF21 increase in response to fasting, starvation and physical exercise which is associated with a lower body weight, insulin levels and plasma triglycerides and improved metabolic health and longevity.

Most studies report beneficial metabolic effects associated with (mildly) increased UCB concentrations. However, it is important to note that under physiological situations, UCB is rapidly conjugated by UGT1A1. Studies investigating administration of bilirubin do not consistently report the UCB versus conjugated bilirubin fractions in the circulation, nor the kinetics of UCB in the circulation. It is therefore difficult to draw firm conclusions on the efficacy and longevity of the beneficial effects. Future studies could make use of labelled bilirubin in order to gain insight about UCB kinetics and allow to estimate for how long the administration of bilirubin will be effective.

A humanized mouse model of GS has a transgenic expression of the human UGT1A1*28 allele (HuUGT*28) and shows mildly increased plasma UCB levels. These HuUGT*28 mice were used in a study where the sensitivity for high-fat induced insulin resistance and hepatic lipid accumulation was investigated. The percentage and total fat mass, as well as hepatic lipid and cholesterol accumulation was decreased after 36 weeks of a 60% HFD in HuUGT*28 mice compared to wild type littermates. These findings were associated with decreased hepatic expression of de novo lipogenesis genes Fas, Srebp1 and Acc. Hepatic expression of PPARα target gene Fgf21 was increased in HuUGT1*28 mice compared to wild type animals.

PPARα is involved in fat oxidation in the liver, as well as brown adipose tissue (BAT) and heart, and the role of PPARα in lowering body weight associated with hyperbilirubinemia has been investigated by Gordon et al. through several approaches. They investigated if bilirubin can affect adipocyte functioning and browning of white adipose tissue (WAT) through PPARα regulation. In line with earlier reports, bilirubin was found to directly bind to PPARα and resulting in enhanced fatty acid oxidation and reduced lipid accumulation in 3T3-L1 cells (murine pre-adipocyte cell line). This was thought to be due to an increased expression of fat-burning genes Cpt1 and Ucp1 in 3T3-L1 cells.
L1 cells. Administration of the bilirubin precursor biliverdin to BAT cells improved mitochondrial function but did not result in lowering of lipid content of BAT. Additionally, obese and hyperbilirubinemic humanized Ugt1a1 mice (HuUGT*28) fed with high fat diet showed increased levels of the coactivator peroxisome-proliferator activated receptor gamma coactivator 1-alpha (PGC1α). The PGC1α protein is involved in several metabolic pathways such as reducing inflammation, but can also increase mitochondrial biogenesis and function. PGC1α is also an important downstream target of the AMPK pathway.

AMPK is a member of the protein kinase family and recognizes depletion of ATP, thereby inhibiting energy consumption. Activated AMPK decreases expression of cholesterol and fatty acid biosynthesis genes as well as post-translational phosphorylation of PGC1α, subsequently followed by activation of PPARs. Mölzer et al. assessed several energy, carbohydrate and lipid metabolic biomarkers to investigate the role of the complex AMPK pathway in male and female individuals with or without GS. Phosphorylated AMPK and its downstream targets PPARα, PPARγ and PGC1α were higher in GS individuals compared to control subjects. Interestingly, no significant lower levels were found in plasma TC, HDL and LDL-C in GS individuals, also not when corrected for gender, compared to control individuals. This indicates that increased AMPK signaling cannot completely explain the lipid-lowering effect of bilirubin.

An interaction between bilirubin and another member of the PPAR family, namely PPARγ, has been found as well. PPARγ plays a significant role in adipocyte differentiation, adipogenesis and lipid metabolism. In addition, PPARγ was also found to participate in regulation of energy balance and insulin sensitivity and is therefore an interesting target for treatment of insulin resistance, obesity and cardiovascular diseases.

Bilirubin was found activate PPARα as well as PPARγ, resulting in beneficial effects on lipid metabolism and glucose homeostasis. The potential of mildly elevated bilirubin levels through exogenous administration of UCB or UGT1A1 inhibitors as a new therapeutic strategy to stimulate these beneficial metabolic effects could be investigated in the future. However, it is important to keep in mind that complete inhibition of UGT1A1 can result in severe unconjugated hyperbilirubinemia what can have toxic effects including kernicterus. UGT1A1 is not only responsible for UCB conjugation, but can also glucuronidate exogenous compounds (for example ezetimibe). Therefore, caution should be taken when using UGT1A1 inhibitors.

3. Transintestinal pathway for bilirubin and cholesterol

During unconjugated hyperbilirubinemia, around 2-15% of intestinal UCB is derived from biliary secretion whereas the major part (85-98%) is derived from transintestinal bilirubin secretion. A transintestinal excretion route also exists for cholesterol (TICE) and can be increased through activation of several NRs including LXR, FXR and PPARδ,
resulting in an increased fecal neutral sterol (FNS) excretion and lower plasma cholesterol levels. The apparent existence of transintestinal pathways for UCB and cholesterol led to the research question whether these are quantitatively related and if stimulation of FNS excretion could lower plasma bilirubin in hyperbilirubinemic Gunn rats (chapter 3). We found that stimulation of FNS excretion through activation of FXR or inhibition of intestinal cholesterol absorption did not lower plasma UCB levels in Gunn rats. From this we can conclude that the intestinal bilirubin excretion pathway and the TICE pathway are not linked in rats.

Although underlying mechanisms are not fully elucidated for the TICE pathway, the ABCG5/G8 transporter was found to be important for stimulation of TICE. ABCG5/G8 can be stimulated by LXR and FXR and intestinal ABCG5/G8 is at least partially responsible for TICE. Therefore, the TICE pathway is considered to be an active process, highlighted by the study of Le May et al., in which ATP-dependency of the TICE pathway was demonstrated by an ex vivo approach using intestinal perfusion of mouse and human intestinal explants, where incubation of these intestinal explants at 4°C resulted in a decreased cholesterol transport into the lumen. Besides active cholesterol export into the intestinal lumen through ABCG5/G8, the TICE pathway is also strongly depending on (partial) reabsorption of cholesterol. Van de Peppel et al. demonstrated that under physiological conditions, the cholesterol secreted into the intestinal lumen is mainly reabsorbed by enterocytes, thereby counteracting net cholesterol disposal from the body via FNS excretion. It is proposed that TICE is the result of transepithelial excretion of cholesterol and (partial) reabsorption.

Possible underlying mechanisms responsible for transintestinal bilirubin excretion (TIBE) have not been elucidated yet and it is not known if TIBE is regulated in a passive or active manner since no transporters have been characterized.

Next to the transporter ABCG5/G8, hydrophilic bile acids (BAs) were found to be involved in regulation of FNS output as well. The hydrophobicity of BAs affects the intestinal cholesterol absorption: muricholic acids (MCA) are hydrophilic BAs and very potent inhibitors of cholesterol absorption, whilst cholic acid (CA) has a more hydrophobic character and stimulates intestinal cholesterol absorption. The primary BAs alpha-muricholic acid (αMCA) and beta-muricholic acid (βMCA) are specific to rodents and are in the murine intestine converted by intestinal microbiota into the secondary BAs hyocholic acid (HCA), murideoxycholic acid (MDCA), omega-muricholic acid (ωMCA), and hyodeoxycholic acid (HDCA). Furthermore, the pharmacological compound PX is a ligand for FXR and administration of PX was associated with a more hydrophilic BA pool and a reduced cholesterol absorption.

Intestinal microbiota produce hydrophilic BAs but are also important in the metabolism of bilirubin into breakdown products. During the neonatal period, intestinal microbiota have not been fully developed yet and therefore, neonatal feces contains more UCB compared to adult feces where urobilinoids are the predominant bilirubin form.
immaturity of microbiota in neonates increases the availability of UCB for intestinal reabsorption and thus contributes to higher total bilirubin levels in plasma. Vitek et al. demonstrated that in Gunn rats, a complete abolishment of intestinal microbiota through administration of antibiotics, significantly increased plasma UCB levels which was associated with a decreased fecal bilirubin output. Recent studies have demonstrated that administration of the BAs cholic acid (CA) and ursodeoxycholic acid (UDCA) alone or combined with phototherapy could be used to treat unconjugated hyperbilirubinemia in Gunn rats. UDCA can alter the intestinal BA pool and both UDCA and CA increase the fecal excretion of UCB together with urobilinoids. The hypobilirubinemic effect of UDCA can be due to stimulation of TIBE or through inhibition of intestinal reabsorption of UCB, whilst CA was found to stimulate the fractional biliary UCB excretion. However, the results obtained with CA on bilirubin levels in plasma seem to contradict the results regarding how CA affects intestinal cholesterol absorption; CA is a rather hydrophobic BA that increases intestinal cholesterol absorption. To elucidate the effect of microbiota on the BA pool and the production of hydrophilic BAs and subsequently on the transintestinal excretion pathways of UCB and cholesterol, as well as on intestinal absorption, this could be studied using labelled cholesterol and UCB. Ideally, this would need to be performed both in conventionalized and in germfree hyperbilirubinemic Gunn rats.

Studies in the past have showed that TIBE could be stimulated by increasing intestinal fat content through administration of the lipase inhibitor orlistat or by feeding a HFD. These treatments increased both the fecal UCB and fat content and it is hypothesized that intestinal fat can ‘capture’ the intestinally present UCB due to the hydrophobic character of both compounds. However, the molecular nature of the association between UCB and unabsorbed fat has not been elucidated yet.

Another explanation for the increase in the TIBE pathway under unconjugated hyperbilirubinemic conditions could be that UCB was found to increase the intestinal permeability. To investigate if an increased intestinal permeability contributes to the TIBE pathway, a transwell system with human intestinal CaCo2 cells could be used to assess intestinal permeability under unconjugated hyperbilirubinemic conditions. Furthermore, this system could also be used to investigate how UCB is transported over the intestinal wall, for example in the presence/absence of intestinal permeability inhibitors or bile acids.

4. Peroxisomal function and lipid metabolism

The transintestinal cholesterol excretion (TICE) pathway has been a topic of interest in the search for therapeutic targets for atherosclerosis. Although recent literature has elucidated more of the TICE pathway, the pathway has not yet been fully elucidated in detail. Comparative transcriptome analysis of intestinal samples from experiments in which
TICE was stimulated revealed an association with the expression of peroxisomal membrane protein 4 (PXMP4, PMP24).

Since its discovery in 1999\textsuperscript{107}, still not much is known about the (metabolic) function of PXMP4. The \textit{Pxmp4} gene is a target of PPAR\(\alpha\) and in \textbf{chapter 4}, we aimed to determine the functional role of PXMP4 under a PPAR\(\alpha\)-stimulated and phytol-loaded condition, using a full body PXMP4 knockout mouse model. Several peroxisomal (membrane) proteins have been characterized in the recent years, however the function of many peroxisomal membrane proteins has remained unidentified. Regarding PXMP4, our studies indicate that PXMP4 is a redundant protein of which its function can be compensated, for example by other peroxisomal proteins, a finding that has been reported for other peroxisomal proteins before\textsuperscript{108–110}. The virtually complete lack of a phenotype in mouse models with a single-peroxisomal protein deficiency is not an unknown phenomenon in the peroxisomal protein field.

The group of Rokka was the first to investigate the function of the peroxisomal membrane protein 2 (PMP22, PXMP2) and showed that PXMP2 deficiency in C57BL/6J mice did not affect serum free fatty acid levels, total biliary BAs concentrations, tissue morphology nor peroxisomal enzyme activity\textsuperscript{111,112}. However, \textit{Pxmp2}\(^{-/-}\) mice had increased levels of uric acid in serum and urine compared to wild type mice under basal conditions (respectively 1.4 and 3.1-fold increase). In addition, female \textit{Pxmp2}\(^{-/-}\) mice displayed reduced mammary glands and had a lower milk production, resulting in a disability to nurse their pups\textsuperscript{111,112}. Similar to what we applied in our study, \textit{Pxmp2}\(^{-/-}\) animals were exposed to two dietary therapeutic strategies to elicit a more severe phenotype regarding peroxisomal functions: the PPAR\(\alpha\) ligand clofibrate (0.5% w/w) for 14 days or phytol (0.5% w/w) for 12 weeks. However, neither clofibrate nor phytol administration did result in a severe phenotype in \textit{Pxmp2}\(^{-/-}\) mice or showed differences in affected serum levels of the phytol-metabolites phytanic and pristanic acid between \textit{Pxmp2}\(^{-/-}\) and wild type mice\textsuperscript{112}. In our study, plasma phytanic and liver pristanic acid levels were slightly increased in \textit{Pxmp4}\(^{-/-}\) mice compared to wild type littermates, suggesting that PXMP4 is involved to some extent in the oxidation pathways of these branched-chain fatty acids (BCFA). Lismont et al. suggested that PXMP2 functions as a channel-forming protein in mammalian peroxisomal membranes, but is not essential for the transport of \(\text{H}_2\text{O}_2\) over the peroxisomal membrane and can be taken over by other candidates, possibly PXMP4 or SCL25A17 (PMP34)\textsuperscript{113}.

PMP34 is a peroxisomal membrane protein belonging to the family of mitochondrial solute carriers\textsuperscript{114}. The first-ascribed function of PMP34 was as a peroxisomal ATP transporter, providing ATP for the oxidation of fatty acids in the peroxisome\textsuperscript{115,116}. Besides being responsible for the transport of ATP and other cofactors such as CoA, FAD and NAD\(^+\) into the peroxisome\textsuperscript{117}, PMP34 was also found to be involved in the conversion of phytic and pristanic acid\textsuperscript{118,119}. Similar to \textit{Pxmp4}\(^{-/-}\) and \textit{Pxmp2}\(^{-/-}\) mice, PMP34-deficient mice (\textit{Pmp34}\(^{-/-}\)) did not exhibit differences in lipid profile, BAs levels, organ morphology and phytic and pristanic acid levels under basal conditions\textsuperscript{119}. In line with our study, total
BAs concentrations as well as VLCFA were not changed by administration of 0.5% w/w phytol (20-30 days) in Pmp34−/− mice. However, the phytol-treatment resulted in a mottled appearance of the liver, increased hepatic inflammation and hepatic steatosis. In addition, livers of Pmp34−/− mice had increased cholesteryl esters and triacylglycerol levels, as well as more phytanic and pristanic acid compared to wild type mice under phytol-fed conditions. Van Veldhoven et al. hypothesized that PMP34 -deletion results in an impaired intraperoxisomal α-oxidation cycling. An explanation for the differences in the effects of phytol administration to Pmp34−/− mice or in Pxmp4−/− mice could be related to the phytol dosages used. In our study, we have chosen a concentration of 0.25% w/w phytol because we wanted to prevent hepatotoxicity. A concentration of 0.5% w/w phytol in female mice and 1% w/w phytol in male and female mice has been shown to cause necrosis and a loss of hepatocytes. However, we did not investigate the individual metabolites of phytol, phytanic and pristanic acid and it is therefore difficult to pinpoint where in this metabolic pathway PXMP4 is functional. For future studies, a more comprehensive analysis of these metabolites as well lipodomics could be interesting to determine if, and where, PXMP4 is involved in the breakdown of phytol.

The single-deletion of PXMP4, PXMP2 and PMP34 has not exhibited a distinct phenotype under basal conditions, and neither after PPARα activation by fibrate administration. Dietary treatment of phytol only resulted in an increase in phytanic and pristanic acid in these animal models. Although various somatic mutations and hypermethylation resulting in silencing of PXMP4 in humans has been reported for several types of cancer, its role in tumor development as well as its physiological function has remained unknown. Hypothetically, these three PMPs could all have a redundant function by themselves, but as part of a bigger complex could exert functions in metabolic degradation of phytanic and pristanic acid. It will be interesting to develop a double or triple-knockout model of these three PMPs to investigate whether under those conditions peroxisomal functions such as the α- and β-oxidation pathways are affected. This could be studied under basal conditions, as well as under phytol-loading because standard rodent diet does not, or in a very low amount, contain phytol.

As discussed in chapter 4, the limited amount of literature concerning PXMP4 suggests a function of PXMP4 in adaptive and innate immune system and as a tumor suppressor gene in prostate cancer. The Pmp34 and Pxmp4 genes show partial protein homology, and although homology does not necessarily result in a comparable function, it could be hypothesized that PXMP4 exerts a similar function as Tmem135. It has been proposed that Tmem135 is not essential for peroxisomal function, but is involved in cholesterol transport between lysosomes and peroxisomes and import of matrix enzymes into the peroxisome, thereby stimulating peroxisomal functions such as β-oxidation.
• Chapter 6

We cannot exclude that under different experimental conditions, PXMP4 can have a more distinct function. Suggestions for future studies include challenging Pxpmp4−/− mice with HFD for a long period to see if deletion of PXMP4 results in hyperlipidemia, accumulation of VLCFA and BCFA due to dysfunctional α- and β-oxidation. Opposed to this, (long-term) fasting activates peroxisomal α-oxidation and other peroxisomal functions and could therefore be an interesting option to investigate the function of PXMP4 under more extreme experimental conditions.

5. Protein restriction and ageing

The prevalence of CVD as well as other chronic metabolic disorders such as T2D, NAFLD, obesity and insulin resistance is higher in aged individuals. In addition, aging itself is a big risk factor for the development of these chronic metabolic disorders due to considerable changes or dysfunction in metabolically active organs including the liver and adipose tissue. Ageing is associated with a reduction in subcutaneous white adipose tissue (WAT) and brown adipose tissue (BAT) function, causing decreased lipid handling, defective thermogenesis and de novo adipogenesis. Dietary protein restriction has been demonstrated to improve metabolic health under various conditions. However, the relevance of ageing and age-related decline in metabolic flexibility on the effects of dietary protein restriction have not been addressed. In Chapter 5, administration of a low protein diet (LPD) to 18-months old mice for a period of 12 weeks improved energy expenditure, increased circulating FGF21 levels and browning of subcutaneous white adipose tissue (scWAT). No deterioration in muscle function or physical health were reported in our old mice. Our findings indicate that interventions based on protein restriction have the potential to improve metabolic health when started at an older age in mice and that this could be a promising therapeutic strategy in elderly human patients. However, to be able to apply a short-term dietary protein restriction in elderly patients, it is important to keep track of the physical activity by means of monitoring the muscle strength or other measurements to assess sarcopenia.

6. Conclusion and future perspectives

The association between elevated UCB levels and the beneficial effects on lipid and glucose metabolism has gained much interest in the recent years, with a suggested role for the nuclear receptors PPARα and PPARγ in the underlying mechanisms. The functions of bilirubin as a signaling molecule has been investigated in more detail, although many aspects of this are still not fully elucidated. Furthermore, the transintestinal excretion pathways for cholesterol and UCB were shown not to be quantitatively linked to each other, and activation of the LXR or FXR transporters are not very potent therapeutic targets to stimulate the transintestinal excretion pathway of bilirubin. Although the intestinal
excretion of bilirubin and cholesterol do not seem to be related, this does not exclude the possibility that stimulation of NRs involved in fatty acid oxidation could regulate the intestinal ‘capturing’ of UCB resulting in a higher fecal UCB excretion. Future studies could investigate if the transintestinal UCB secretion is altered after PPAR activation.

The role of hydrophilic bile acids on intestinal UCB absorption or the effect of UCB on intestinal permeability and therefore the possible effects on the enterohepatic circulation of UCB could be interesting topics to elucidate in future studies. However, it is important to note that in rodents the prominent lipoprotein class is HDL, whilst in humans this is LDL. In humans, the cholesteryl ester transfer protein (CETP) is responsible for the transport of cholesteryl esters from HDL to apoB-containing lipoproteins including very low-density lipoprotein (VLDL) and LDL. CETP is absent in mice and rats and therefore most cholesterol is present in the HDL fraction as compared to the LDL-C in humans. This implicates that studies investigating the effects of bilirubin on cholesterol and lipoprotein metabolism should keep in mind that species-specific effects exist.

The field of peroxisomal disorders is a relatively young field and peroxisomal disorders can be caused by single protein deletions, resulting in disorders with varying clinical manifestations. The function of many peroxisomal proteins have not been described yet. In this thesis, we showed that PXMP4 is a redundant protein that could possibly be involved to a limited extent in the breakdown pathway of phytanic and pristanic acid. Future studies could use other experimental conditions such as long-term fasting could to possibly elucidate the function of PXMP4.

In conclusion, the studies described in this thesis have provided more insight in the mechanism of transintestinal bilirubin excretion: the process is independent from transintestinal cholesterol secretion and stimulation of LXR and FXR (which stimulate the TICE pathway) does not ameliorate unconjugated hyperbilirubinemia in Gunn rats. Regarding the physiological role of a specific peroxisomal protein, we provided data to suggest that PXMP4 is largely redundant. We feel that the present studies and the detailed characterizations of (partly new) animal models will be useful to further delineate and explore the mechanisms underlying the different bilirubin and lipid disorders.
References


178


30. Sakamoto S, Kasuhara H, Miyata K, et al. Glucuronidation converting methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) to a potent apical sodium-dependent bile acid transporter inhibitor, resulting in a hypocholesterolemic action. J Pharmacol Exp Ther. 2007;322(2):610-618. doi:10.1124/jpet.106.116426


179


50. Sinal CJ, Bend JR. Aryl hydrocarbon receptor-dependent induction of Cyp1a1 by bilirubin in mouse hepatoma hepa 1c17 cells. Mol Pharmacol. 1997;52(4):590-599. doi:10.1124/mol.52.4.590


180


64. Senkeko-Effenberger K, Chen S, Bracce-Sinnokrak E, et al. Expression of the human UGT1 locus in transgenic mice by 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (WY-14643) and implications on drug metabolism through peroxisome proliferator-activated receptor α activation. Drug Metab Dispos. 2007;35(3):419-427. doi:10.1124/dmd.106.013243


181


75. Zhang X, Yeung DCY, Karpisek M, et al. Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. Diabetes. 2008;57(5):1246-1253. doi:10.2337/db07-1476


183


General discussion and conclusion


140. Renquist BJ, Madanayake TW, Ghimire S, Geisler CE, Xu Y, Bogan RL. TMEM135 is an LXR-inducible regulator of peroxisomal metabolism. 2018;47. doi:https://doi.org/10.1101/334979


185


