Chapter 1

General introduction

1. Understanding metabolic disorders

Metabolic homeostasis is defined as the balance between catabolic (energy-producing) and anabolic (energy-consuming) processes. In the past century, the large increase in availability of food together with a reduced level of physical activity has led to a positive energy balance, driving the increased prevalence of obesity-related (chronic) disorders [1]. Obesity affects most physiological functions of the human body, illustrated by a variety of obesity-related (chronic) disorders targeting different (metabolic) organs. Examples of these metabolic disorders or conditions are insulin resistance, dyslipidemia and high blood pressure [2]. This cluster of metabolic conditions are termed Metabolic Syndrome (MetS) and increases the risk of chronic metabolic diseases including type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), cardiovascular disease (CVD) and certain types of cancer. The prevalence of diseases are expected to continue to rise [3]. Chronic metabolic disorders significantly decrease the quality of life and are associated with high healthcare costs, resulting in a higher economic burden [4,5].

These developments have spurred research aimed to elucidate mechanisms underlying the development of metabolic disorders. A close link between metabolic homeostasis and genetic mutations and (dysfunctional) transcriptional regulation by nuclear receptors (NRs) was found, for example in diseases such as T2DM, CVD and in ageing processes [6–9]. Subsequently, activation of NR pathways has been discovered as a therapeutic target for these conditions. The family of NRs consists of ligand-activated transcription factors and is involved in many biological processes including cell growth, stress responses and a plethora of metabolic pathways [1]. The increasing prevalence of metabolic disorders as well as the knowledge about the role of NRs in metabolism has led to a great interest in developing new ligands targeting NRs to treat these disorders.

In our lab we are generally interested in the etiology of disorders of (energy) metabolism, and in this thesis we specifically focus on the relation between dyslipidemia and unconjugated hyperbilirubinemia. To this end, we characterized novel animal models and investigated the potential role of several NRs in the pathophysiology of these disorders and whether or not targeting NR could be a potential therapeutic intervention strategy.

2. Nuclear receptors as therapeutic targets

2.1. Nuclear receptor structure and target gene regulation

The family of NRs consist of 48 members which act as receptors for a variety of lipophilic compounds including steroid hormones, thyroid hormone, vitamins A and D, lipids, bile acids and xenobiotics [10,11]. Regulation of target genes by activated NRs can be executed in different ways. Some NRs are resident in the cytosol as a complex with chaperone proteins, and translocate upon activation by their ligands to the nucleus where they can bind to the promotor region of target genes to regulate transcription. These include

the steroid receptors such as the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and progesterone receptor (PR) and typically regulate transcription of downstream targets by acting as homodimers [6,10,12].

Most of the NRs, however, reside on the chromatin in an inactive state, forming a complex with histone deacetylases (HDACs), and are activated upon ligand binding. Ligand binding causes dissociation of the HDAC complex and formation of a new complex with histone acetyltransferases (HAT) such as p300 and CREB binding protein (CBP). This results in a higher chromatin accessibility and increased transcription of the target genes [13,14]. NRs that are bound to the chromatin such as the liver X receptor (LXR), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPAR), constitutive androstane receptor (CAR) or pregnane and xenobiotic receptor (PXR) generally form a heterodimer with the retinoid X receptor (RXR) in order to regulate transcription of downstream target genes (reviewed in [6,10]). This heterodimerization with RXR allows the heterodimer to function as a reversible switch. In absence of a ligand, the heterodimer binds corepressors resulting in repression of target genes of the specific NR. Conversely, binding of a ligand to either the specific NR or RXR causes a conformational change of the heterodimer thereby liberating the corepressors and subsequently recruitment of coactivators, which perform biochemical reactions required for augmenting transcription of the target genes [15,16]. These target genes are involved in a broad variety of metabolic pathways and are expressed in different cell types and tissues [10].

A common feature of all NR family members is the three-dimensional structure consisting of several functional domains (*Figure 1*). The N-terminal domain (NTD, or A/B domain) contains a ligand-independent AF-1 transcriptional activation domain (AF-1), and the more central region (C-domain) is constituted of 2 zinc fingers forming the highly-conserved DNA-binding domain. The COOH-terminal region (D/E domain) contains the ligand-binding domain (LBD) and a ligand-dependent activation function domain (AF-2) [17,18]. Some NRs contain at the very end of the COOH-terminal a variable stretch of amino acids called the F-domain [19]. The AF-2 domain is responsible for activation or repression of the NR through binding to recruited coactivators or corepressors.
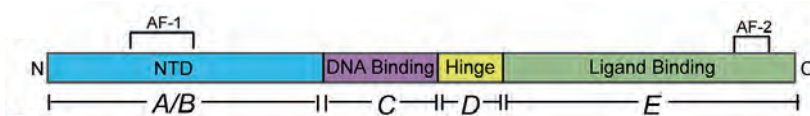
Figure 1. Schematic representation of the general domain structure of NRs. The A/B domain is located on the N-terminus region and contains the ligand-independent transcription activation function region 1 (AF-1). The central C-domain contains the DNA-binding domain (DBD). Finally, the COOH terminal region (D/E domain) contains the ligand-dependent transcriptional activation functional region 2 (AF-2) and is involved with recruitment of cofactors and heterodimerization with RXR. Adapted from [6].

Individuals with MetS often show comorbidities such as NAFLD and T2DM [20], and although the etiology is different, various NRs including PPARs play a role in the underlying

pathogenic pathways of these disorders. The important role of NRs in many metabolic pathways, together with the discovery that dysfunctional transcriptional regulation by NR could contribute to metabolic disorders, has led to extensive research on NRs as new therapeutic targets. Currently, around 13% of the Food and Drug Administration (FDA) approved drugs target NRs, including drugs targeting metabolic disorders such as insulin resistance (TZDs), dyslipidemia (fibrates) and inflammation (dexamethasone) [21]. However, unwanted side-effects have also been reported for NR-targeting drugs because NRs have a complex network of transcriptional downstream targets along with partial agonism of ligands [22]. The effects of individual NRs as well as interaction between NRs are dependent on the activated (metabolic) organ.

2.2. The role of NRs in metabolic processes

The liver plays a central role in glucose and lipid homeostasis, protein synthesis and in detoxification of endogenous and xenobiotic compounds. Activated NRs play an important coordinating role in these metabolic pathways and multiple NRs can be involved in one metabolic pathway and can perform overlapping or opposite functions. The NR family members LXR, FXR, CAR, PXR and PPARs all heterodimerize with RXR and are important players in metabolic pathways in the liver, as well as the gut, pancreas, adipose tissue and muscle [23,24]. They provide coordination between metabolic responses across organ systems during the fed and fasted states [10].

Under fasting conditions, energy is mainly retrieved from fatty acid oxidation (FAO) in muscles, heart and liver [25]. Fatty acids (FAs) derived from adipose lipolysis can in turn activate peroxisome proliferator-activated receptor alpha (PPARα) in the liver, thereby inducing hepatic FAO in order to produce energy in the form of ATP and ketone bodies. Besides stimulation of FA oxidation, fasting-induced activation of PPARα also stimulates gluconeogenesis which is driven by the obtained energy from FAO [26]. Activated PPARα also stimulates the production of the hepatokine fibroblast growth factor 21 (FGF21) which functions as a stress-signal to other organs to prepare them for an approaching energy-deprivation state [10,27].

During the fed state, NRs such as FXR, LXR and PPARs are responsible for extracting nutrients from the gut, nutrient transportation to the liver and storage in adipose. A post-prandial increase in glucose availability increases the concentration of insulin and insulin plays an important role in the fat storage and mobilization by the adipose tissue, as it suppresses the lipolysis of TAG [28,29]. Furthermore, the post-prandial rise in bile acids (BAs) activates FXR which in turn exerts a negative feedback on their synthesis. FXR also suppresses gluconeogenesis and lipogenesis [30]. After hepatic metabolism, transport to and utilization of lipids by peripheral tissues including adipose tissue and muscles is exerted to an important extent by PPARβ/δ and PPARγ [24]. LXR, FXR and PPARα are all involved in cholesterol and bile acid (BA) homeostasis, fatty acid metabolism and glucose and insulin sensitivity. The involvement of these NRs in the metabolism of cholesterol, BA, FAs, triglycerides as well as hepatic detoxification will be discussed below.

2.2.1. Cholesterol metabolism

Cholesterol is an indispensable molecule for vertebrates due to its function as a major component of cell membranes and precursor for steroid hormones and BAs. Although cholesterol can be synthesized by many tissues, the liver is quantitatively the major production site. Cholesterol can be secreted in its sterol form via the bile or directly into the intestinal lumen or as a BA via the bile after a multi-enzymatic conversion, including 7α-hydroxylase (CYP7A1) [31]. The direct secretion of cholesterol across the intestinal epithelium into the intestinal lumen is called the Trans Intestinal Cholesterol Excretion (TICE). The TICE pathway was found to be regulated by different NRs which will be discussed in more detail in section *2.2.2*.

Maintenance of cholesterol homeostasis is under regulatory control of several NRs including LXR, FXR and PPARs as well as other transcription factors such as sterol regulatory element binding proteins (SREBPs) [32–34]. Both LXR and FXR are highly expressed in the liver and intestine, organs that are important for cholesterol homeostasis [35,36]. Endogenous ligands for LXR are oxidized forms of cholesterol called oxysterols and synthetic compounds including T0901317 (T09) and GW3965 [37,38]. LXR exists in two isoforms, LXRα (NR1H3) and LXRβ (NR1H2), which have a distinct tissue expression pattern [39]. LXRα is highly expressed in the liver, intestine, adipose tissue and macrophages, while LXRβ is ubiquitously expressed in the body [40]. LXR is considered a cellular sterol-sensor and activates pathways to eliminate or metabolize excess cholesterol, such as the process of reverse cholesterol transport (RCT). RCT is the transport pathway of excess cholesterol in the form of high-density lipoprotein (HDL) cholesterol from the peripheral tissues back to the liver [31,41]. Excess cholesterol is then excreted from the body as neutral sterols (NS) or BAs via the bile. The ATP-binding cassette transporter A1 (ABCA1) is the rate-limiting step in the formation of HDL particles and transports cholesterol to ApoA1. Another ABC half-transporter, ABCG1, is also involved in RCT and transports cholesterol from macrophages to HDL-2 and HDL-3 particles [42–44]. HDL can appear in several degrees of density, with HDL-3 being more dense than HDL-2 [41]. Both ABCA1 and ABCG1 are transcriptionally regulated by LXR [44–46]. After being taken up by the liver via the Scavenger receptor class B type 1 (SR-B1), excess cholesterol can be converted into BAs or secreted as free cholesterol into the biliary tract.

FXR also plays a role in cholesterol homeostasis, although this is complex and incompatible findings have been reported in literature. FXR knockout mice (FXR^{-/-}) display increased hepatic and plasma levels of total cholesterol (TC). The increased TC levels in FXR^{-/-} mice correspond with elevated plasma levels of very-low density lipoprotein (VLDL), low-density lipoprotein (LDL) and HDL [47–49]. The increase of HDL-C due to FXR deficiency is suggested to be attributable to decreased hepatic cholesterol uptake, through reduced expression of SR-B1 [49]. Activation of FXR by BAs or synthetic ligands such as GW4064 or obeticholic acid (OCA) decreased plasma TC, HDL, VLDL and LDL

in mice [47,49–52]. Furthermore, activation of hepatic FXR increased the expression of genes involved in lipoprotein metabolism and RCT including SR-B1 [53–55]. However, in the study of Zhang et al., administration of the FXR ligand GW4064 to wild type mice did not affect plasma VLDL and LDL cholesterol levels, but TC and HDL-C levels were decreased in plasma [56]. In contrast to results in mice, administration of the synthetic FXR ligand OCA to patients with NASH and healthy volunteers has been shown to increase plasma TC, LDL-C levels and decreased HDL-C [57,58]. This increase in TC and LDL could be explained by an inhibited hepatic conversion of cholesterol into BA by FXR activation [57,58]. The differences in response to OCA between mice and humans could be due to species-specific differences between humans and rodents; rodents mainly have HDL-C and to a lower extent LDL-C.

Not only LXR and FXR but also PPARs were found to be an important therapeutic target for the treatment of hypercholesterolemia [59]. PPARs can be activated by various species of lipids as well as chemicals specified as peroxisome proliferators. PPARs can be classified into three subtypes: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) and these subtypes differ in tissue expression and metabolic function [60–62]. The group of fibrates are ligands for PPARα and are used in the clinic as lipid-lowering drugs [63]. Administration of fibrates resulted in a lower plasma LDL-C and increase in HDL in patients with dyslipidemia [64,65]. Thiazolidinediones (TZD) are ligands for PPARγ and used as insulin-sensitizing drugs, but were also found to increase plasma HDL and decrease TG levels in patients with T2DM [66,67].

The process of cholesterol efflux and absorption is regulated by several NRs. The heterodimer ABC sub-family G5/G8 (ABCG5/G8) is expressed on the canalicular membrane of hepatocytes and the apical membrane of enterocytes and regulated by LXR and FXR, thereby coordinating apical cholesterol efflux from these cells [54,68–71]. The Niemann-Pick C1 like 1 (NPC1L1) protein is expressed in the small intestine and is critically involved in intestinal cholesterol absorption [72]. Around 80% of the intestinal cholesterol content is reabsorbed by the NPC1L1 transporter [73]. The *Npc1l1* gene was found to be directly downregulated by LXR in mice as well as in the human enterocyte cell line Caco-2/TC7, thereby decreasing cholesterol absorption and increasing the fecal disposal of neutral sterols [74]. The human *Npc1l1* gene also contains a PPAR-response element (PPRE) indicating that PPARs can directly regulate human *Npc1l1* expression [75]. Activation of PPARα as well as PPARβ/δ reduced expression of *Npc1l1* [76].

2.2.2. *The transintestinal cholesterol excretion (TICE) pathway*

The TICE pathway can be stimulated through activation of LXR, FXR and PPARδ, although the underlying mechanisms are not fully understood [54,69,77–80]. Increased intestinal expression of ABCG5/G8 has been suggested to be involved in the increase of TICE upon

treatment with the LXR ligand T09 in mice [69,77]. However, mice deficient of ABCG5/G8 still showed around 60% of the fecal neutral sterol (FNS) excretion compared to their wild type littermates, implicating that ABCG5/G8 is not fully responsible for the TICE pathway [69,81,82]. A still unresolved question refers to the mechanism of cholesterol transport for the TICE pathway from the liver to the proximal intestine. Le May et al. suggested that HDL and/or LDL could function as a cholesterol-carrier, demonstrated by *in vivo* and *ex vivo* data in mice [83]. Le May et al. found that TICE was increased by lovastatin in wild type mice, but this effect was absent in LDL-receptor deficient (LDLR^{-/-}) mice. Mice deficient for proprotein convertase subtilisin/kexin type 9 (PCSK9), a protein causing breakdown of the LDL-receptor, showed an increased TICE pathway [83]. Surprisingly, LDLR^{-/-} mice did not have a decreased TICE, despite the lower hepatic LDL content [83]. Taken together, the contribution of LDL and HDL can only partially explain TICE is and more studies are needed in order to elucidate how cholesterol delivery to the TICE pathway is performed.

Van de Peppel et al. showed in mouse studies that under physiological conditions, cholesterol excreted via TICE is largely reabsorbed by NPC1L1 [84] (*Figure 2*). Decreasing the intestinal cholesterol reabsorption can be performed by inhibition of NPC1L1 by ezetimibe [78,79,84,85], by decreasing the biliary secretion rate of BAs or by increasing the hydrophilicity of the BAs in the intestinal lumen [54]. These strategies appeared effective to increase the net excretion of cholesterol.
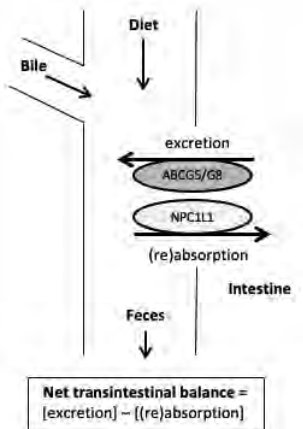
Figure 2. Cholesterol fluxes and the involved transporters ABCG5/G8 and NPC1L1 in the intestine. ABCG5/G8 is involved in apical cholesterol efflux into the intestinal lumen, and NPC1L1 is responsible for cholesterol (re)absorption in the small intestine. However, an ABCG5/G8-independent influx of cholesterol into the intestinal lumen has also been demonstrated [69,81,82]. Net intestinal cholesterol balance can be calculated by subtraction of mean dietary cholesterol intake and biliary cholesterol excretion from the FNS excretion. Adapted from [86].

2.2.3. *Bile acid metabolism*

BAs are natural ligands of the FXR and the Takeda G protein-coupled receptor 5 (TGR5 or G-Protein Coupled Bile Acid Receptor (Gpbar1)) in the intestine, and function as

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important signaling molecules through their capacity to activate these receptors [31,87]. FXR is ubiquitously expressed but is the highest in the intestine and liver [88–90]. Four splice-variants of FXR originating from one single gene are known in rodents and humans: FXRα1, FXRα2, FXRα3 and FXRα4 [89,91,92]. FXR can not only be activated by BAs but also by some hydrophobic compounds such as FAs, steroids and hormones [90]. Binding of BAs to intestinally expressed FXR causes transcriptional upregulation of amongst others the gene encoding fibroblast growth factor 15 (FGF15) in the terminal ileum [31,93]. Subsequently, FGF15 travels to the liver, where it binds to the membrane-bound FGF receptor 4 (FGFR4). The activated FGFR4 cooperates with the co-protein β-Klotho in order to downregulate genes involved in BA synthesis, including the rate-controlling enzyme cytochrome P450 7A1 (CYP7A1) [93–96]. The human liver produces the primary BAs chenodeoxycholic acid (CDCA) and cholic acid (CA), whilst in rodents primary BAs exist of CA and muricholic acids (MCAs) [97]. These BAs can be synthesized through two pathways: a classical and alternate (acidic) pathway. CYP7A1 is the rate-limiting enzyme in the classical BA synthesis pathway, accounting for around 75% of the hepatic BA production [98]. The alternate BA synthesis pathway is regulated by the sterol-27-hydroxylase (CYP27A1), followed by sterol 7α-hydroxylase (CYP7B1) and CDCA is mainly produced through the alternative pathway [97,98]. The feedback regulation of the homeostasis of its own ligands by activated FXR is an example of the control of fed-state metabolism by NRs. Activation of LXR in rodents has been shown to induce the expression of *Cyp7a1* [99]. However, this is not conserved in humans [99,100]. The *Cyp7a1* and *Cyp27a1* were also found to be downregulated by the PPARα ligand fibrate, thereby lowering BA synthesis [101].

2.2.4. *Lipid metabolism*

Opposite roles for LXR and FXR have been described in lipid metabolism. LXR can directly bind and activate the Sterol Regulatory Element Binding Transcription Factor 1 (SREBF1 or SREBP-1C), one of the master regulators of fatty acid and triglyceride biosynthesis [102]. Therefore, administration of LXR ligands such as T0901317 (T09) often results in hypertriglyceridemia in rodents as well as in humans. In contrast, activation of FXR was found to increase hydrolysis of triglycerides by downregulation of SREBP-1C, resulting in lowered triglyceride levels [32,103,104]. In the liver, the PPARα-activating fibrates decrease the expression of apolipoprotein C-III (ApoCIII) and increase the expression of lipoprotein lipase, resulting in a decrease in serum triglyceride concentration [64,105]. Fibrates are therefore often used to treat hypertriglyceridemia and also are effective in decreasing the risk of cardiovascular disease (CVD) [62,106].

2.2.5. *Detoxification*

The liver is an important site for detoxification of xeno- and endobiotics and this process is under regulation of several NRs including LXR, PXR and CAR [107–109]. Recently, a

function for LXR and PPARα in the detoxification and secretion of bilirubin has been reported in mice [107,110,111]. FXR-induced activation of the enzyme uridine diphosphoglucuronosyl transferase (UGT1A1) was first described by Lee et al. in wild type mice where UGT1A1 contains an FXRE upstream of the transcriptional start site [112]. The UGT1 family is responsible for detoxification of endogenous and xenobiotics through glucuronidation. An intronic FXRE was also found in human and mouse UGT1A1 promotor [113].

3. Nuclear receptors and bilirubin disorders

3.1. Bilirubin metabolism

3.1.1. Synthesis and transport

The liver is of great importance for the detoxification of endogenous and xenobiotic toxic compounds including unconjugated bilirubin (UCB) [114]. The liver has a high expression of metabolizing enzymes and proteins which are under transcriptional regulation of several NRs [115]. UCB is a breakdown product of heme, mainly derived from hemoglobin in erythrocytes [116], but UCB can also be derived in a smaller extent from mitochondrial heme components and myoglobin located in muscle tissue [117,118]. Erythrocyte degradation is primarily performed by the spleen, although degradation can also take place in the liver. Heme is converted into the non-toxic molecule biliverdin by the enzyme heme oxygenase (HO) [119]. In humans as well as rats and mice, biliverdin is then further metabolized into the toxic and hydrophobic compound UCB by the enzyme biliverdin reductase [120]. Because the hydrophobic character complicates transport of free UCB throughout the blood, binding of UCB to the carrier albumin is required. This UCB-albumin complex is transported to the liver where UCB is released from albumin, followed by uptake into the hepatocytes. The UCB-albumin ratio can be disturbed under several conditions for example when UCB levels are extremely high, in case of hypoalbuminemia or with a lower binding capacity of albumin [121]. This increases the concentrations of free UCB in the plasma and free UCB can diffuse over the blood-brain barrier, causing UCB deposition in the brain [122].

3.1.2. Hepatic metabolism

Hepatic uptake of UCB can occur actively by the organic anion transporting polypeptides (OATP)1B1/1B3 transporters in humans and OATP1B2 in rats [123] (*Figure 3*). Deficiencies or mutations in human OATP1B1/B3 results in the Rotor syndrome, a disease characterized by mildly increased levels of conjugated bilirubin (CB) and UCB in the serum [124,125]. The presence of CB in bile and plasma in patients with Rotor syndrome illustrates that hepatic UCB uptake can also take place passively [126]. In the liver, UCB is conjugated by the enzyme uridine diphosphoglucuronosyl transferase (UGT1A1) into bilirubin

monoglucuronide (BMG) or bilirubin diglucuronide (BDG). The conjugation of UCB with one or two glucuronyl groups gives it a more hydrophilic character and facilitates secretion into the bile. Mutations in the *Ugt1a1* gene can result in a complete or partial absence of the UGT1A1 protein, respectively called Crigler-Najjar type 1 (CN-1) and 2 (CN-2) [127]. A residual activity of UGT1A1 of 20-30% caused by additional TA repeats in the promotor region of the *Ugt1a1* gene also impairs UCB glucuronidation, resulting in mild unconjugated hyperbilirubinemia. This disease is called Gilbert Syndrome (GS) [128]. These disorders will be explained in more detail in section 3.2.

The translocation of CB across the canalicular hepatocyte membrane into the bile is largely performed by ATP-binding cassette transporter 2 (ABCC2, MRP2) [129,130]. A hereditary recessive mutation in the *ABCC2* gene encoding the transporter MRP2 causes Dubin-Johnson syndrome and patients display both CB and UCB accumulation [131,132]. During bile duct obstruction or other conditions where CB cannot be transported into the bile, the basolateral transporter ABCC3 transports CB back into the blood. Expression of ABCC3 is low under physiological conditions but was found to be upregulated in MRP2 deficient rats, patients with Dubin-Johnson syndrome and individuals with a cholestatic liver [133–135].

When UGT1A1 expression is absent, an alternative metabolic pathway can be upregulated in order to decrease the accumulating levels of UCB in the body. The cytochrome P450 family 1A1 (CYP1A1) and 1A2 (CYP1A2) can oxidize UCB and its oxidation products are secreted into the bile, although these compounds are not fully characterized yet and further research is necessary to determine their contribution under these conditions [136].
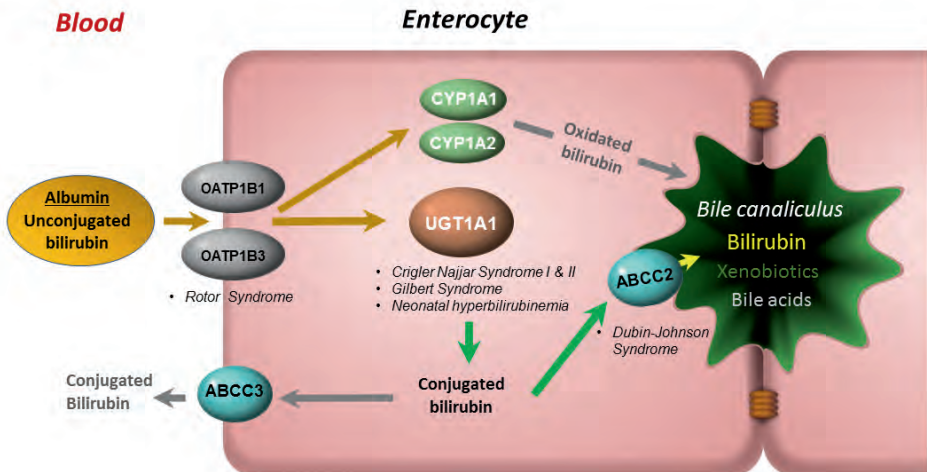
Figure 3. Schematic overview of hepatic metabolism of bilirubin. Unconjugated bilirubin (UCB) is transported to the liver as an albumin-bilirubin complex. The human transporters OATP1B1 and OATP1B3 (OATP1B2 in rats) transport (free = not-albumin-bound) UCB into the hepatocyte, where the enzyme UGT1A can convert UCB into mono- and diconjugated bilirubin (CB). Subsequently, CB is transported via ABCC2 into the bile canaliculus or alternatively, particularly upon accumulation by a defective biliary route of secretion, by ABCC3 back to the

bloodstream. An alternative pathway of bilirubin metabolism involves oxidation by Cyp1a1 and Cyp1a2, a pathway that has a limited activity upon absence of UGT1A1. Oxidation products of bilirubin are also secreted into the bile. Adapted from [312].

3.1.3. Intestinal metabolism

CB is secreted via the bile into the intestinal lumen, where most of the CB is deconjugated into UCB by mucosal β-glucuronidase [137–139]. From the intestinal lumen, UCB can be taken up again by enterocytes and transported back to the liver via the bloodstream, a process called the enterohepatic circulation (EHC) [140,141]. Non-absorbed intestinal UCB can be metabolized into non-toxic urobilinoids by intestinal microbiota. These urobilinoids can also be reabsorbed by the intestine in order to be secreted by the kidneys, or are excreted into the feces [142,143].

UGT1A1 is mostly present in hepatocytes, but was also found to be expressed by the intestine. In preterm neonates as well as humanized UGT1A (*hUGT1*1*) mice, an animal model used for neonatal unconjugated hyperbilirubinemia, hepatic *Ugt1a1* expression is delayed in the first postnatal days [144]. In *hUGT1*1* mice, the expression of *Ugt1a1* increases in the small intestine between PD14 and 21 and in the same time the serum total bilirubin (TB) decreases to adult levels [144,145]. Induction of intestinal *Ugt1a1* expression in *hUGT1*1* mice by agents such as obeticholic acid (OCA) or cadmium increases the clearance of serum bilirubin and counteracted systemic bilirubin accumulation in the absence of hepatic *Ugt1a1* expression [146–148]. An *in vivo* study performed with hyperbilirubinemic Gunn rats, a rat model representative for CN-1, showed that transplantation of the small intestine from Wistar rats to Gunn rats decreased serum bilirubin levels in the latter, demonstrating that the intestinal expression of *Ugt1a1* can aid in the clearance of serum unconjugated bilirubin [149]. The feces contains several breakdown products of (unconjugated) bilirubin, such as urobilinoids and include metabolites such as mesobilirubin, urobilinogen and stercobilirubin [150]. This group of urobilinoids forms the majority of molecules in the feces originating from bilirubin; the parent molecule UCB is only present in small amounts [142]. During the neonatal period, *Ugt1a1* expression is low and the intestinal microbiota have not been fully developed yet [143,151]. This increases the intestinal reabsorption of UCB, contributes to higher bilirubin levels in plasma (neonatal jaundice), together with the high metabolism of fetal hemoglobin in the neonatal period [142]. Accordingly, neonatal feces contains more UCB compared to adult feces where urobilinoids are the predominant bilirubin form.

3.1.4. Transintestinal bilirubin excretion

Under physiological conditions, around 98% of the bilirubin secreted into the bile is CB and less than 2% is UCB [152]. Upon accumulation of UCB in the body, UCB can also be excreted in small amounts into the bile despite its hydrophobic character, as well as across the intestinal epithelium into the intestinal lumen [153]. The transintestinal secretion route

comprises the direct transport of UCB from the plasma over the cell wall of enterocytes into the intestinal lumen, thereby bypassing the hepatobiliary route. In Gunn rats, an animal model for CN-1, around 2 – 15% of intestinal UCB is derived from biliary secretion whereas 85 – 98% is coming from transintestinal bilirubin secretion [154]. Transintestinal bilirubin excretion was thus found to be the major secretion route under unconjugated hyperbilirubinemic conditions, suggesting that stimulation of transintestinal bilirubin excretion (and/or with prevention of its reabsorption) might be a good strategy to prevent or treat unconjugated hyperbilirubinemia [154–156].

3.2. Unconjugated hyperbilirubinemia

Unconjugated hyperbilirubinemia is a common condition in infants, especially in preterm infants, and mainly occurs throughout the first 2 weeks of life [157]. Levels of bilirubin in plasma, bile and tissues are a result of a balance between bilirubin production and breakdown or excretion. The production of UCB is higher in neonates compared to adults due to a high breakdown rate of fetal erythrocytes. In addition, the glucuronidation pathway of UCB in the liver which facilitates removal from the body is not fully matured in neonates because the *Ugt1a1* gene is under developmental regulation [158–160]. Fetuses between gestational weeks 17 and 30 have a low expression of hepatic *Ugt1a1* (~ 0.1%), and between gestational week 30 and 40 the hepatic *Ugt1a1* expression is around 1% of adult expression levels [158]. After postnatal day (PD) 14, hepatic *Ugt1a1* expression reaches levels as seen in adults [159]. Therefore, the combination of a high production rate of UCB and a low hepatic *Ugt1a1* expression results in neonatal unconjugated hyperbilirubinemia. In hUGT1A1 mice it was found that intestinal *Ugt1a1* expression is already present before PD14, whereas hepatic expression is not detectable yet [144,145]. Toxic accumulation of UCB can enter the brain, especially in neonates due to their high permeable blood-brain barrier, causing severe symptoms including central nervous system toxicity and brain damage. When left untreated, this can eventually lead to death [161].

Unconjugated hyperbilirubinemia can also be caused by mutations in the *Ugt1a1* gene resulting in a complete or partial deficiency in the UGT1A1 protein, respectively called CN-1 and CN-2. CN-1 is a rare autosomal recessive inborn disorder with an estimated prevalence around 1:1000 000 [127,162]. No detectable levels of UGT1A1 activity are present in patients with CN-1 and plasma UCB levels in untreated CN-1 patients range from 300 to 800 μM [163,164]. The incidence of CN-2 is also rare (1:100 000) and CN-2 patients are characterized by moderate unconjugated hyperbilirubinemia with plasma levels ranging from 100 to 350 μM [165–167].

Additional TA repeats, often 7 or more, in the TATA box of the gene promotor of *Ugt1a1* (UGT1A1*28 allele) cause a polymorphism of *(TA)7/(TA)7* instead of *(TA)6/(TA)6*. This mutation is called Gilbert Syndrome (GS) and results in a decreased expression and

activity of UGT1A1 [128]. A number of other polymorphisms in the promotor region of UGT1a1 exist in the Asian population and UCB concentrations in the body depend on the specific polymorphism [128]. Individuals diagnosed with GS show mildly unconjugated hyperbilirubinemia (plasma UCB concentrations > 17.1 μM), but also can remain undiagnosed [168]. The prevalence of GS is around 10% in the population and a mild jaundice often only is visible under fasting conditions or during sickness [169,170]. Interestingly, individuals with GS were found to have a leaner phenotype and lower total plasma cholesterol as well as LDL-C levels compared to matched control individuals [171–173]. Recently, a link between mildly elevated (unconjugated) bilirubin levels and protection against cardiovascular disease (CVD) has been found [171–176]. Suggested underlying mechanisms for the protective effect of bilirubin are anti-inflammatory effects, lowering endoplasmic reticulum (ER) stress as well as lowering of total and LDL-C [171,177]. Therefore, strategies that can mildly increase endogenous bilirubin levels as well as exogenous administration of bilirubin can be interesting to explore as new therapeutic therapies for CVD and metabolic syndrome. Plasma UCB levels around 30-50 μM have been associated with beneficial effects, although future studies should investigate what the safe threshold is to increase endogenous bilirubin concentrations.

3.3. Animal models for unconjugated hyperbilirubinemia

3.3.1. Gunn rats

In the last few decades, several animal models have been used to study unconjugated hyperbilirubinemia *in vivo*. The best known animal model is the Gunn rat, a rat strain with a spontaneous mutation in the *Ugt1a1* gene, resulting in a complete absence of UGT1A1 activity [178]. These animals display non-hemolytic jaundice and are therefore a model for patients with CN-1 and are used for studies investigating treatments for unconjugated hyperbilirubinemia [178,179]. Several Gunn rat strains exist with different genetical backgrounds and increased plasma UCB levels. The R/APfd-j/j strain characterized by the group of Leyten et al. displayed an average serum bilirubin concentration ~150 μmol/L, and the RA/jj rat strain and the RHA/jj strain respectively presented plasma levels of ~80 μmol/L and ~121 μmol/L [154,180,181]. A more recent Gunn rat strain is the Gunn-Ugt1a1[i/BluHsdRrrc] strain showing varying levels of UCB, from a mean serum UCB concentration of ~177 μmol/L as well as levels ~46 μmol/L in rats in this same strain [182,183].

Gunn rat pups have been used to study neonatal unconjugated hyperbilirubinemia because, in accordance to human neonates, Gunn rat pups show a neonatal peak in plasma UCB. After this, plasma UCB levels decrease within days to levels observed throughout adult life, to gradually increase again during ageing [181,184,185]. Untreated Gunn rats have severe unconjugated hyperbilirubinemia throughout their life and show mild neurotoxic signs. These signs include stunting, ataxia, delay in motor development and cerebellar

hypoplasia [185,186]. In **chapter 2**, we assessed the bilirubin and plasma lipid phenotype in wild type, heterozygous and homozygous Gunn-Ugt1a1[i/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.

3.3.2. *Ugt1a1 knock-out mice*

Ugt1a1^{-/-} mice have a comparable mutation in the *Ugt1a1* gene as Gunn rats [187]. However, these mice display higher plasma UCB levels and when left untreated, *Ugt1a1^{-/-}* mice die between PD5-11 and are therefore in constant need of UCB-lowering therapy to prevent lethality [187]. The small size of *Ugt1a1^{-/-}* mice complicates the assessment of tissues and the constant need of therapy limits the usability of the *Ugt1a1^{-/-}* mouse to study new treatments for adult hyperbilirubinemia. Nevertheless, the *Ugt1a1^{-/-}* mouse model has been shown to be very useful to study developmental effects of bilirubin and bilirubin-induced brain toxicity [188,189].

3.3.3. *Humanized UGT1A mice*

Recently, humanized UGT1A (*hUGT1*1*) mice were developed by deleting the complete murine UGT1A family, followed by replacement with the human UGT1A locus consisting of 9 UGT1A family members [144,190]. In contrast to the *Ugt1a1^{-/-}* mouse model, *hUGT1*1* mice show a milder hyperbilirubinemia and these mice do not die prematurely. Because UGT1A1 in these mice is under control of the endogenous human promoter, the UGT1A1 expression profile of *hUGT1*1* mice resembles the human expression profile. Neonatal *hUGT1*1* mice have a peak in plasma UCB around PD14 and after PD21, the *hUGT1*1* mice become normobilirubinemic when reaching adulthood [144]. This model contains an important beneficial feature because it allows the investigation of human UGT1A1 stimulation during neonatal unconjugated hyperbilirubinemia. Human *Ugt1a1* can be upregulated by the constitutive androstane receptor (CAR), pregnane X-receptor (PXR), aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR) and PPARα [109,191,192]. Administration of ligands for these NRs in *hUGT1*1* mice can be used to investigate how UCB levels are affected by activation of these NRs and could potentially lead to new therapeutic strategies to ameliorate (neonatal) unconjugated hyperbilirubinemia [107].

3.4. Therapeutic interventions for unconjugated hyperbilirubinemia

3.4.1. *Phototherapy*

Over the years, different therapeutic strategies for unconjugated hyperbilirubinemia have been developed and these strategies can be targeted to different causes of this disorder. Phototherapy (PT) is the golden standard for unconjugated hyperbilirubinemia in patients with CN-1 and preterm neonates and has been used for many years [193]. During PT, blue

light-emitting diodes (LED) with a range of 450 – 470 nm is used to permeate the skin to reach UCB in the superficial capillaries and interstitial spaces [194–196]. When exposed to light, bilirubin can undergo three different processes [197]. The first is photo-oxidation of bilirubin into polar molecules that are more water-soluble and therefore are excreted from the body via the urine [198]. The second process is the conversion of the toxic bilirubin isomer (4Z,15Z) into more water-soluble and less toxic isomers (4Z,15E, 4E,15Z and 4E,15E) through configurational isomerization [195,199]. The third process is the structural isomerization of bilirubin into the compound lumirubin which is irreversible, in contrast to the reversible process of configurational isomerization [200,201]. The reversion of photosiomers into 4Z, 15Z isomers makes them prone for reabsorption from the intestinal lumen, undergoing enterohepatic circulation [140]. On the other hand, the generation of irreversible lumirubin, what also can be secreted into the bile, comprises quantitatively the main route of bilirubin disposal from the body after exposure to light or phototherapy [202].

Especially for patients with CN-1, the long-lasting exposure to PT between 10 – 14 hours a day is a serious burden and has profound effects on their social life [203,204]. Furthermore, PT becomes less effective over the years due to increased skin thickness or body surface to weight ratio, as well as by decreased hepatic clearance of lumirubin [203,205]. Therefore, alternative or adjuvant strategies for PT have been studied in the recent years. Eventually, liver transplantation is the inevitable treatment for patients with CN-1, but this is obviously still a treatment with associated morbidity and even mortality. Recently, the possibility of adeno-associated virus (AAV) vector-mediated gene therapy for CN-1 has been investigated [206,207]. Liver-specific gene transfer of the human *Ugt1a1* gene in Gunn rats as well as in *Ugt1* mutant mice has been effective in lowering plasma UCB [208–211]. This therapy appears very promising for CN-1 patients, however around 30% of CN-1 patients show anti-AAV immunity which decreases the efficacy of gene therapy and limits the use in the clinic [212].

3.4.2. Stimulation of UGT1A1 activity

The *Ugt1a1* gene is under transcriptional control of several NRs and other transcription factors [213,214]. CN-2 patients have a remaining UGT1A1 activity between 4-10% and treatment with phenobarbital has been used for many years in CN-2 patients to ameliorate unconjugated hyperbilirubinemia through upregulation of the expression and activity of UGT1A1 [127,213]. The underlying mechanism of phenobarbital was later found to be through activation of the constitutive active receptor (CAR) by binding to the phenobarbital-responsive enhancer module of UGT1A1 (gtPBREM) [108,215]. The beneficial effect of phenobarbital as a supplemental treatment besides PT on (neonatal) unconjugated hyperbilirubinemia has been demonstrated in several clinical trials [216–221]. Although phenobarbital alone or combined with PT is very effective in lowering serum bilirubin, it has adverse sedative and behavioral effects [222]. UGT1A1 activity can also be increased by dexamethasone, a ligand for the GR, as well as through activation of PXR [223].

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor in different tissues including the liver, intestine, lungs and lymphocytes [224]. A role for AhR in bilirubin metabolism was proposed after the discovery that both biliverdin and bilirubin are ligands for AhR and that AhR can bind to the promotor region of *Ugt1a1* [225,226]. One of the main target genes of AhR is the *cytochrome P450 family 1A (Cyp1a)* which was found to be involved in the hepatic oxidation of UCB as an alternative catabolic pathway under hyperbilirubinemic conditions [136]. Activation of *Ugt1a1* by CAR, PXR, GR and AhR is exerted through binding of these transcription factors to the gtPBREM [213].

3.4.3. *Inhibition of enterohepatic UCB reuptake*

The intestine is an important site in bilirubin metabolism where reuptake, deconjugation as well as conjugation (intestinal epithelium) and conversion of UCB into urobilinoids (intestinal lumen) takes place. Intestinal excretion of UCB and urobilinoids into the feces is a very efficient pathway to lower bilirubin levels in the body, but this is counteracted by intestinal reabsorption of UCB. During fasting, the motility of the intestine is decreased and this can result in a reduced fecal output of compounds including bile salts and bilirubin metabolites [227]. Furthermore, a decreased motility is accompanied by a higher intestinal transit time for compounds including UCB, promoting the possibility for reuptake of UCB in the intestinal lumen for enterohepatic circulation (EHC) back to the liver [228]. A higher EHC of UCB causes accumulation of UCB in the blood, and it has been shown that fasting was associated with increased plasma UCB levels and decreased fecal bilirubin excretion in Gunn rats, as well as in patients with hemolysis, obstructive jaundice and individuals with Gilbert Syndrome [228–231]. Several strategies have been used to interrupt the EHC of bilirubin to reduce hyperbilirubinemia. Shortening of the intestinal transit time for UCB by administration of polyethylene glycol (PEG) decreased plasma UCB levels and increased fecal UCB output in Gunn rats [230]. Administration of PEG in addition to PT even further decreased plasma UCB levels compared to control Gunn rats or rats treated with PEG alone. Interestingly, while short-term administration of PEG (36 hours) increased fecal UCB output, a steady-state in fecal UCB excretion was reached only after 2 weeks of PEG administration [230].

A different strategy to decrease intestinal reabsorption of UCB is through intestinal entrapment of UCB by compounds including agar, cholestyramine, charcoal, calcium phosphate and zinc salts [153]. Agar and cholestyramine are often used as binders of bile salts, but are also effective in lowering plasma UCB in Gunn rats, although conflicting results were found in neonatal studies [232]. Charcoal functions as a binding matrix for UCB and reduces plasma UCB in the first postnatal days. However, charcoal is a non-selective binder and binds to essential nutrients in the intestine as well and often causes obstipation. These severe side effects of charcoal limits its clinical application in humans [153].

Calcium phosphate has a high affinity for bilirubin in the intestine and the decrease in plasma bilirubin levels in Gunn rats could be ascribed to an increase in fecal UCB, especially during the first three days of treatment [233,234]. After this period, fecal bilirubin excretion reached a steady-state while plasma UCB remained lower compared to controls [233]. The application of zinc salts inhibits the EHC of UCB in hamsters [235], Gunn rats [236] and individuals with Gilbert's Syndrome [237]. However, PT is associated with already increased serum zinc levels in neonates with severe unconjugated hyperbilirubinemia (total serum bilirubin > 18 mg/dL) [238]. Therefore, administration of zinc salts in combination with PT could possibly lead to zinc toxicity, making it not suitable as a therapeutic strategy for severe unconjugated hyperbilirubinemia. The EHC of bilirubin was also found to be interrupted by increasing fecal fat excretion through administration of a high fat diet (HFD) or orlistat, an inhibitor of lipases [155,164,239,240]. This will be discussed in more detail below (section *3.4.5*).

3.4.4. Bile acids

The transintestinal excretion of bilirubin is considered as the main elimination pathway under unconjugated hyperbilirubinemic conditions, although the efficiency of this pathway is counteracted by intestinal UCB reabsorption [154–156]. Decreasing the EHC of UCB can be achieved by intestinal 'entrapment' of UCB as discussed above, or through increasing intestinal fat content. Intestinal absorption of fats and lipids are regulated by the total BA pool, as well as the composition of the BA pool. Cholic acid (CA) is a very hydrophobic bile acid and can stimulate intestinal cholesterol absorption through forming mixed micelles [241]. Hydrophilic BAs such as muricholic BAs, have a lower solubilization capacity and, accordingly, increasing the amount of hydrophilic BA by FXR activation has been associated with a higher fecal neutral sterol (FNS) output [54].

UCB is a hydrophobic compound and bile salts were found to bind to UCB *in vitro* and in the bile [242]. It has been demonstrated that administration of the hydrophilic bile acid ursodeoxycholic acid (UDCA) alone or combined with phototherapy lowered plasma bilirubin levels in hyperbilirubinemic Gunn rats [156,234]. Administration of UDCA lowered plasma bilirubin levels [156,243] and has been used as a therapy for cholestatic liver diseases as well as neonatal unconjugated hyperbilirubinemia [243,244].

3.4.5. Dietary fat and intestinal fat content

A transintestinal secretion pathway has been described for both cholesterol (TICE) and UCB [78,239,245]. The TICE pathway can be stimulated by activation of LXR, FXR, PPARδ and plant sterols, thereby lowering plasma cholesterol levels and increasing fecal neutral sterol (FNS) output [54,69,77–80]. An increased fecal fat and neutral sterol secretion can also be achieved by administration of respectively the lipase inhibitor orlistat or a high dietary fat intake (HFD). Recently, we demonstrated that increasing fecal fat excretion could lower plasma UCB levels in Gunn rats [155,239,240] as well as in CN-1 patients [164]. It has been hypothesized that the increase in fecal UCB and subsequent decrease in plasma UCB levels

upon higher intestinal fat concentrations is the result of UCB "capturing" by fatty acids, meaning that the reabsorption of UCB is decreased upon its association with non-absorbed fat in the intestinal lumen [155,171].

The underlying mechanisms for the transintestinal bilirubin excretion and its possible interaction with the TICE pathway have not been fully elucidated yet. Based on the findings that LXR and FXR activation can stimulate TICE, we hypothesize that this might also hold true for the stimulation of transintestinal bilirubin excretion. In **chapter 3** we investigated if stimulation of the FNS output by activation of LXR and FXR could also stimulate transintestinal bilirubin excretion, resulting in hypobilirubinemic effects in Gunn rats.

3.5.   Metabolic functions of bilirubin

Recently, the involvement of bilirubin in several metabolic pathways including cholesterol metabolism, inflammation, fat oxidation and glucose and insulin homeostasis has been reported [177,246–249]. Together with the finding that mild unconjugated hyperbilirubinemia, as seen in individuals with GS, decreases the risk of cardiovascular disease this led to the hypothesis that administration of (unconjugated) bilirubin can be used as a new therapeutic strategy for metabolic disorders. The study of Stec et al. showed that bilirubin can directly bind to PPARα and increases its transcriptional activity [111]. This is ascribed to the structure of bilirubin, containing a pyrrole-ring like structure, resembling other ligands for PPARα such as WY-14643 and fenofibrate. In this study, wild type (WT) and PPARα knock-out (KO) mice on HFD were treated with bilirubin, and WT mice showed a reduced body fat percentage, a phenomenon which was blunted in PPARα KO mice.

The protein AMP-activated ser/thr kinase (AMPK) functions as an important energy sensor in eukaryotic cells and plays a role in a plethora of metabolic pathways. Depletion of the energy source ATP activates AMPK, which subsequently suppresses the synthesis of cholesterol and fatty acids, as well as gluconeogenesis [250]. Additionally, the PPAR-gamma coactivator 1 alpha (PGC-1α) is activated by AMPK and regulates browning of adipose tissue and thermogenesis [251]. In the diet-induced obesity (DIO) mouse model, administration of bilirubin could reduce body weight, blood glucose levels as well as cholesterol levels. These beneficial effects of bilirubin were ascribed to an upregulated expression of PPARγ [252]. Upregulation of PPARγ is accompanied by an increase in adiponectin, a hormone that is produced by the adipose tissue and that increases insulin sensitivity and FAO. In this study it was observed that adiponectin was increased acutely and remained increased up to 7 weeks after two weeks of bilirubin administration, together with beneficial effects on plasma lipid profile and insulin sensitivity. PPARγ plays a significant role in adipocyte differentiation, adipogenesis and lipid metabolism as well as in insulin sensitivity, making PPARγ an interesting target for treatment of insulin resistance, obesity and cardiovascular diseases [253,254]. The study of Mölzer et al. showed that levels of several biomarkers of energy metabolism (PPARα, PPARγ, PGC-1α and AMPK) were

higher in individuals with GS compared to healthy control subjects [251]. However, a recent paper by Gordon et al. showed that bilirubin selectively binds to the LBD of PPARα and not to PPARβ or PPARγ [255]. When bound to PPARα, bilirubin causes a switch from corepressors to co-activators resulting in higher mitochondrial activity in an adipose cell line as well as in white adipose tissue (WAT) of DIO mice [255] and remodeling of WAT. Taken together, these findings suggest that bilirubin can be a promising new therapeutic target for the treatment of metabolic diseases.

4. The role of NRs in dyslipidemia and peroxisomal function

4.1.   NRs and dyslipidemia

Interaction between organs such as the liver, intestine and adipose tissue is very important for the maintenance of energy homeostasis. This maintenance is for an important part coordinated by the NRs LXR, FXR, PPARs, PXR and CAR. As stated above, altered transcriptional regulation by NRs can be involved in the pathophysiology of metabolic disorders such as insulin resistance, dyslipidemia and high blood pressure [2,25]. On the other hand, NRs can also be the target of therapeutic intervention. The cluster of these conditions are termed MetS which is characterized by abdominal obesity, increased triglyceride levels, lower (HDL) cholesterol, elevated blood pressure and fasting glucose [256]. Dyslipidemia is defined by an increase in total cholesterol, increased serum triglycerides (TG) and apolipoprotein B, as well as increased small dense low-density lipoprotein cholesterol (sdLDL-C), TG and a decrease in HDL-C [256,257]. Atherogenic dyslipidemia increases the risk to develop atherosclerotic cardiovascular disease (CVD), a disease with a high mortality rate worldwide [258,259].

4.1.1.   PPARs as therapeutic targets

The role of NRs in lipid homeostasis has been a great point of interest and led to new insights for the use of NRs as therapeutic targets for metabolic disorders. The family of PPARs are known for their important role in lipid metabolism, but are also involved in many other metabolic pathways including carbohydrate metabolism, immune response, cell growth, differentiation and apoptosis [260]. The group of thiazolidinediones (TZD), including pioglitazone and rosiglitazone, are pharmacological agonists for PPARγ and have clinically been used as insulin sensitizers in patients with T2D [261,262]. In addition, piaglitazone has been shown to ameliorate non-alcoholic hepatic steatosis (NASH) [263,264]. Statins as well as fibrates have been used in the clinic to treat dyslipidemia [265,266]. Fibrates are agonists for PPARα and showed to be effective in lowering hypertriglyceridemia as well as LDL-C, but increased plasma HDL-C levels [64,106]. Activation of PPARα could increase plasma fibroblast growing factor 21 (FGF21), which functions as a stress-signal to other organs to prepare

them for an approaching energy-deprivation state [10,27]. Upregulation of FGF21 increases fatty acid oxidation rates and decreases VLDL-receptor expression, thereby protecting against hepatic steatosis in mice [267]. PPARs also exert their metabolic effects by upregulation of peroxisomal biogenesis and stimulation of peroxisomal functions.

4.2. Metabolic functions of peroxisomes

4.2.1. *Peroxisomes as multifunctional cellular organelles*

Peroxisomes were discovered in 1954 as single-membrane organelles and described as 'microbodies' and were later termed peroxisomes [268]. Because peroxisomes do not contain their own DNA, peroxisomal (matrix) proteins have to imported into the peroxisomes. Peroxisomal proteins involved in peroxisome biogenesis and protein import machinery organelles are termed peroxins and are encoded by PEX genes. Peroxisomal biogenesis include targeted protein import into the peroxisomal matrix, as well as insertion of peroxisomal membrane proteins (PMP) [269].

Although peroxisomes are present in virtually all cells of the body, the highest numbers of these organelles can be found in tissues with a high rate of fatty acid or lipid oxidation [270,271]. Peroxisomes are involved in various anabolic and catabolic metabolic pathways, but the specific metabolic function differs per organism, tissue and cell type [272]. Examples of these functions are biosynthesis of ether phospholipids, BAs and docosahexaenoic acid, α- and β-oxidation of branched-chain fatty acids and very long chain fatty acids (VLCFA) [272]. These functions will be explained in short below.

4.2.2. *β-oxidation*

Peroxisomes are not able to produce proteins themselves and therefore rely on import of proteins from the cytosol. Peroxisomes are in close contact with the endoplasmic reticulum (ER), mitochondria, lysosomes and cytosol in order to accurately perform their metabolic function. Overlapping functions between peroxisomes and mitochondria have been described in higher eukaryotes, such as β-oxidation of several fatty acids [273–275]. However, substrates that exclusively undergo peroxisomal β-oxidation are saturated very long-chain fatty acids (VLCFA) (>C22 atoms), hexacosanoic acid, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA respectively) and long-chain dicarboxylic acids. After several cycles of β-oxidation in peroxisomes, the formed medium-chain fatty acids (MCFA) are transported to mitochondria for further oxidation and processing.

Another molecule that undergoes peroxisomal β-oxidation is pristanic acid. Pristanic acid is a metabolite of phytanic acid formed after one round of peroxisomal α-oxidation. It was found that pristanic acid can go through three rounds of β-oxidation in the peroxisome and eventually is converted to 4,8-dimethylnonanoyl-CoA together with two molecules of

propionyl-CoA and one unit of acetyl-CoA. These metabolites are transported as a carnitine ester or in their free form to mitochondria where they are further metabolized [276].

4.2.3. a-oxidation

Not all molecules are compatible with β-oxidation and need a conformational change in order to be further metabolized in peroxisomes or mitochondria. The saturated branched-chain fatty acid phytanic acid is a metabolite of phytol, a widely abundant compound in nature and derived from chlorophyll from green plants and planktonic algae [277]. Phytanic acid contains a methyl group at the 3-position making it not compatible for β-oxidation. Therefore, oxidative decarboxylation at the α-carbon of phytanic acid takes place (α-oxidation) to form pristanic acid. The first enzymatic step of α-oxidation is the activation of phytanic acid to phytanoyl-CoA, performed by the enzymes ACSL1 and ACSVL1 localized outside of the peroxisome [277]. Subsequently, phytanoyl-CoA is converted into 2-hydroxyphytanoyl-CoA by the enzyme phytanoyl-CoA 2 hydroxylase (PHYH) and further metabolized in pristanal by the enzyme 2-hydroxyacyl-CoA lyase (HACL1). The last step of α-oxidation is conversion of pristanal into pristanic acid by a so far unknown enzyme [277]. However, pristanic acid needs activation to a CoA ester in order to be metabolized by β-oxidation (*Figure 4*) [277,278].
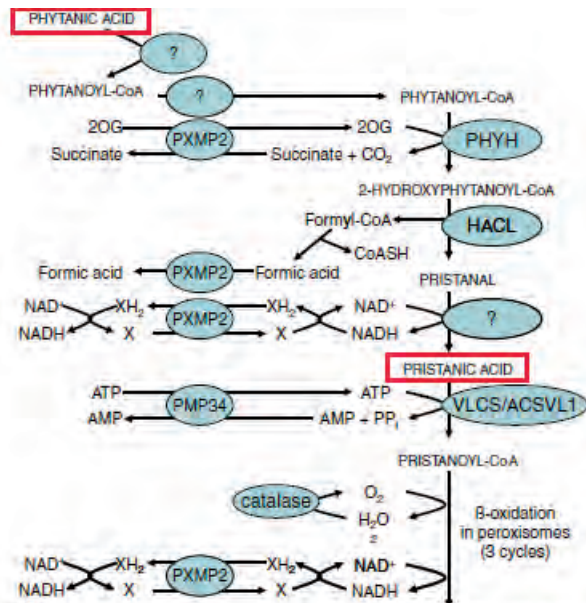

Figure 4. Peroxisomal *a* -oxidation of phytanic acid in peroxisomes. Adapted from [277].

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4.2.4. Synthesis and conjugation of bile acids

One of the main elimination pathways of cholesterol from the body is the conversion into bile acids in the liver. Cholesterol contains 27 carbon atoms and is converted into the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA respectively) in hepatocytes. Both DHCA and THCA contain 24 carbon atoms and are direct precursors of the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). In order to be β-oxidized by peroxisomes, DHCA and THCA are first activated to CoA esters at the ER membrane, followed by entering the peroxisome through uptake by the peroxisomal half ABC-transporter PMP70 [279]. The enzyme CYP27A1 produces only (25*R*)-stereoisomers of DHCA and THCA, however the peroxisomal β-oxidation is only able to handle (25*S*)-stereoisomers. Racemization of the (25*R*)-isomers into (*S*)-isomers is done by the enzyme α-methylacyl-CoA (AMACR) thereby allowing β-oxidation forming choloyl-CoA and chenodeoxycholoyl-CoA. After oxidation, the enzyme bile acyl-CoA:amino acid *N*-acyltransferase (BAAT) conjugates the formed bile acids with a taurine or glycine group [280]. The taurine- or glycine-conjugated bile acids are exported out of the peroxisome and outside of the hepatocytes, which is mediated by the bile salt export pump (BSEP).

4.2.5. Other peroxisomal functions

Reactive oxygen species (ROS) are a product of oxidative metabolism in mitochondria, ER and peroxisomes and include radical species such as superoxide anion as well as hydrogen peroxide (H_2O_2). Peroxisomes were found to produce several types of ROS but also ROS-metabolizing enzymes [281–285]. The peroxisomal processes responsible for the production of H_2O_2 are β-oxidation, as well as enzymatic reactions of the flavin oxidases and breakdown of superoxide radicals. Because most of the ROS are toxic, scavenging of these ROS is an indispensable process and peroxisomes are important for the production of ROS-degrading compounds including superoxide dismutase 1 and catalase. Catalase is the best-known enzyme and often used as a marker for the presence of peroxisomes. The discovery that catalase and H_2O_2 were colocalized in peroxisomes indicated that these organelles play an important role in the metabolism of oxygen breakdown [285,286].

Peroxisomes also produce ether phospholipids, a special class of phospholipids characterized by an alkyl or alkenyl bond. Plasmalogens are a subgroup of ether phospholipids and are solely produced by peroxisomes by the enzymes glyceronephosphate O-acyltransferase (GNPAT) and alkylglycerone phosphate synthase (AGPS) [287]. Plasmalogens exert various functions, such as acting as an endogenous antioxidant, mediators of membrane structure or as storages of polyunsaturated fatty acid and lipid mediators [288]. Other functions executed by peroxisomes are glyoxylate detoxification, as well as metabolism of oxygen and reactive nitrogen species [272].

4.3. Peroxisomal disorders

4.3.1. Peroxisomal biogenesis disorders (PBD)

The biogenesis of peroxisomes consists of different processes including membrane formation, import of peroxisomal membrane and matrix proteins, growth, division and proliferation [289–291]. Mutations in peroxisome biogenesis (PEX) genes can cause severe inborn disorders including Zellweger Spectrum Disorders (ZSD) and rhizomelic chondrodysplasia type 1, disorders characterized by an absence of functional peroxisomes [272,292,293]. ZSD are further divided into Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease. Although there is a high heterogeneity in the symptoms of ZSD, a common feature is an impaired lipid metabolism illustrated by accumulation of VLCFA and the branched-chain fatty acids phytanic and pristanic acid in plasma and tissues of patients [294]. Other metabolic abnormalities in patients in ZSD are accumulation of C27 bile acid intermediates as well as higher urinary levels of oxalate and glycolate [295].

4.3.2. Single peroxisomal enzyme deficiencies (PED)

Single peroxisomal enzyme deficiencies are caused by a defect in a single peroxisomal protein. This group comprises proteins involved in membrane transport, as well as executing enzymatic reactions in the peroxisomal matrix [272]. Therefore, the symptoms of a PED depend strongly on the function of the affected or absent peroxisomal protein, for example in peroxisomal β-oxidation, α-oxidation, glyoxylate metabolism, ether phospholipid biosynthesis, BA synthesis and H_2O_2 breakdown. One of the most frequently occurring peroxisomal disorders is X-linked adrenoleukodystrophy (X-ALD), caused by a mutation in the peroxisomal membrane half ABC transporter encoded by the *Abcd1* gene. The ABCD1 transporter is responsible for the import of VLCFA in the peroxisomal matrix, and therefore when ABCD1 is mutated, no VLCFA can enter the peroxisome to undergo β-oxidation. As a consequence, X-ALD patients show an accumulation of VLCFA in plasma and tissues [295]. Other PED affecting the peroxisomal β-oxidation are D-bifunctional protein deficiency, AMACR deficiency as well as Sterol carrier protein X (SCP-X) deficiency. Refsum disease is characterized by an accumulation of phytanic acid due to a deficiency of the peroxisomal enzyme PHYH, involved in α-oxidation. Patients with Refsum disease show cerebellar ataxia, polyneuropathy and progressive retinitis pigmentosa [296,297].

4.4. Mouse models for peroxisomal disorders

The use of genetically manipulated mouse models has given more insight in the etiology of peroxisomal disorders and the clinical phenotype. Depending on the specific peroxisomal protein that has been manipulated, the phenotypes of these animals differ

dramatically. Mice lacking the Pex5 gene died within 24 hours and *Pex2^{-/-}* mice survived only up to 6 weeks [271]. In contrast, the *Phyh^{-/-}* mouse model is representative for Refsum disease and showed a mild phenotype with moderate accumulation of phytanic acid in plasma under chow diet conditions [297]. But when fed with 0.1% w/w phytol, *Phyh^{-/-}* mice showed an aberrant gait as the peripheral nervous system was affected and levels of plasma phytanic acid increased. Mice lacking the *Scp-x* gene challenged with a phytol-enriched diet presented an unsteady gait, developed ataxia and peripheral neuropathy [298]. The deletion of the gene encoding ACOX1, a protein involved in the β-oxidation, led to a dramatically severe hepatic phenotype with increased VLCFA levels. Furthermore, these animals were not fertile and showed growth retardation [299].

Examples of mouse models with a deficiency in peroxisomal proteins that do not show a distinctive phenotype are mice lacking 2-hydroxyacyl-CoA lyase (HACL1), a key enzyme in α-oxidation of phytanic acid. These *Hacl1^{-/-}* mice display no divergent phenotype under dietary chow conditions [300]. *Amacr^{-/-}* mice have increased biliary and serum C27-intermediates and lower C24 bile acids, but no alterations in phytanic or pristanic acid compared to wild type animals under chow conditions [301]. Despite the changes in bile acid metabolism, no clinical phenotype was found in *Amacr^{-/-}* mice [301].

A plethora of peroxisomal proteins have not been characterized yet and this remains a young and relatively unexplored scientific field.

4.5. Peroxisomal membrane protein 4 (PXMP4)

Peroxisomal membrane proteins (PMPs) are inserted into the peroxisomal membrane by import machineries formed by peroxins (PEX) [269]. Three PEX proteins are involved in peroxisomal membrane biogenesis; PEX3, PEX16 and PEX19 of which PEX19 is considered as a receptor for newly synthesized PMPs [302]. PEX3 is located in the peroxisomal membrane and functions as a docking station for PEX19 and its accompanied protein. The PEX3/PEX19 import machinery is the most-used import pathway for PMPs as well as PEX proteins. The peroxisomal membrane contains several metabolite transporters in order to process these compounds in the peroxisome [303,304]. Mutations in peroxisomal transporters or PMPs can result in accumulation of C27-intermediate bile acids, pristanic and phytanic acid and VLCFA levels [278,305,306].

PXMP4 is an integral membrane protein of 212 amino acids and has a molecular mass of 24 kDa and was first isolated from rat hepatocytes [307]. It is a member of the Tim17 family and has been linked to the development of several types of cancer [308–310]. However, its precise role in tumor development as well as its physiological function has remained unknown [307]. PXMP4 has found to be a target of PPARα in both mouse and human hepatocytes [311]. Activation of PPARs was shown to stimulate the TICE pathway, however the exact regulation of the TICE pathway is still not fully understood. Therefore, a microarray was performed on several experiments where the TICE pathway was stimulated by activation of several nuclear receptors, including FXR, PPARδ- and α. This revealed an

upregulation of PXMP4 (unpublished data). In **chapter 4**, we aimed to address the metabolic function of PXMP4, using mice with a genetic deficiency in PXMP4 in combination with pharmacological approaches to stimulate peroxisomal activity.

5. Aim and outline of this dissertation

In the recent years, the role of NRs in a plethora of metabolic pathways including lipid metabolism, glucose homeostasis and detoxification has been described, although many mechanistic pathways remain to be elucidated. In our lab, we are interested in disorders of (energy) metabolism including unconjugated hyperbilirubinemia and dyslipidemia. Recent literature has shown associations between bilirubin and cholesterol and lipid homeostasis. The aim of this thesis is 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.

The Gunn rat is a widely used animal model for unconjugated hyperbilirubinemia and several strains are available that exhibit differences in bilirubin levels and response to treatment. Recently, 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 [172,173]. In **chapter 2,** we assessed the bilirubin and lipid phenotype in wild type, heterozygous and homozygous Gunn-Ugt1a1j/[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 as well as the interaction between UCB and lipids.

Bilirubin detoxification and excretion is under regulatory control of several NRs including PXR, CAR and AhR. The transintestinal bilirubin excretion was found to be the major secretion route under unconjugated hyperbilirubinemic conditions and a similar excretion route is found for cholesterol (TICE). We previously showed that transintestinal UCB excretion was stimulated by increasing fecal fat excretion in Gunn rats, whereas TICE is stimulated through activation of nuclear receptors LXR and FXR. We hypothesized that transintestinal excretion of bilirubin and cholesterol are interrelated. Accordingly, we determined in **chapter 3** whether stimulation of transintestinal or FNS excretion by NRs activation, could also be a therapeutic target to ameliorate unconjugated hyperbilirubinemia.

The peroxisome proliferator-activator receptor alpha (PPARα) is involved in fatty acid oxidation and metabolism of cholesterol and bile acids and, therefore, could be involved in the TICE pathway. Based on transcriptome data of several experiments where the TICE pathway was induced, Peroxisomal Membrane Protein 4 (PXMP4) was identified as a potential new target. In **chapter 4**, we characterized the function of PXMP4 using a full-

body knockout mouse model generated by the CRISPR/Cas9-mediated gene editing. Up to date, information regarding the function of this peroxisomal protein is scarce.

Many processes involved in lipid metabolism are not stable during the life course, but may alter upon ageing. During ageing, the metabolic flexibility and functionality of organs including the liver and intestine decrease. Lipid handling, glucose utilization as well as insulin sensitivity are altered, which may result in development of CVD and T2DM. Although ageing in itself was not the main topic of this thesis, it is relevant to understand to what extent the different processes change over the life course, and to what extent a dietary intervention could be beneficial. Dietary protein restriction has been demonstrated to improve metabolic health under various conditions. In **chapter 5** we studied if decreasing the dietary protein content affects the metabolic flexibility in aged mice.

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- Chapter 1

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