Gut microbiota and nuclear receptors in bile acid and lipid metabolism

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CHAPTER 8

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GENERAL DISCUSSION

Metabolic diseases, associated with obesity, are world-wide health problems. Obesity is one of the hallmarks of the metabolic syndrome, which increases the risk for diabetes and cardiovascular diseases (1). Traditional approaches, such as diet and physical activity, have been unsuccessful in decreasing the prevalence of metabolic diseases. Therefore, novel science-based strategies are explored for prevention and therapy, such as pharmaceuticals targeting nuclear receptors, bile acid signaling or modulation of gut microbiota. To do so it is crucial to understand the molecular signaling pathways involved in regulation of metabolism.

Bile acids are generated in the liver and are traditionally recognized for their role in bile formation, cholesterol solubilization and lipid absorption. Recently, bile acids emerged as signaling molecules that, as ligands for the bile acid receptors FXR and TGR5, activate and integrate multiple complex signaling pathways involved in lipid and glucose metabolism and energy homeostasis.

This thesis focuses on bile acid and lipid metabolism and the diverse cues in response to which they are regulated, including: signaling via ligand-activated nuclear receptors such as LRH-1, bile acids, glucocorticoids and gut microbiota.

Regulation of bile acid synthesis

Bile acids are synthesized in the liver by a complex multi-enzyme process. The rate-controlling step in the classic pathway of bile acid synthesis – as explained in **Chapter 1** – is catalyzed by the enzyme Cyp7a1, which converts cholesterol to 7α-hydroxycholesterol (2). Subsequently, 7α-hydroxycholesterol is converted into 7α-hydroxy-4-cholesten-3-one, which can be converted by the enzyme Cyp8b1 into 7α,12α-dihydroxy-4-cholesten-3-one, ultimately leading to synthesis of the primary bile acid cholic acid (3). Alternatively, cholesterol can be converted to chenodeoxycholic acid via multiple enzymes, independent of Cyp8b1. The expression of Cyp7a1 follows a circadian pattern and is tightly regulated by complex feedback mechanisms. Glucose and insulin are major postprandial factors that induce Cyp7a1 activity (4), whereas bile acids down-regulate their own synthesis (5). Several hepatic nuclear receptors and transcription factors are implicated in regulating the expression of Cyp7a1, including Lxr, Shp, Fxr and Hnf4α (6).

In addition, Lrh1 binding sites have been identified in the proximal promotor parts of the *Cyp7a1* gene (7, 8). *In vitro* studies showed that Lrh1 is able to induce its expression (7, 9, 10). However, in subsequent *in vivo* studies in mice, hepatocyte-specific *Lrh1* knockout strategies did not reduce *Cyp7a1* mRNA levels, nor protein activity (11, 12). Unexpectedly, heterozygous *Lrh1* knockout mice exhibited 5-7-fold higher *Cyp7a1* expression levels and increased total bile acid pool sizes. It was suggested this effect was due to increased occupation of the overlapping Lrh1/Hnf4α binding site in the *Cyp7a1* promotor by Hnf4α (13). Therefore, the proposed role of Lrh1 in regulating Cyp7a1 expression remained uncertain.

In **Chapter 3** we used a novel conditional *Lrh1* knockdown (LRH-1-KD) mouse model to evaluate the dependency of bile acid synthesis and composition on Lrh1. In LRH-1-KD and wildtype mice we performed an experiment under normal feeding conditions and during conditions with high fecal bile acid loss, induced by giving a bile acid sequestrant. Under these conditions bile acid synthesis must be enhanced to maintain homeostasis. We show that under physiological conditions, *i.e.* without the sequestrant, knockdown of *Lrh1* slightly increased *Cyp7a1* expression, with a concomitant increase in fecal bile acid excretion. In contrast, in LRH-1-KD mice the mRNA expression of *Cyp8b1* was reduced, with an according shift towards CDCA-derived bile acids versus CA-derived ones in the bile acid pool. Thus, under these conditions Lrh1 determines pool composition, but does not limit bile acid synthesis rate. However, when a bile acid sequestrant was used to deplete the bile acid pool by enhancing their fecal excretion, *Cyp7a1* expression did not further increase in LRH-1-KD mice and neither did fecal bile acid excretion. Therefore, Lrh1 does function as a critical factor for adequate induction of hepatic *Cyp7a1* expression and bile acid synthesis under conditions of high bile acid loss. Yet, Lrh1 is dispensable for *Cyp7a1*, but not for *Cyp8b1*, expression under normal conditions.

These observations strongly suggests that compensatory mechanisms or redundant transcription factors exist for maintenance of *Cyp7a1* expression under physiological conditions. Recently, it was shown that Lrh1 cooperates with Hnf4α in maintaining basal *Cyp7a1* expression (14). The nature of the differential regulation of *Cyp7a1* and *Cyp8b1* under normal conditions remains obscure. Cyp7a1 expression is rate-limiting for bile acid synthesis, which is of vital importance to sustain life (15), and is regulated by various transcription factors in response to a broad range of external cues. Therefore,

Cyp7a1 expression may be maintained constant by the concerted action of various factors and is less likely vulnerable to stochastic perturbation, *e.g.* loss of signaling by one factor.

The increase in *Cyp7a1* expression under normal conditions was likely due to suppressed ileal *Fgf15* expression, possibly caused by a decrease in Lrh1-dependent Asbt expression in the small intestine. The same mechanism could also explain the increased *Cyp7a1* expression levels in heterozygous *Lrh1* knockout mice. Taken together, these results indicate that Lrh1 modulates *Cyp7a1* expression and bile acid synthesis via two different pathways at different sites in the enterohepatic system, with a reciprocal outcome on *Cyp7a1* expression. Hepatic Lrh1, together with other factors, positively regulates *Cyp7a1* expression, whereas intestinal Lrh1 causes an opposing effect by stimulating the expression of *Fgf15* in enterocytes resulting in a repression of hepatic *Cyp7a1*. Moreover, it was recently shown that hepatic Lrh1 is involved in FGF19-mediated repression of *Cyp7a1* expression (14). The fact that *Cyp8b1* expression was decreased upon *Lrh1* knockdown supports data from Kim *et al.* (16), who showed that *Cyp7a1* is suppressed much more efficiently compared to *Cyp8b1* by Fgf15 signaling, and supports that Lrh1 is important for *Cyp8b1* expression, as shown before (11, 12).

The role of gut microbiota in the regulation of bile acid synthesis

In the intestinal lumen bile acids emulsify and solubilize dietary lipids so that the lipids can be digested and absorbed by intestinal epithelial cells. More and more it is recognized that the intestine also plays an important regulatory role in controlling hepatic bile acid synthesis. In the ileal enterocytes bile acid bind and activate Fxr, which induces the expression of the hormone Fgf15. Consequently, Fgf15 is released into the circulation and inhibits bile acid synthesis via binding the hepatic FGF receptor 4.

In the intestine, bacteria also influence bile acid homeostasis. The human intestinal tract is colonized by a huge bacterial population, consisting of several thousands of species (17). Certain bacteria can deconjugate bile acids and metabolize primary bile acids into secondary ones (18-20). It has been shown that germfree conditions and antibiotic treatment decrease fecal bile acid excretion (21-24). Studies using radiolabelled or stable isotopes showed that germfree conditions decrease the turnover of bile acids and cholesterol, whereas bile acid pool size increased (25-28). The underlying regulatory mechanisms have remained unidentified so far. It has been suggested that

the gut microbiota interfere with intestinal Fxr activation (29, 30). However, antibiotic treatment in *Fxr*-KO mice induced similar effects as in wildtype mice (23, 24).

In **Chapter 5** we aimed to elucidate the mechanism(s) by which absence of gut microbiota affects bile acid metabolism. In germfree and conventional mice, and in mice treated with antibiotics we studied bile acid metabolism. We show that germfree and antibiotic-treated mice reabsorb bile acids more efficiently than control animals do, leading to a larger bile acid pool. Induction of Asbt expression in the ileum and a proximal shift in protein expression mediates the effects on bile acid absorption. Subsequent studies in *Asbt*-KO mice showed that in these mice only a small effect of antibiotic treatment remained, which could be related to enhanced uptake of bile acids by the colon. The increased venous return of bile acids to the liver upon antibiotic treatment resulted in increased biliary bile acid secretion. More effective intestinal bile acid conservation led to a compensatory decrease in bile acid synthesis, as apparent from decreased fecal bile acid excretion, which in steady state equals *de novo* synthesis, and bile acid kinetic studies. The increased bile acid pool size may reflect a new steady-state after an initial increase in bile acid reabsorption, with spillover of bile acids to the systemic circulation, before *de novo* synthesis is decreased.

In the antibiotic-treated and germfree animals there is a discrepancy between *in vivo* bile acid synthesis and *Cyp7a1* mRNA expression. Antibiotic treatment increased hepatic *Cyp7a1* expression, whereas it actually decreased bile acid synthesis, measured by fecal bile acid excretion and turnover of labeled cholic acid. Previous studies on bile acid synthesis under different conditions have shown that altered bile acid synthesis is not always correlated to changes in *Cyp7a1* mRNA expression (31-33), but the reason for this apparent discrepancy has remained puzzling. Possibly, post-transcriptional regulation of *Cyp7a1* expression may play a role. Unfortunately, Cyp7a1 protein expression is unknown in these experiments. These findings underscore that changes in the expression and/or abundance of metabolic enzymes may not always truly reflect actual metabolic fluxes. This highlights the importance of physiological measurements, in particular metabolic fluxes, to properly study bile acid homeostasis *in vivo*.

In **Chapter 5** we also show that Gata4 is crucial in mediating the effects that gut microbiota exert on host bile acid reabsorption via Asbt. Antibiotic treatment in Gata4-iKO mice did not increase *Asbt* expression, neither did it change fecal bile acid excretion, bile flow rate or plasma bile acid concentrations. Thus, under physiological

conditions, gut microbiota induce Gata4-dependent Asbt repression, thereby inhibiting enterohepatic recycling of bile acids. The exact mechanism by which intestinal bacteria regulate Gata4 remains to be elucidated. An interaction between the gut microbiota, the immune system and Gata4-dependent metabolic processes within epithelial cells has been described (34).

The immune system in bile acid and cholesterol homeostasis

To protect the enormous epithelial surface of the intestinal mucosa that is responsible for the absorption of nutrients and separates outside from inside, the intestinal mucosa contains the largest number of immune cells in the body (35). The challenge for this extensive immune system is to distinguish harmless food antigens and commensal bacteria from potentially dangerous pathogenic bacteria, parasites, and viruses. Therefore, the gut immune system is tightly regulated to prevent excessive immune responses to foods and gut bacteria. A critical factor for the symbiotic relationship between the gut microbiota and the host is the emergence of adaptive immunity. Failure to control T cell activation in the intestine leads to the development of intestinal inflammation (36). T cell activation triggers immunological responses, which impair the intestinal epithelial barrier function (37) and further accelerate inflammatory responses.

The metabolic and immune systems are closely integrated and functionally dependent on each other. Activation of inflammatory responses can lead to chronic low-grade inflammation, which is associated with various metabolic diseases. Inflammation robustly downregulates of the expression of a number of nuclear receptors, including *Fxr* (38, 39). Because the regulatory effects of nuclear receptors expressed in the intestine may not remain confined to the intestine, dysregulation might have systemic effects on metabolic processes. One example could be intestinal Fxr, which can regulate hepatic bile acid synthesis via induction of intestinal Fgf15 expression. However, little is known about the influence of the immune system on bile acid homeostasis. Therefore, the question arises whether modulation of the immune system may also impact on bile acid metabolism.

Glucocorticoids are steroid hormones that are produced in response to inflammation, with strong anti-inflammatory and immunosuppressive effects, such as inhibition of T cell activation (35). Glucocorticoids are common first line therapy options in non-infectious inflammatory diseases. Recently, it has been shown that the glucocorticoid

receptor (GR) may also regulate several genes involved in murine bile acid and cholesterol metabolism (40, 41), yet, the physiological relevance hereof is controversial. It has been suggested that GR blocks the transcriptional activity of Fxr in the liver (42). However, Fxr also exerts major control on bile acid homeostasis via the small intestine. Also, two functional glucocorticoid response elements have been identified within the promoter region of *Asbt* (43), and glucocorticoids enhance taurocholic acid absorption in *in situ* ileal loops (44). Therefore, glucocorticoids may have a dual action on bile acid metabolism via hepatic and intestinal signalling, yet, the complex nature of bile acid metabolism and its regulation prevents prediction of actual consequences on the basis of gene expression analysis only.

In **Chapter 7** we aimed to provide a mechanistic analysis of the effects of glucocorticoids on bile acid and cholesterol homeostasis in mice. Sustained prednisolone treatment induced the expression of Asbt in the ileum and stimulated bile acid absorption. Higher portal influx of bile acids to the liver induced biliary bile acid secretion and spillover to the peripheral circulation resulted in elevated plasma bile acid levels. In response, hepatic bile acid synthesis was lowered, as is evident from decreased *Cyp7a1* expression, decreased plasma C4 levels and decreased fecal bile acid excretion. Despite the increased bile acid uptake by enterocytes, ileal expression of bile acid-responsive genes such as *Fgf15* markedly decreased. It seems plausible that this is due to inhibition of the transcriptional activity of intestinal Fxr by prednisolone-activated GR, as described by Lu *et al*. (42). Decreased intestinal Fxr activity may also contribute to increased *Asbt* expression (45, 46). Apparently, the strongly increased influx of bile acid into the liver overruled GR inhibition of Fxr activity in the liver as well as the putative stimulating consequences on bile acid synthesis of reduced Fgf15 production in the ileum, since the hepatic expression of *Cyp7a1* was decreased. Taken together, we now show that enhancing bile acid reabsorption in the small intestine via induction of Asbt expression is the principal effect that glucocorticoids exert on bile acid homeostasis *in vivo*. In future experiments, it would be interesting to test whether glucocorticoids still affect bile acid homeostasis in Asbt-KO mice.

Changing bile acid metabolism can have great impact on cholesterol turnover. Moreover, glucocorticoids are also known to directly influence the expression of several genes involved in cholesterol metabolism (47, 48). The impact of cholesterol and different classes of lipoproteins on the development of atherosclerosis is well known

(49). However, the role of glucocorticoids in the development of atherosclerosis is not yet clearly established. In **Chapter 7** we therefore also investigated the effects of prednisolone treatment on cholesterol homeostasis. The increased enterohepatic cycling of bile acids upon prednisolone treatment induced biliary cholesterol secretion. In addition, prednisolone treatment increased plasma HDL-cholesterol levels, probably attributable to increased hepatic *ApoA-I* expression. In contrast, systemic inflammation is invariably associated with reduced HDL cholesterol and ApoA-I levels (50, 51). Prednisolone treatment increased fecal neutral sterol excretion. Together, the increased cholesterol transport capacity and increased hepatic secretion into bile resulted in increased movement of cholesterol from macrophage foam cells to the feces, indicating stimulated macrophage-derived reverse cholesterol transport. This may contribute to the anti-atherosclerotic effects of glucocorticoids, at least in animal models.

Taken together, inflammation can downregulate the expression of several nuclear receptors, including Fxr, which may be due to the actions of glucocorticoids. Glucocorticoids induce the expression of Asbt, which has systemic effects on bile acid homeostasis. The question arises why one receptor may regulate the immune system and metabolic functions simultaneously. Perhaps during times of inflammation there is an increased energy need, which asks for simultaneous adaptation of fuel metabolism.

In **Chapter 7** we have shown that synthetic glucocorticoids exert their principal effect on bile acid homeostasis via the small intestine. Besides synthesis in the adrenal glands, glucocorticoids are also synthesized by intestinal epithelial cells (35). Their intestinal synthesis is regulated in a circadian manner (52), is ACTH-independent and critically regulated by Lrh1 (53-55). Recently, it has emerged that the diurnal production of corticosterone in the ileum is controlled by gut microbiota via Toll-like receptor (TLR) signaling (56). TLRs are cell surface receptors that recognize molecular patterns expressed by bacteria. TLR expression by intestinal epithelial cells is rhythmically regulated by the circadian clock. As a consequence, signaling through these innate immune receptors triggered by the constant exposure to the host microbiota also follows a rhythmic pattern. These signals prevent the production of corticosterone by the ileal intestinal epithelial cells. Thereby, the absence of cues from the microbiota disrupts the circadian rhythm and phasic production of corticosterone by the ileum, resulting in hypercorticosterolism (56). Thus, intestinal microbiota exert a significant effect on host immune response via TLR signaling and inhibiting glucocorticoid pro-

duction. In turn, glucocorticoids repress a large set of inflammatory response genes, involving disruption of TLR-4 of TLR9-dependent transcriptional activation (57). The immune system, in turn, shapes the commensal microbial communities, as exemplified by aberrant microbiota compositions in several TLR-deficient mice (58), which affects host metabolic homeostasis (59). Taken together, the immune system and the gut microbiota are tightly interconnected and can influence host metabolism. We have seen that both gut microbiota (**Chapter 5**) as well as glucocorticoids (**Chapter 7**) are able to modulate bile acid metabolism via regulating Asbt expression. Moreover, gut microbiota inhibit intestinal glucocorticoid synthesis. These observations may imply that gut microbiota inhibit Asbt expression via inhibition of intestinal glucocorticoid synthesis. In **Chapter 5** we have also seen that Gata4 is crucial in mediating the effects that gut microbiota exert on Asbt expression and suggested that gut microbiota induce Gata4. It is therefore not surprising that glucocorticoids can inhibit the expression of Gata4 (60). These observations may therefore suggest that microbial signaling inhibits intestinal glucocorticoid synthesis, resulting in decreased induction of Asbt expression and increased Gata4 expression, which limits Asbt expression to the distal small intestine. Together, this promotes fecal excretion of bile acids. It is known that ileal Asbt expression follows a diurnal rhythm (61), it would be interesting to see whether the expression also diurnally shifts between the distal and proximal intestine.

Gut microbiota and glucose homeostasis

Besides regulation of bile acid metabolism, glucocorticoids and gut microbiota are both associated with alterations in glucose homeostasis. Previous studies suggested that gut microbiota contribute to the development of obesity and systemic insulin resistance (62-65). Germfree mice are protected from high fat diet-induced obesity and insulin resistance (66, 67) and transfer of lean donor fecal microbiota to obese subjects with the metabolic syndrome significantly altered the composition of the intestinal microbiota and improved peripheral insulin sensitivity (68). The mechanism(s) underlying the effects of gut microbiota on glucose homeostasis are poorly understood. One hypothesis involves the role of gut microbiota on bile acid homeostasis. Several gram-positive bacterial species, such as *Lactobacilli*, are able to deconjugate primary bile acids (69). The subsequent formation of secondary bile acids is only carried out by a minor population of gram-positive anaerobic *Clostridium* species (70-73). Bile acids

can act as regulators of systemic energy homeostasis and are known to affect glucose homeostasis (74).

We hypothesized that short term administration of oral antibiotics in humans would affect intestinal microbiota composition and subsequently bile acid and glucose metabolism. In **Chapter 6** we performed a single blinded randomized controlled trial in male obese subjects with metabolic syndrome. These subjects were randomized to either 7 days of amoxicillin or 7 days of vancomycin treatment. Vancomycin treatment reduced fecal microbial diversity, with a decrease in gram-positive bacteria (mainly *Firmicutes*). Concomitantly, vancomycin treatment decreased peripheral insulin sensitivity and fecal excretion of secondary bile acids, with a simultaneous postprandial increase of primary bile acids in plasma. Amoxicillin did not affect any of these parameters. The distinct effects of the antibiotic therapies may be explained by the difference in bacterial killing. Vancomycin mostly affects gram-positive bacteria, whereas amoxicillin affects more gram-negative/anaerobic bacteria (75). Gram-positive bacteria belonging to the *Clostridium* clusters IV and XIVa of the *Firmicutes* phylum are known to metabolize primary bile acids into secondary ones (69-73), but until today no gram-negative bacterium has been reported to dehydroxylate primary bile acids. Therefore, the results presented in **Chapter 6** indicate that gram-positive intestinal microbiota, particularly of the *Firmicutes* phylum, increase insulin sensitivity in humans by affecting bile acid homeostasis.

Bile acids can influence energy expenditure and glucose homeostasis by activating bile acid receptors and triggering downstream signaling pathways, *e.g.*, via their effects on gluconeogenesis, glycogenolysis, insulin secretion and insulin sensitivity (74, 76-78). Although various bile acid species differ in their *in vitro* capacity to activate specific receptors such as FXR or TGR5, it is not known which subclasses of bile acids are responsible for these effects in humans. Nevertheless, the secondary bile acids LCA and DCA were found to have a much stronger activating capacity for TGR5 than the primary bile acids CDCA and CA (76, 77, 79). Thus, theoretically, the decrease in secondary bile acids after vancomycin treatment could lead to decreased TGR5 activation, thereby affecting glucose homeostasis via GLP-1, PYY and/or modulation of energy expenditure in peripheral tissues (80-82). However, plasma GLP-1 levels were unchanged after vancomycin treatment, indicating unchanged intestinal TGR5 activation.

Vancomycin treatment decreased the plasma levels of FGF19 and increased the levels of 7α-hydroxy-4-cholesten-3-one (C4), a marker of hepatic bile acid synthesis. These observations may suggest decreased activation of FXR in the intestine. FXR is strongly activated by hydrophobic deconjugated bile acids, especially deconjugated CDCA binds with high affinity, whereas conjugated bile acids bind FXR in a reverted orientation with lower affinity (83). Thus, possibly, a decrease in deconjugated bile acids in the intestinal lumen during antibiotic treatment could contribute to decreased FXR activation. Fxr-deficiency in mice reduces peripheral insulin resistance with a reduction of glucose disposal and decreased adipose tissue and skeletal muscle insulin signaling (74). Decreased Fgf15/FGF19 levels have been associated with increased plasma glucose levels (84-87), whereas administration of obeticholic acid, a semisynthetic derivative of the human FXR-agonist CDCA, increased insulin sensitivity and plasma FGF19 levels (88). Fgf15/FGF19 influences glucose homeostasis by inhibiting hepatic gluconeogenesis and stimulating glycogen synthesis (89, 90), furthermore it increases glucose uptake in 3T3-L1 adipocytes (91) and increases energy expenditure (85). Decreased FXR activation in pancreas and in white adipose tissue may impact on glucose homeostasis as well (74, 92), which is explained in **Chapter 2**.

Gut microbiota and bile acids may also impact on glucose homeostasis via regulation of low-grade inflammation. It has been shown that FXR is required for immune-regulatory activities of TLR-9 in intestinal inflammation (39). Through FXR and TGR5 signaling, bile acids can regulate the intestinal barrier and immune function (93). They regulate maintenance of cell integrity, prevent pathogenic bacterial invasion and inhibit inflammation (94). Bile acid deficiency results in increased intestinal permeability, dysregulation of the immune response (95-98) and intestinal inflammation (93). Increased gut permeability and activation of inflammatory signaling pathways can lead to chronic low grade inflammation, a condition associated with obesity and insulin resistance (93).

Taken together, changes in bile acid signaling resulting from antibiotic treatment may affect glucose homeostasis. Metabolization of bile acids by gut microbiota has several effects: it alters the activation of bile acid receptors, which influences the expression of genes involved in glucose homeostasis. In addition, it modulates gut permeability and activates inflammatory signaling pathways (99, 100), resulting in low-grade inflammation, which influences insulin resistance (101). However, impaired gastrointestinal barrier function and chronic inflammation in obesity and insulin resistance may be

both cause and effect, the precise molecular mechanisms are yet unknown. We cannot exclude that other mechanisms also contribute to the effects of vancomycin treatment on insulin sensitivity. Several other hypotheses by which gut microbiota may influence glucose homeostasis have been suggested, including short-chain fatty acids (SCFAs) such as butyrate (101, 102). In rodents there is evidence that SCFAs are able to modulate low-grade inflammation, influence satiety and increase energy expenditure (101). However, there is conflicting evidence in humans.

The data discussed above suggest that administration of specific probiotics may be beneficial for the prevention and treatment of a number of health disorders (94). By re-establishing intestinal bile acid deconjugation and metabolization via delivering probiotics capable for these functions, normal bile acid homeostasis may be restored, which may reduce cholesterol levels, improve glycemic control and re-establish disrupted intestinal homeostasis in inflammation. In contrast, gut microbiota may contribute to atherosclerosis through promoting atherosclerotic plaque formation via TMAO production from dietary phosphatidylcholine (103-105).

In both mouse (**Chapter 5**) and human studies (**Chapter 6**) antibiotic treatment decreased the concentration of fecal and plasma secondary bile acids and plasma levels of Fgf15/FGF19. However, in mice antibiotic treatment decreased total fecal excretion of bile acids, in contrast to unchanged excretion levels in the human experiment. In the human study plasma C4 levels increased, suggesting enhanced hepatic bile acid synthesis, whereas synthesis was decreased in the mouse studies. In the human experiment it could be that steady state was not (yet) reached due to the short experimental time. Possibly, differences in bile acid species and intestinal microbiota between mice and humans could also contribute to this apparent contrast.

Regulation of hepatic triglyceride metabolism

As stated above, bile acids are important for intestinal lipid absorption and bile acid synthesis represents a major pathway of cholesterol catabolism. Manipulation of bile acid metabolism by bile acid sequestration significantly impacts on systemic lipid concentrations. In **Chapter 2** we review the impact bile acid sequestrants have on lipid and glucose metabolism and the roles of several nuclear receptors herein. Bile acid sequestrants interrupt the enterohepatic circulation of bile acids and decrease plasma total and LDL cholesterol while increasing levels of HDL cholesterol and triglycerides

(106). The conversion of cholesterol into bile acids is increased, whereas increased hepatic Ldl receptor expression harvests more cholesterol from the systemic circulation, which accounts for the decline in total and LDL cholesterol. Bile acids can influence triglyceride levels via activation of Fxr, which modulates triglyceride production and increases triglyceride clearance by influencing lipoprotein lipase activity (106).

Recently, it has been shown that Lrh1 is involved in the control of lipid metabolism as well (107). Lrh1 binds in proximity of genes related to lipid metabolism and is required for the anti-steatotic effects of the phospholipid DLPC (108), a natural Lrh1 agonist (109). These data suggest involvement of Lrh1 in the regulation of hepatic triglyceride metabolism. To further delineate the effects of Lrh1 on lipid metabolism, we analyzed lipid metabolism in conditional whole-body *Lrh1* knockdown mice and compared them to wildtype mice in **Chapter 4**. We show that conditional *Lrh1* knockdown mice develop a phenotype characterized by low circulating ketone bodies, high levels of plasma non-esterified fatty acids and medium- and long-chain acylcarnitines, and hepatic steatosis specifically in periportal areas. *Lrh1* knockdown impaired hepatic Pparα signaling and decreased fatty acid β-oxidation and ketogenesis, whereas *Lrh1* overexpression *in vitro* induced *Ppara* expression. We show that Lrh1 is able to directly bind and activate Pparα, a major regulator of hepatic fatty acid metabolism. Thus, *Lrh1* knockdown disturbs hepatic triglyceride homeostasis in mice primarily via downregulation of Pparα, resulting in decreased fatty acid oxidation and ketogenesis.

Since we used a conditional whole-body *Lrh1* knockdown mouse model, the contribution of decreased *Lrh1* expression in other tissues, besides the liver, to the overall phenotype cannot be ruled out. Also changes in CA-derived bile acids, which are decreased in LRH-1-KD mice, have been linked to lipid metabolism (110, 111). In addition, recently it was suggested that Lrh1 recruits Fxr to lipid metabolic genes, thereby regulating genes of lipid metabolism in concert with Fxr (107). Alterations in Fxr activity may therefore also contribute to the observed phenotype.

We also show in **Chapter 4** that expression of the LRH-1 gene in liver biopsies of obese human subjects correlates negatively with the extent of NAFLD and NASH. These results indicate that LRH-1 may also play a crucial role in human hepatic triglyceride metabolism. The influence of Lrh1 on inflammation, described previously (112-114) and also evident from upregulated genes involved in inflammatory responses in LRH-1-KD mice, might contribute to progression of hepatic steatosis to NASH. Taken together, we

show in **Chapter 4** that LRH-1 plays a pivotal role in the metabolic network controlling hepatic triglyceride levels.

Nutrient-sensing transcription factors are active in complex networks that regulate multiple metabolic pathways. In **Chapter 4** we have shown that Lrh1 is important in the regulation of bile acid metabolism. Lrh1 interacts with different nuclear receptors, transcription factors and target genes involved in lipid and bile acid metabolism. The coordinated regulation of both lipid and bile acid metabolism couples intake of lipid substances to synthesis and secretion of bile acids to ensure proper lipid solubilization in the intestine. Several nuclear receptors show a strong circadian expression pattern and their ligands, including lipids, bile acids and glucocorticoids, also exhibit strong daily fluctuation, thereby linking nutrient sensing to circadian control of metabolism. Therefore, we propose Lrh1 may be a key component of the coordinated response necessary to relay circadian signals into metabolic responses.

FUTURE PERSPECTIVES

The metabolic syndrome is characterized as a clustering of health disorders associated with an increased risk of cardiovascular disease and type 2 diabetes, and is becoming more and more common. Nuclear receptors are ligand-dependent transcription factors that control a diverse set of biological activities by translating dietary and endocrine signals into changes in expression of gene networks. Nuclear receptors are attractive therapeutic targets for the treatment of metabolic syndrome because their dysfunction – due to naturally occurring mutations – can result in metabolic disorders and their activity can be modulated by small molecules that can be substituted by synthetic ones, despite the challenge of achieving functional selectivity.

Characterization of new receptors and further elucidation of the roles of known nuclear receptors and their ligands in bile acid, glucose and lipid homeostasis and the intestinal immune system will contribute to our understanding of metabolic pathways and their interactions in health and disease. The development of pharmacological compounds that specifically activate or inhibit proteins, enzyme activities, cell receptors or transcription factors will provide valuable insight in their roles in the system and may help to combat disease. The discovery of key roles played by gut microbiota in inter-organ crosstalk and in the development of metabolic diseases, opens new windows for

better understanding of microbiota-to-host signaling pathways, the pathophysiology of diseases and potential starting points for studying possible therapeutic intervention.

To further understand the link between gut microbiota, bile acid metabolism and disease, large-scale metagenomic sequencing studies in both healthy and diseased populations should continue to evaluate the microbial community structure. In the future, specific microbial fingerprints may be able to reliably predict disease risk in the host. In addition, knowledge on the ability of specific bacteria to influence host metabolism, will contribute to targeted therapy to either eliminate or stimulate these species. Therefore, probiotics should continue to be explored to help restore bile acid metabolism and potentially aid in the treatment of obesity, type 2 diabetes, atherosclerosis and gastrointestinal disease. Towards understanding the functional metabolic interactions between gut microbiota and the host, the use of metatranscriptomics, metaproteomics and metabolomics could provide information on genetic potential, transcripts, proteins and metabolites. However, changes in the expression of transcription factors and/or abundance of metabolic enzymes may not always truly reflect the actual metabolic fluxes. Therefore, *in vivo* evaluation of metabolic fluxes is critical to obtain a complete physiological picture.

The role of diet in shaping the microbiota and influencing disease states should be further explored to uncover more details about the influence of specific food components upon the gut microbiota, which may lead to the development of specific dietary interventions to prevent and treat disease. Also the impact of antibiotic therapy, glucocorticoids and other commonly used medications upon the gut microbiota and metabolic homeostasis remains to be fully established. In turn, the ability of an individual's microbiota to influence the pharmacokinetics of drugs will contribute to personalized medicine.

Finally, the role of membrane or intracellular receptors in various tissues in binding microbial- or nonmicrobial-derived physiological ligands and their connection with the peripheral circadian clocks should also be further explored. It is insufficiently known how arrhythmic signals or the oscillation of (nuclear) receptors are converted into circadian rhythmic outputs, which time homeostatic functions with physiologically-relevant circadian events, such as food intake, exposure to infection or variation in light and dark. Elucidation of the molecular mechanisms that underlie the dialog between ligands,

receptors and the peripheral circadian clock, may pave the way to new therapies aimed at treating pathologies originating from disruption of these interactions.

CONCLUSIONS

Nutrient-sensing transcription factors play key roles in the maintenance of organismal energy homeostasis and are active in complex networks. Upon ligand binding they translate dietary and endocrine signals into changes in expression of gene networks.

Bile acids are among the substances that can act as signaling molecules and thereby exert diverse endocrine and metabolic actions by activating receptors such as FXR and TGR5. In addition, the nuclear receptor LRH-1 also plays an important role in bile acid and lipid metabolism. Regulation of bile acid homeostasis in mammals is complex and regulated via extensive cross-talk between liver, intestine and gut microbiota. Understanding the relationship between the gut microbiota and human health is of utmost importance since our health appears to be partly dependent upon the balance within this ecosystem. A disbalanced microbiotic flora impairs bile acid metabolism and gastrointestinal barrier function and activates inflammatory signaling pathways, leading to altered activation of bile acid receptors and chronic low grade inflammation. Altered activation of bile acid receptors can influence gene expression involved in various metabolic processes, *i.e.* glucose homeostasis, whereas low-grade inflammation has also been associated with insulin resistance and obesity. These observations highlight the importance of a balanced intestinal bacterial flora. The function of the gut microbiota to transform bile acids may be of pivotal importance to maintain bile acid signaling and energy homeostasis.

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