Gut microbial regulation of bile acid metabolism and signaling

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To my Father

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ABSTRACT

The collection of microbes in our gastrointestinal tract, the gut microbiota, is an environmental factor that has profound impact on host health and disease. Bile acids are endogenous cholesterol-derived molecules that can be modified by the gut microbiota and function as signaling molecules in regulation of host metabolic processes. This thesis investigates the role of the gut microbiota on bile acid metabolism and signaling by comparing mice that lack microbiota with their conventionally-raised counterparts.

We found that the gut microbiota regulates bile acid metabolism at several levels, including proportionalities of individual bile acid species and expression of genes involved in bile acid homeostasis. Specifically, the gut microbiota decreased levels of mouse primary bile acid tauro-beta-muricholic acid (T-βMCA), which we identified as an antagonist of the nuclear receptor farnesoid-x-receptor (FXR). FXR mediates negative feedback regulation of bile acid homeostasis, as well as regulation of several physiological processes. Hence, we identified the molecular mechanism behind microbial regulation of bile acid homeostasis as T-βMCA mediated inhibition of FXR activity. Since humans lack T-βMCA, this thesis plays an important role in explaining the existing discrepancies between mouse and human studies targeting FXR for treating gastrointestinal diseases. Furthermore, in order to better understand the effect of the microbiota on FXR signaling, we re-derived mice that lacked functional FXR as germ-free and mapped microbial regulation of genes through FXR. We found that the microbiota can regulate expression of FXR target genes through direct FXR binding to promoters in the intestine, while protein-protein interactions between FXR and other co-regulators are likely regulated in the liver.

In conclusion, this study establishes the microbiota as a key player in bile acid metabolism and FXR signaling in the liver and the intestine. The findings from this thesis implicate the microbiota as an important factor that needs to be taken into consideration in treating gastrointestinal diseases by targeting bile-acid mediated FXR signaling.

Keywords: gut microbiota, bile acids, tauro-beta-muricholic acid (T-βMCA), farnesoid-x-receptor (FXR)

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SAMMANFATTNING PÅ SVENSKA

Samlingen av bakterier i vår mag-tarmkanal, tarmfloran, är en miljöfaktor som har direkt inverkan på både hälsa och sjukdom. Gallsyror är molekyler deriverade från kolesterol som kan ändras av tarmfloran och fungerar som signalmolekyler i regleringen av människans metaboliska processer. Denna avhandling studerar tarmflorans roll i gallsyra metabolism med hjälp av bakteriefria möss som jämförs med normalt uppfödda möss med tarmflora.

Vi upptäckte att tarmfloran minskar total nivåerna av gallsyror hos möss genom att specifikt minska den primära gallsyran tauro-beta-muricholic syra (T-βMCA). Vidare identifierade vi att T-βMCA verkar som en hämmare av den nukleära receptorn farnesoid-x-receptor (FXR), som reglerar gallsyra produktion och är involverad i reglering av många fysiologiska processer. Följaktligen identifierade vi den molekylära mekanismen bakom bakteriell reglering av gallsyrametabolism, som sker via T-βMCA medierad hämning av FXR aktivering. Eftersom människor saknar T-βMCA spelar vår studie en viktig roll när det gäller att förklara skillnaderna mellan studier på möss och kliniska prövningar där behandling av magtarmsjukdomar riktas mot ändring av FXR-aktivitet. Därefter studerade vi FXR beroende reglering av genuttryck via tarmfloran i tarmen och levern, organ där FXR uttrycks väldigt högt och de viktigaste organen i FXR-medierad reglering av gallsyrametabolism. Vi fann att mikrobiell reglering av genuttryck sker via direkt FXR-bindning till dess målgener i tarmen, men i levern upptäckte vi att indirekta mekanismer existerar.

Studien etablerar tarmfloran som en nyckelaktör i gallsyra metabolism och FXR signalering. Resultaten från denna avhandling implicerar tarmfloran som en viktig faktor som måste beaktas då gallasyra medierad FXR signalering blockeras i syfte för behandling av gastrointestinala sjukdomar.

Nyckelord: tarmfloran, gallsyror, tauro-beta-muricholic syra (T-βMCA), farnesoid-x-receptor (FXR),

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Sayin, SI. Wahlström, A. Felin, J. Jäntti, S. Marschall, HU. Bamberg, K. Angelin B. Hyötyläinen, T. Oresic, M. Bäckhed, F.

Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist

Cell Metabolism 2013; 17: 225-235

II. Sayin, SI. Sommer, F. Chevalier, J. Rosenstiel, P. Staels, B. Eeckhoute, J. Bäckhed, F.

Differential FXR-mediated regulation by the gut microbiota in the liver and the intestine

Manuscript

CONTENT

ABBREVIATIONS

αMCA	Alpha muricholic acid
ApoE	Apolipoprotein E
Asbt	Apical sodium dependent bile acid transporter (see also Ibat)
βMCA	Beta muricholic acid
Bsep	Bile salt export pump
BSH	Bile salt hydrolase
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CONV-R	Conventionally raised
Cyp7a1	Cholesterol 7-alpha hydroxylase
DCA	Deoxycholic acid
Fgf15/19	Fibroblast growth factor 15/19
FXR	Farnesoid X receptor
FXR-KO	Farnesoid X receptor knockout
GF	Germ-free
Gly-βMCA	Glycine conjugated beta muricholic acid
GO-term	Gene ontology term
HDL	High density lipoprotein
Hnf4α1	Hepatocyte nuclear factor 4 alpha 1
HSDH	Hydroxysteroid dehydrogenase

Ibat	Ileal bile acid transporter (see also Asbt)
IBD	Inflammatory bowel disease
LCA	Lithocholic acid
LDL	Low density lipoprotein
Ldlr	Low density lipoprotein receptor
Lrh-1	Liver receptor homolog 1
MCA	Muricholic acid
Mdr3	Multidrug resistance protein 3
Mrp2	Multidrug resistance-associated protein 2
Mrp3	Multidrug resistance-associated protein 3
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
Oatp1	Organic anionic transporting polypeptide 1
ωMCA	Omega muricholic acid
Ostα/β	Organic solute transporter alpha/beta
PBC	Primary biliary cirrhosis
Shp	Small heterodimer partner
T-βMCA	Tauro-beta muricholic acid
UDCA	Ursodeoxycholic acid

1 INTRODUCTION

1.1 Gut microbiota

We have developed a symbiotic relationship with the microbes inhabiting our intestinal tract, the gut microbiota, throughout evolution; we provide them with a stable environment with nutrients and constant temperature, while they assist in metabolism of nutrients and xenobiotics that we cannot metabolize ourselves [1, 2]. Importantly, the microbes in our gut have evolved to control our physiology beyond the intestinal lumen, and they can signal to cells throughout our body and regulate their activity [3-6]. Products of microbial metabolism – microbial metabolites – are taken up from the intestinal lumen into our bloodstream and can be transported to any cell in the body. Many of the microbial metabolites, such as bile acids and short chain fatty acids, are signaling molecules and several organs in our bodies express receptors for these molecules [7]. These receptors regulate cellular pathways and are under the influence of their signaling molecules. Through modulation of these signaling molecules for cellular receptors, the microbiota has intertwined its role in our physiology as key regulators of our health and disease [8, 9].

Together with the microbiota's intrinsic role in regulating host physiological processes, what makes the microbiota a topic of intense research focus, is the fact that the gut microbiota is easily modifiable through environmental factors such as dietary components [10-12], probiotics [13, 14], and antibiotics [15]. This, together with the fact that the microbiota is a key regulator of host biology, makes it a novel and powerful candidate for treating human disease[16].

1.1.1 Gut microbiota – at a glance

The mammalian gut is colonized by bacteria, archaea, viruses and fungi [17]. The non-bacterial members of the microbiota probably also play significant roles in host physiology [18] but research on gut microbiota so far has focused mainly on the bacterial population and these members of the microbial community are the focus of this thesis, and will henceforth be referred to as the "gut microbiota". There are 10^{14} bacterial cells in our gastrointestinal tract, weighing approximately 1 kg and are estimated to outnumber our own cells 10 to 1 [1]. The gut microbiota consists of more than 1000 different bacterial species [19], and while they vary extensively between individuals, a core microbiota consisting of a fraction of these bacterial species can be identified in large cohorts of healthy individuals [20].

At the phylum level, 90% of the species in our gut microbiota belong to the phyla Firmicutes and Bacteroidetes. The remaining 10% belong to the phyla Proteobacteria, Acinetobacteria, Fusobacteria and Verrucomicrobia[19].

Although we are colonized throughout our gastrointestinal tract by the microbiota, the members in the small and large intestine seem to play an important role in regulating our metabolism, probably due to the close contact between microbes in the gut and our diet. The lumen of the intestine is an anaerobic environment and the vast majority of the gut microbiota consists of obligate anaerobes. In adult humans, there is an increasing gradient of density of microbial population from the proximal small intestine (duodenum), which is colonized by 10^3 bacteria/ml belonging to the genera *Lactobacillus* and *Streptococcus* to the most distal part of the small intestine (ileum) with a bacterial density of 10^6-10^8 bacteria/ml and consisting of *Enterobacteria, Enterococcus, Bacteroides, Clostridium, Lactobacillus* and *Veilonella.* In the large intestine, the bacterial population increases dramatically, with 10^{11} bacteria per gram content in the colon belonging to the genera *Bacteroides, Eubacterium, Bifidobacterium, Ruminococcus, Peptostreptococcus, Propionibacterium, Clostridium, Lactobacillus, Escherichi* and *Streptococcus* [21].

1.1.2 Gut microbiota and host physiology

Actions of the gut microbiota are important for several aspects of host biology [22]. Together with absorption of micronutrients from food that are otherwise unavailable to us and vitamin synthesis, the gut microbiota has been shown to regulate a variety of physiological processes such as development and differentiation of our intestinal epithelium and immune system [23-25], protection again pathogenic bacteria [26], maintaining effective barrier and motility functions in the gut [27], regulating bone homeostasis [28] and even host behavior [29, 30].

A particular effect of the microbiota on host biology that has been studied extensively is its effect on host metabolism. Since the 1950s, we have been able to rear animals in completely microbe-free environments, called germ-free (GF) animals, which has enabled several mechanistic studies to be done on defining the role of the microbiota in metabolism [31, 32]. In the beginning of the past decade, two landmark studies established a solid role of the microbiota in obesity where GF mice were found to gain less weight than their conventionally-raised controls (CONV-R), even though they ate more [33] because the microbiota influenced both energy expenditure and uptake

[34]. Several studies have since suggested a role of the microbiota in obesity in humans [35].

Among the most important mechanisms through which the gut microbiota contributes to host metabolic dysfunction is its ability to regulate metabolic processes beyond the intestinal epithelium [36]. As discussed earlier, the gut microbiota is able to influence metabolic processes in all cells of the host through production of metabolically active microbial metabolites that function much like hormones in our body. These microbial metabolites are taken up in the bloodstream and transported through all of the host's organs, many of which express cognate receptors. Mapping of the wide variety of biologically active microbial metabolites is the topic of intense research and the number of metabolites known to us is likely to increase in the coming years. Examples of microbial metabolites that have been shown to bind to receptors in host cells and regulate metabolism are : short chain fatty acids, catecholamines, amino acids, steroid hormones, and for the purpose of this thesis, most importantly, bile acids.

Bile acids are endogenous molecules that are metabolized by the gut microbiota and that can signal to cells throughout the body and alter metabolic functions. This thesis will explore the role of the microbiota in regulating bile acid composition and signaling capacity.

1.2 Bile acids

Bile acids are amphipathic (with both hydrophobic and hydrophilic properties) molecules that are produced in parenchymal cells of the liver (hepatocytes) through chemical modification of cholesterol (Figure 1) [37]. Through a series of enzyme catalyzed reactions, primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in the human liver. In the mouse, ursodeoxycholic acid (UDCA) is also a primary bile acid, while CDCA is further modified in the liver into the mouse-specific primary bile acids alpha and beta-muricholic acids (αMCA, βMCA). Bile acids are hepatotoxic in excess and are immediately conjugated to the amino acids taurine or glycine in the liver upon synthesis. In humans, majority of the primary bile acids are conjugated to glycine and in mice to taurine [37]. The conjugated primary bile acids are then secreted into the bile canaliculi through active transport and flow to the gallbladder in bile, together with bilirubin, phospholipids and cholesterol [38, 39]. Bile is stored in the gallbladder until its contents are emptied into the duodenum upon meal ingestion. Once in the intestine, mixed micelles of bile acids emulsify dietary lipids and facilitate fat absorption (Figure 2).

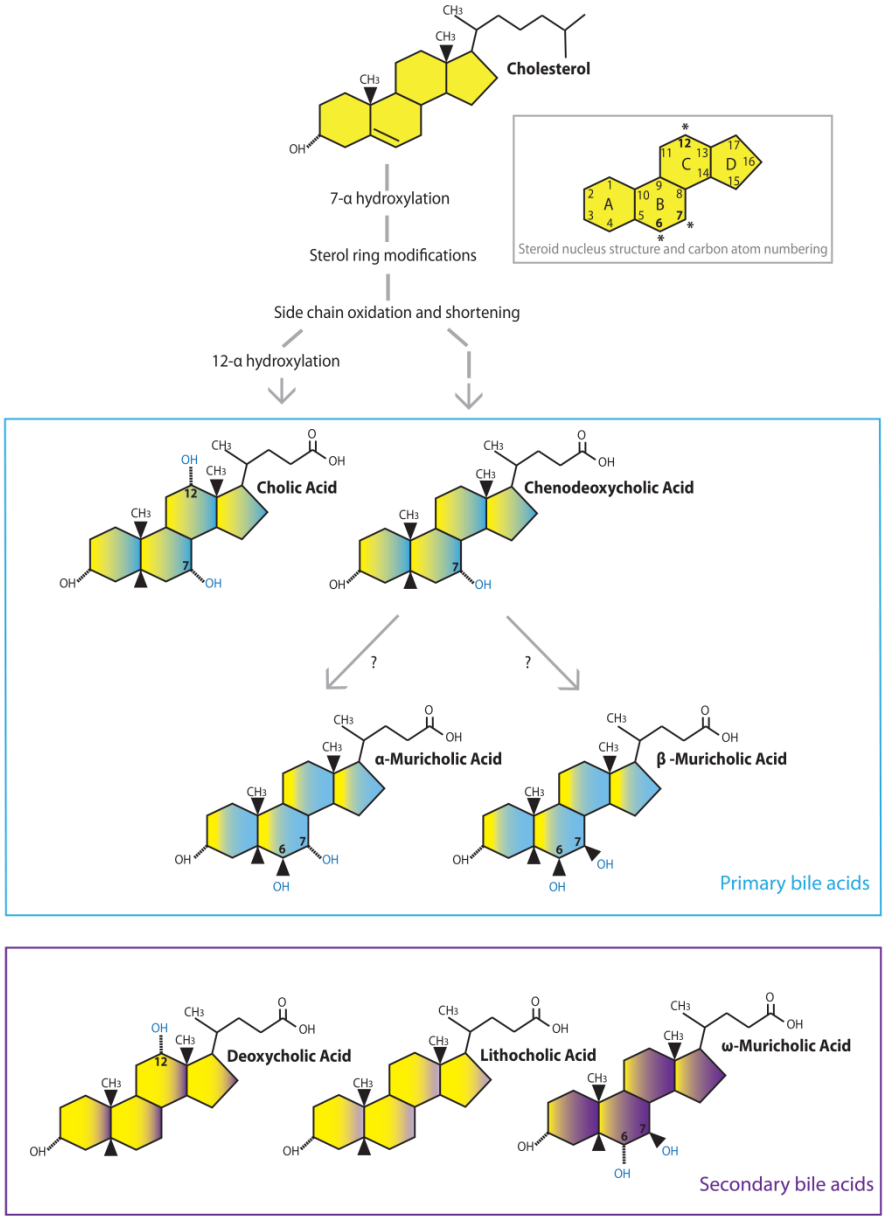

Figure 1. Bile acids: structure and synthesis. Schematic representation of primary bile acid synthesis from cholesterol is rendered at top. For individual bile acid species, amount of yellow is proportional to hydrophobicity. Hydroxyl groups (-OH) contributing to hydrophilicity are rendered in blue. Bonds in the steroid nucleus in alpha and beta configurations are rendered with dotted lines and triangles, respectively.

Upon entering the duodenum (most proximal part of the small intestine), primary bile acids come into contact with the gut microbiota, which deconjugates primary bile acids from their taurine or glycine conjugates (Figure 2). Further down in the small intestine and mainly in the colon, gut microbes possess various enzymes that further chemically modify primary bile acids into secondary bile acids [21]. Many species of secondary bile acids have been identified so far, and the most common ones are deoxycholic acid (DCA) and lithocholic acid (LCA), in addition to omega-muricholic acid (ωMCA) in mice.

The amphipathic nature of bile acids provides them with detergent properties. The steroid nucleus derived from cholesterol gives them their hydrophobic property, while the number of hydroxyl groups on the steroid nucleus and their orientation determines the hydrophilicity of bile acids. An individual bile acid's degree of hydrophobicity/hydrophilicity determines its fat emulsification property, toxicity, method of uptake as well as ligand binding to its target receptors.

1.2.1 Bile acid physiology – the enterohepatic circulation

The intestine and the liver are part of an organ system within which bile acids are circulated, called the eneterohepatic circulation [40]. The enterohepatic circulation consists of liver, gallbladder, the bile duct connecting the liver to the duodenum, the small intestine, and the portal vein system leading blood back from the intestine to the liver (Figure 2).

Like many other molecules in the body that have strong regulatory roles in various physiological processes, bile acid levels are under tight negative feedback regulation. Only 5% of bile acids secreted into the intestine are excreted in feces daily, whereas 95% are reabsorbed under normal conditions. For primary bile acids, reabsorption mostly occurs in the distal small intestine (ileum) where active transporters in the apical surface of enterocytes, such as apical sodium dependent bile salt transporter (Ibat/Asbt), absorb them from the intestinal lumen. The bile acids are then transported to the basal border of the enterocytes where active transporters, such as the organic solute transporters α/β (Ost-α/β) pump them out into the portal circulation [39, 41]. Most reabsorption of secondary bile acids, which are more hydrophobic than primary bile acids, occurs through passive diffusion in the colon where they are synthesized [42].

The portal circulation transports the reabsorbed bile acids back to the liver, where active transporters on the surface of hepatocytes import them. Upon entering the hepatocyte, reabsorbed bile acids are immediately conjugated to taurine or glycine and released back to the bile canaliculi, to be secreted into the intestine again through the gallbladder [42].

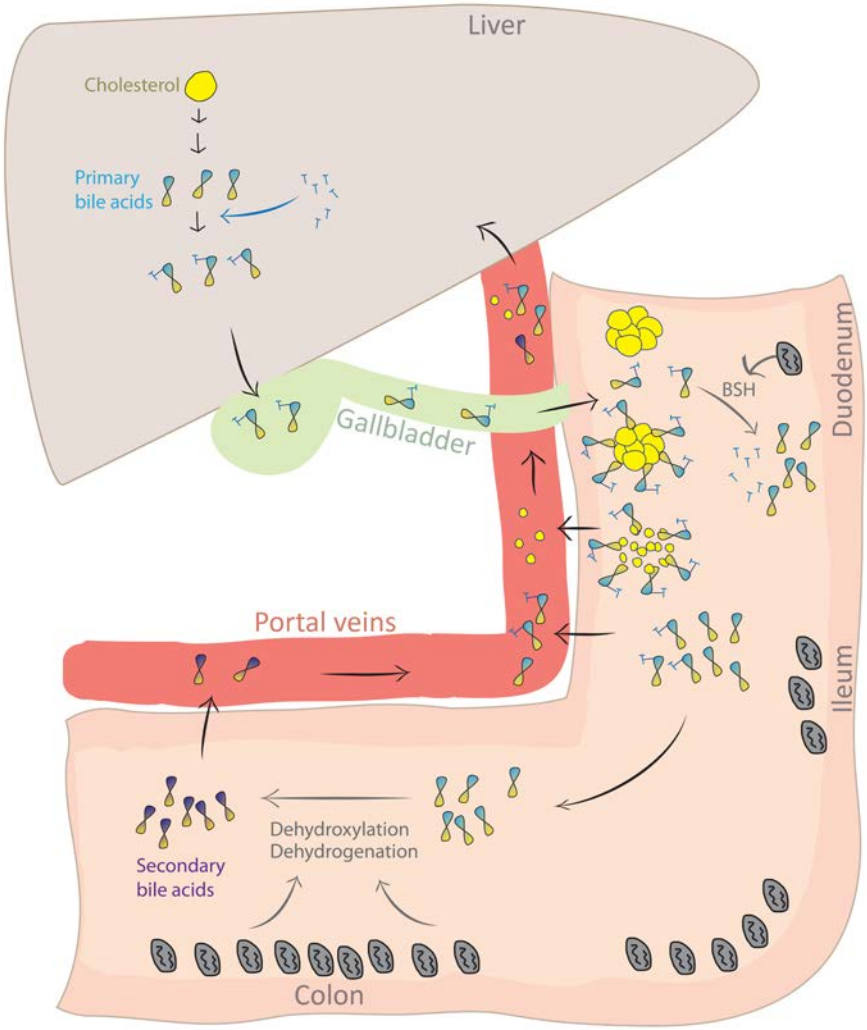

Figure 2. The enterohepatic circulation and bile acid physiology. Black arrows represent enterohepatic circulation path of bile acids. Grey arrows represent microbial bile acid modulation. Yellow aggregates in intestine are dietary lipids, grey ovals represent the microbiota.

1.2.2 Gut microbial biotransformation of bile acids

The gut microbiota possesses many enzymes that are able to perform a plethora of modifications of bile acids, and researchers are still mapping these various microbial modifications. Here, I will discuss the three most abundant and physiologically relevant modifications that have been studied to date.

The most abundant microbial modification of bile acids in the gut is deconjugation, which is the first step of microbial modification of bile acids [21]. The enzyme needed for deconjugation, bile salt hydrolase (BSH) is abundantly expressed by all major phyla in the gut [43]. Already upon entering the duodenum, bile acids come into contact with BSH containing genera like *Lactobacillus* and *Streptococcus*. It is unclear why gut microbes express BSH, but since deconjugation reduces antimicrobial properties of bile acids, it is speculated to decrease the toxicity of bile acids to the microbiota. Deconjugation also makes bile acids available for further modifications by the microbiota.

Microbial 7α-dehydroxylation is required for synthesis of secondary bile acids DCA and LCA. Unlike BSH, 7α-dehydroxylation enzymes have only been found in a few Clostridium species (clostridum cluster XI, XVI) that are low abundant taxa in both ileum and colon [44].

Hydroxysteroid dehydrogenase (HSDH) is also an abundantly present bile acid biotransformation enzyme present in the gut microbiota. It catalyzes the oxidation of bile acids to form oxo-bile acid intermediates [21, 45]. The exact function of HSDH is still unknown but it is speculated to generate energy for the bacteria through producing reducing equivalents for cellular biosynthetic reactions.

1.2.3 Bile acids – functions in the body

Fat absorption

Bile acids are crucial for the extraction and absorption of fat from the food we eat. The hydrophobic side of bile acids binds to fat aggregates in food content in the duodenum, while the hydrophilic ends face outwards (Figure 2). This process emulsifies the fat droplets and makes them accessible by pancreatic lipases. In addition, bile acids form mixed micelles with products of lipid digestion such as fatty acids and monoglycerides, as well as cholesterol and phospholipids, which solubilizes them and makes them

available for absorption by enterocytes. In fact, patients fed with CA have increased cholesterol absorption through increased cholesterol solubilization in micelles [46].

As stated before, the degree of hydrophilicity of a bile acid is determined by the number of hydroxyl groups on the steroid nucleus and their orientation. In addition, conjugated bile acids are ionized in physiological pH, which increases hydrophilicity. MCA and UDCA, which are primary bile acids in mice and UDCA is found as a secondary bile acid in humans, are the most hydrophilic bile acid species, and the secondary bile acids DCA and LCA are the most hydrophobic bile acids, with CA and CDCA having a more even hydrophobic/hydrophilic ratio. Because the fat emulsification and mixed micelle formation properties of bile acids are dependent on their detergent property, the hydrophobicity/hydrophilicty of the bile acid pool is crucial in their function of fat absorption from the intestinal lumen. In addition, since more hydrophilic bile acids are more easily cleared from the enterohepatic circulation while a more hydrophobic pool contributes to formation of gallstones, the hydrophobicity/hydrophilicity quotient of the total bile acid pool is important in terms of cholestatic liver disease and gallstone disease.

Cholesterol clearance

Cholesterol is used as the substrate for biosynthesis of bile acids in hepatocytes. The transformation of cholesterol into soluble bile acids and their excretion in feces accounts for 90% of cholesterol clearance from the body [47]. In fact, substances that bind bile acid molecules in the intestinal lumen and prevent them from being reabsorbed - bile acid sequestrants – lower low-density lipoprotein (LDL) cholesterol in the blood of patients with hyperlipidemia [48]. They are however not used as a first line of treatment for lowering blood lipids since they also lower the beneficial high-density lipoprotein (HDL) cholesterol, and in addition triglyceride levels are increased, as well as cholesterol biosynthesis, likely due to compensatory mechanisms. Bile acid sequestrants do, however, improve glycemic control in patients with type 2 diabetes and can be used as adjunct therapy for lowering blood glucose levels in these patients [49, 50].

Signaling molecules in metabolic regulation

Bile acids can function as signaling molecules and regulate various metabolic processes such as lipid metabolism, glucose metabolism and energy expenditure [51-54]. This function of bile acids is relatively newly discovered and is what makes bile acids the key molecules of interest in this field of research and in this thesis. The ability of bile acids to bind to and regulate several metabolically active receptors throughout the body allows them to be

key players in metabolic regulation [42, 55]. Bile acid receptors that have been identified so far are the nuclear receptors farnesoid-x-receptor (FXR), pregnane-x-receptor (PXR), vitamin D_3 receptor (VDR) and constitutive androstane receptor (CAR), as well as the cell-surface receptor G-protein coupled bile acid receptor 1 (TGR5) [56]. As discussed earlier, the fact that microbially modulated bile acids are reabsorbed in the intestine and bind to receptors throughout the body makes bile acids a key microbial metabolite that modulates host metabolism [50, 52, 53, 57-59].

1.3 Farnesoid-x-receptor

The farnesoid-x-receptor (Nr1h4/FXR) is currently the most extensively studied bile acid receptor due to its regulation of many metabolic processes and the availability of several drugs targeting FXR to modulate metabolism [56]. FXR is a nuclear receptor that regulates the expression of its target genes and is involved in regulating a vast number of metabolic processes, such as bile acid metabolism, lipid metabolism, glucose metabolism and energy homeostasis [60-62].

The FXR protein is expressed in highest concentrations in the intestine and liver, but is also present in kidneys, adrenal glands, white adipose tissue, stomach, pancreas, endothelial cells, vascular smooth muscles cells and cells of the immune system [62-68]. Although many of these tissues are metabolically active, FXR in the intestine and liver have been the focus of most studies on FXR due to their presence in the enterohepatic circulation and their exposure to high levels of bile acids.

Bile acids can bind to and modulate the activity of FXR. CDCA, DCA, LCA and CA are known FXR agonists [69-72] while taurine and glycine conjugated βMCA (T-βMCA and Gly- βMCA) in mice [73-75] and UDCA in humans [76] are FXR antagonists. By binding to FXR, bile acids can increase or decrease the expression of FXR target genes and thereby regulate metabolic processes controlled by those genes.

1.3.1 Regulation of gene expression by FXR

Regulation of gene expression by FXR is complex. It involves a large machinery of coactivators and corepressors, recruitment of chromatin modifying proteins and the RNA polymerase complex, protein-protein interactions with other nuclear receptors, and several overlapping pathways are involved in the process [60]. Like other nuclear receptors, the FXR protein has a ligand binding domain (LBD) and a DNA binding domain

(DBD), and activation of FXR by its agonists is traditionally measured in the literature in terms of increased expression of its downstream genes. In this process, docking of an activating ligand to its LBD causes structural changes in the protein that leads to shedding of co-repressors, enabling the DBD to bind to specific promoter region of genes and induce expression through recruitment of coactivators and the RNA polymerase complex [77]. In contrast, activation of FXR can also lead to suppression of gene expression. FXR can induce the expression of other nuclear receptors such as the small heterodimer partner (Shp) [78], which acts as a co-repressor for the nuclear receptor liver receptor homologue-1 (Lrh-1) by binding to it and preventing it from inducing expression of target genes [79, 80]. In addition, FXR activation in the liver has been shown to induce expression of the transcriptional repressor MafG which, like Shp, also represses bile acid synthetic genes and alters bile acid composition [81]. Alternatively, FXR has been shown to bind directly to the nuclear receptor Lrh-1 upon activation and both proteins together induced genes involved in lipid metabolism [82, 83]. FXR has also been shown to have synergistic relationship with the nuclear receptor hepatocyte nuclear factor-4α (Hnf4α1), which has been showed to bind to the genome near FXR-responsive elements upon FXR activation [84]. These differential protein-protein interactions between FXR and its co-players may explain why different FXR agonists have different effects on FXR mediated gene regulation.

Less is known about the mechanism of action of FXR antagonists due to the relatively new discovery of endogenous antagonists of FXR [73]. The endogenous antagonist T-βMCA is thought to be a competitive inhibitor since computer modelling shows that it is able to dock to the ligand binding pocket of FXR, where an agonist would otherwise bind, and because the inhibitory effect of T-βMCA on FXR can only be seen in the presence of an activating agonist. Hence, it is presumed that T-βMCA replaces an agonist such as CA or CDCA in the LBD and prevents FXR activation rather than decrease basal level activity. Recently, glycine-conjugated βMCA (Gly-βMCA) has also been identified as an endogenous antagonist of FXR [75] and high-dose UDCA has been shown to exert FXR antagonistic effects in morbidly obese patients [76] .

In addition to FXR binding to genes in a ligand-specific manner, the expression profile of genes following FXR activation or inactivation is organ specific. Previous studies have shown that FXR binding sites throughout the genome in the liver are mostly different from FXR binding sites in the intestine [85]. This, together with data from tissue-specific FXR knock-out

mice lacking the receptor in the liver or the intestine have shown that FXR regulates different metabolic processes in these organs [86, 87].

What complicates the elucidation of exact mechanisms of action by FXR is that FXR does not only bind to genes that are directly involved in metabolism, it also binds to genes for other transcription factors that have their own effect on the DNA, as well as working together with co-factors that on their own also regulate metabolism [88]. Taken together, FXR seems to be part of a large and complex system of transcription factors and co-factors which are each also part of complex networks on their own and it is therefore too simplistic to directly link specific alterations of FXR to metabolic effects.

1.3.2 Role of FXR in bile acid metabolism

FXR is central to bile acid homeostasis [62]. Bile acids regulate their own synthesis in a negative feedback loop regulated by FXR. When bile acids in the ileum are taken up into the enterocytes they bind to FXR and activate it during normal conditions when the bile acid pool consists of more agonists than antagonists. Upon activation, intestinal FXR induces the expression of fibroblast growth factor 15 (Fgf5) in mice and its orthologue Fgf19 in humans [89]. FXR binds to the second intron of the Fgf15 gene and induces its expression. With the help of a cellular protein Diet1 [90], Fgf15 is then secreted into the portal circulation where it functions as a hormone by traveling in the bloodstream and signaling to the liver. In the liver, Fgf15 binds to membrane bound receptor complex consisting of fibroblast growth factor receptor-4 (Fgfr4) and βKlotho. Fgfr4, in a complex with βklotho activates a cascade of reactions involving the cytoplasmic tyrosine phosphatase Shp2 [91], the Jnk and ERK pathways [92], that ultimately suppresses expression cholesterol-7a-hydroxylase (Cyp7a1), the first and rate limiting enzyme in the bile acid synthesis cascade. In addition, bile acids in the hepatocytes bind to activate FXR, which induces transcription of the atypical nuclear receptor Shp [93-95]. Shp lacks a DNA binding domain and is a potent transcription repressor. Upon its activation in the hepatocyte, Shp is recruited by the nuclear receptors Lrh-1 and Hnf4α to the promoter region of Cyp7a1 [79, 80, 96]. Therefore, bile acids control their own synthesis in the liver through a negative feedback loop mediated by FXR induced expression of Fgf15 in the intestine and Shp in the liver, which downregulates bile acid synthesis in the liver [97, 98]. In addition to regulation of bile acid synthesis, FXR regulates bile acid transport in the enterohepatic circulation by by regulating bile acid transporters in enterocytes and hepatocytes, a tight regulation of which is crucial for bile acid homeostasis [56].

1.3.3 Role of FXR in lipid and glucose metabolism

FXR has extensive physiological functions in addition to maintaining bile acid homeostasis [99, 100]. Mice lacking FXR (FXR KO mice) have elevated serum cholesterol, triglycerides, phospholipids, and low-density and very-low-density lipoproteins, confirming that FXR has a key role in lipid metabolism [62]. In addition, FXR KO mice have glucose intolerance with hepatic and peripheral insulin insensitivity [65, 100, 101] suggesting a role of FXR in glucose metabolism. While it is clear that FXR plays a key role in lipid and glucose metabolism, these findings from lean whole-body FXR KO mice are, however, not consistent with findings from mice with disease phenotypes. While FXR deficiency is associated with a worsened glucose homeostasis in lean mice, the lack of FXR in mouse models of obesity improves glucose homeostasis [102]. Similarly, while knocking out FXR in the apolipoprotein E (ApoE) deficient mice that has a atherogenic phenotype increases atherosclerotic plaques and lipid abnormalities [103], LDLR-deficient mice, which also have an atherogenic phenotype, have reduced atherosclerotic plaques in the absence of FXR [104]. Finally, the role of FXR in targeting lipid and glucose metabolism has been emphasized by studies of bariatric surgery on FXR-deficient mice, which have showed that FXR is required for mediating the metabolically beneficial effects of vertical-sleeve gastrectomy [105-107].

In summary, while data from mouse studies of FXR-deficient mice suggest a crucial role of FXR in lipid and glucose metabolism, the exact mechanisms through which modulation of FXR can lead to beneficial effects on these metabolic parameters are not clear. The inconsistencies in the results from these studies may be explained by differences in diet between studies and the gut microbial profile between different sites, as recent studies have shown strong interactions between microbiota, diet, breeding site, and metabolic phenotype [108].

1.3.4 Targeting FXR for treating metabolic disease

While studies in whole body FXR-deficient mice have left many questions unanswered about how to target FXR in a beneficial way to treat metabolic conditions, several recent studies using organ specific FXR targeting and tissue-specific FXR-deficiency in mice have now established intestinal FXR as a powerful target for treating metabolic disease.

One study has showed that feeding mice the intestine specific FXR agonist fexaramine reduces diet induced weight gain, body wide inflammation, blood

glucose and hepatic steatosis and increases adipose tissue browning [109]. In contrast, another study fed mice the antioxidant tempol, which changed the microbiota and increased T-βMCA, an FXR antagonist [110]. In this case, tempol treatment made the mice resistant to diet-induced obesity and lowered blood glucose, a phenotype that is also seen in intestine-specific FXR-deficient mice.

In addition to the mouse studies, several clinical trials targeting FXR have shown promising results on metabolic parameters. A double blinded multicenter placebo controlled randomized clinical trial has shown that treating patients with the FXR agonist obeticholic acid improved the histological features of non-alcoholic steatohepatitis (NASH) [111], while a phase two clinical trial of treatment with the same agonist increased insulin sensitivity, and reduced markers of liver inflammation and fibrosis in patients with type 2 diabetes mellitus and nonalcoholic fatty liver disease (NAFLD) [112]. Mouse studies suggest that these beneficial effects of FXR on NAFLD and progression to NASH are mediated through anti-inflammatory and anti-fibrotic effects of FXR [113] in addition to its effects on glucose and lipid metabolism [114]. In human obesity, however, antagonizing FXR has been shown to be beneficial [76]. In this study, administration of UDCA to morbidly obese patients awaiting bariatric surgery exerted antagonistic effects on intestinal FXR, as measured by decreased serum Fgf19 and increased bile acid synthesis, and decreased hepatic and LDL cholesterol as well as had positive effects on hepatic and adipose tissue lipid accumulation. Whether the antagonistic effect on FXR was exerted by UDCA directly or through other mechanisms such as modification of microbiota remains to be elucidated.

Hence, while these studies have brought us one step closer to solving the puzzle of targeting FXR in a beneficial way by clarifying the robust role of intestinal FXR in metabolic regulation, at the same time, they show that both activating and inhibiting FXR in the intestine have beneficial metabolic effects in terms of diet induced weight gain and blood glucose levels.

1.3.5 Role of FXR in hepatobiliary and gastrointestinal diseases

Phenotyping FXR deficient mice combined with treatment of mice and patients with FXR agonists [115] have implicated FXR in several hepatobiliary and gastrointestinal diseases[116].

Cholestatic liver diseases are characterized by impaired bile secretion and flow, intrahepatic bile accumulation, inflammation, fibrosis and cirrhosis. FXR plays a role in the severity of the outcome of cholestatic liver disease since treatment of mice and patients with FXR agonists alleviates disease severity [117-119]. Specifically, patients with the cholestatic liver disease primary biliary cirrhosis (PBC) treated with the FXR agonist obeticholic acid in addition to UDCA, which is the standard treatment for PBC otherwise, had 20% decrease in alkaline phosphatase, an indicator of disease severity [120]. Mouse studies suggest that the mechanism for the positive effect of FXR agonists on cholestatic liver disease may be through intestinal FXR mediated decrease in hepatic bile acid pool as a result of decreased bile acid synthesis and increase in bile acid transporters [121, 122].

Gallstone disease is characterized by accumulation of cholesterol (70%) or pigmented (30%) stones in the gallbladder, that can lead to bile flow obstruction and inflammation of the gallbladder. In the Western world, gallstone disease is a major health burden with 10-15% of adults being affected [123]. FXR seems to be involved in cholesterol gallstone disease, as FXR knockout mice on lithogenic diet are highly susceptible to cholesterol gallstone formation, with biliary cholesterol supersaturation and marked gallbladder wall inflammation [124, 125]. In addition, treatment of lithogenic-diet-fed gallstone-susceptible mice with FXR agonist GW4064 prevented cholesterol gallstone formation and increased the expression of bile salt export pump (Bsep/Abcb11) and multidrug resistance protein-3 (Mdr3/Abcb4) transporters, resulting in substantially higher bile salt and phospholipid bile concentrations in gallbladder bile [124]. Treatment of cholesterol gallstone disease by targeting FXR is therefore a promising field and deserves further attention.

Inflammatory bowel disease (IBD) is characterized an imbalance of gut microbiota, epithelial dysfunction, and aberrant mucosal immune response resulting in inflammation of the intestinal tract, and is divided into the diagnoses of Crohn's disease and ulcerative cholitis depending on the anatomical and histological location of the inflammation [126]. Several mouse studies have implicated FXR in the pathogenesis of IBD. FXR has been shown to alleviate inflammation and preserve the integrity of the intestinal epithelial barrier in many ways by regulating the extent of the inflammatory response, maintaining the integrity and function of the intestinal barrier, and preventing bacterial translocation in the intestinal tract [116, 127]. In addition, mice lacking FXR experienced bacterial overgrowth, increased intestinal permeability and contained large amounts of bacteria in mesenteric lymph nodes, as well as inflammation of the intestinal walls

[128]. Importantly, FXR modulates intestinal innate immune response [129], which is an important aspect of IBD. Taken together, these data suggest intestinal FXR as a promising target for treating IBD and clinical trials testing the efficacy of FXR modulation for treating IBD are warranted.

Hepatocellular carcinoma is a frequent complication in patients with cirrhosis due to inflammatory liver diseases. In FXR knockout mice, pronounced inflammation and elevated expression of inflammatory genes in the liver are associated with spontaneous tumor formation [130]. However, this spontaneous hepatocarcinogenesis in FXR knockout mice can be prevented by intestinal-specific FXR reactivation [131]. Further research on the role of FXR in human hepatocellular carcinoma needs to be done to confirm these findings in humans.

Colorectal cancer is the third most common form of cancer and the second most common cause of cancer-death worldwide. Colonic polyps, which are precursors to cancerous lesions, have decreased FXR mRNA expression, and this decrease is even more pronounced in colonic adenocarcinoma [132]. In two experimental murine models for intestinal cancer, FXR deficiency led to significantly increased sizes and numbers of the tumors [133]. In addition, large collections of human frozen colon carcinoma tissues and human cell lines show that FXR expression may be linked to the development of colorectal carcinomas [134].

Hence, FXR is a key regulator of several physiological processes and plays an important role in several diseases. Because the ligands of FXR – bile acids – are microbial metabolites, regulation of FXR signaling by the microbiota's effects on bile acids is a potentially vital mode of microbial regulation of host physiology, and this will be explored in this thesis.

2 AIM

The general aim of this thesis is to investigate the role of the gut microbiota in bile acid metabolism and bile acid signaling through the nuclear receptor FXR.

The specific aims are:

Paper I: **Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist**

The aim of this study was to map the impact of the gut microbiota on bile acids throughout the enterohepatic system and investigate the molecular mechanisms behind microbial regulation of bile acid metabolism through altered FXR signaling.

Paper II: **Differential FXR-mediated regulation by the gut microbiota in the liver and the intestine**

The aim of the second study was to investigate the role of the gut microbiota in regulating the expression of genes in the liver and the intestine through the nuclear receptor FXR.

3 RESULTS AND DISCUSSION

In this section, I will highlight the main findings from Paper I and Paper II to extend the discussion on these findings beyond what the scope of the individual papers allowed for. Here, I will analyze how these findings relate to literature and discuss the implication of microbial regulation of bile acids on host physiology.

3.1 Paper I

3.1.1 Gut microbiota regulates bile acid profile

First we confirmed that the microbiota reduces the size of the total bile acid pool [135]. This effect of the microbiota on the total pool size is achieved through regulation of bile acid metabolism at several levels. First, the microbiota induces increased excretion of bile acids in feces by microbial biotransformation of bile acids, which makes them more hydrophobic and these secondary bile acids are excreted more readily than primary bile acids. Second, this increase in excretion is not compensated for by increasing synthesis in the liver, rather the opposite, in which microbiota inhibits synthesis of bile acids in hepatocytes (Paper I Fig 3a). Activation of FXR in the intestine of CONV-R mice mediates decreased expression of Cyp7a1, the rate limiting enzyme in bile acid synthesis. Finally, the microbiota decreases the total bile acid pool by downregulating expression of bile acid transporters in the ileum and thus reduces bile acid reuptake. In fact, we found that Ibat/Asbt, which accounts for most bile acid absorption from the ileum, is regulated by the microbiota independent of FXR, a finding supported by other studies that have shown FXR-independent regulation of Ibat/Asbt. Role of the microbiota in modulating the size of the bile acid pool has been a crucial finding in the crossroads of linking microbial regulation of host metabolism through bile acids [136, 137].

3.1.2 Microbial modification of the primary bile acid pool

We found that the microbiota altered the composition of the bile acid profile throughout the enterohepatic system, with increased deconjugated and secondary bile acid species in their presence (Paper 1 Fig 2). While the existence of secondary bile acids and increased deconjugation in CONV-R mice is expected due to the action of microbes in the intestinal lumen on

deconjugation of primary bile acids through bile salt hydrolase. However, we observed that microbiota not only affect secondary bile acids but also modulate primary bile acids. The microbiota reduces the CA/MCA ratio significantly by specifically decreasing levels of T-βMCA in CONV-R mice (Paper 1 Fig S3). This effect of the gut microbiota on hepatic bile acid synthesis may be the key point of microbial modulation of bile acid metabolism, the downstream effects of which causes several other changes (increased FXR activation due to decreased T-βMCA (Paper 1 Fig 4, Fig 6), decrease of Cyp7a1 in liver (Paper 1 Fig 3A), consequent changes in the secondary bile acid profile due to changes in the primary bile acid pool (Fig 2)).

This is important because the mechanism behind how the microbiota changes the composition of the primary bile acid pool (decrease of T-βMCA synthesis) is unknown. The problem here stems from the fact that the enzyme responsible for synthesis of βMCA from CDCA is unknown and we can therefore not measure its regulation by the microbiota. However, it appears as the CDCA is almost completely converted to βMCA. Also, because we see the microbiota mediated decrease of T-βMCA also in the FXR-knockout condition (Paper 1 Fig S5) we hypothesize that this regulation, unlike the microbiota's effect on CA, is FXR independent. Our finding of microbial modulation of the bile acid pool through decreasing T-βMCA levels was confirmed in a recent study showing that administration of the antioxidant tempol causes changes in the microbiota that causes T-βMCA levels to increase. Similar to our study they observed that the increase in TbMCA was associated with decreased expression of FXR targets in the intestine.

The issue of microbial regulation of primary bile acid synthesis deserves further attention, since many studies on patients have focused on significant alterations in the secondary bile acid pool and bacteria that synthesize them. For example, the secondary bile acid DCA has been found to be increased in the bile acid pool of individuals on a high-fat diet [138] as well as T2DM [48]. Corresponding changes in the microbiota with increased DCA producing microbes (Clostridium cluster XIVa and XI) have been studied [138]. However, as the dynamics of BA study on T2DM patients shows [48, 50], the initial change is in the primary bile acid pool, with increase in CA and decrease in CDCA that leads to increased DCA. Indeed, the increase of Clostridium XIVa and XI species may be the result of increased supply of the substrate CA which causes the species to flourish and produce more DCA as a consequence. Mechanisms behind microbial modulation of the primary bile acid pool need to be elucidated in order to make sure that we focus future therapy on the point of origin of microbial regulation and not the effects of it.

3.1.3 Gut microbiota regulates gallbladder size

Upon fasting conditions the gallbladder contains the largest proportion of the total bile acid pool, and we found the size of the gallbladder and its contents to be significantly increased in GF compared with CONV-R mice (Paper 1 Fig 1C). The increased volume of the gallbladder content was due to increased bile acids and not cholesterol or phospholipids (other common constituents of gallbladder content) (Paper 1 Fig 1D,E). The increased size is likely due to impaired gallbladder emptying, as it has recently been shown that GF mice have impaired cholecystokinin-induced gallbladder emptying in addition to increased BA synthesis [139]. This finding, together with the fact that GF mice have a more hydrophilic bile acid profile, has important implications for a role of the microbiota in the development of gallstones, which can be caused by decreased outflow of bile from the gallbladder and a more hydrophobic bile acid profile. Studies of the gut microbiota of patients with gallstones are needed in order to study whether the microbiota changes the hydrophobicity of their bile acid profile and contributes to decreased gallbladder emptying in these patients.

3.1.4 Gut microbiota regulates bile acid conjugation

We found that the microbiota reduces levels of the amino acid taurine in the liver (Paper 1 Fig 3C). The largest proportion of bile acid conjugation in mice is to taurine and we found strong upregulation of taurine synthetic enzyme Csd in the liver (Paper 1 Fig 3D), suggesting a compensatory response to decreased taurine levels in the presence of the microbiota. Since the ratio of taurine to glycine conjugated bile acids is solely dependent on the relative abundance of the amino acids[37], microbial regulation of taurine levels in the liver may be yet another way in which the microbiota control metabolism. Indeed, studies have shown that a Western diet causes human bile acid pool to be conjugated to taurine more than to glycine[140] and that the Western diet shifts the composition of the gut microbiota to increases in the species *Bilophila wadsworthia*, which utilizes the sulfite in taurine [141]. This, taken together with our finding that T-βMCA is an FXR antagonist but not βMCA, implicates bile acid conjugation into being yet another mechanism through which the microbiota may modulate FXR activity.

3.1.5 Gut microbiota regulates the expression of genes involved in bile acid metabolism

We found that the gut microbiota regulates expression of several genes involved in bile acid metabolism. In the presence of the microbiota, intestinal

FXR is activated and its target genes including Fgf15, Ibabp and Shp are upregulated (Paper 1 Fig 4A). FXR in the liver is not activated (as measured by Shp expression levels, Fig 4B) but the effects of intestinal FXR activation on the expression of hepatic genes involved in bile acid metabolism are mediated by the microbiota (Fig 3a). This showed that microbial regulation of bile acid metabolism is through regulation of intestinal FXR and not hepatic FXR. Indeed, several studies have shown that bile acid metabolism is dependent on intestinal FXR [86, 92], rather than both intestinal and hepatic FXR, as previously thought. In addition, others have shown that microbial regulation of bile acid metabolism is through FXR in the intestine and not in the liver [110].

In addition to regulating bile acid synthesis, we found that the microbiota regulates bile acid transporters in the intestine and the liver (Paper 1 Fig 3E, 3F). In the intestine, apical transporters Ibat/Asbt and multidrug resistance-associated protein-2 (Mrp2) are downregulated while basolateral transporters Mrp3 and Ostα are upregulated. The reduction in apical transporters facilitates reduced uptake of the more toxic bile acid pool from the intestinal lumen in the presence of the microbiota, combined with faster clearance of these more toxic bile acids from the enterocytes through increased efflux by basolateral transporters into the portal circulation.

Interestingly, a similar pattern of regulation is seen in the liver where the portal uptake transporter organic anion transporting polypeptide-1 (Oatp1) is downregulated while the canalicular transporter Bsep is upregulated. In addition, Mrp3, which is a protein on the sinusoidal side of hepatocytes that aids in systemic bile acid efflux and is important in the context of cholestasis [142-144], is upregulated in hepatocytes by the microbiota. This suggests that the gut microbiota may be important in the context of hepatocyte injury during cholestatic liver disease, additionally since FXR is under microbial regulation and is of importance in reducing hepatic injury from primary biliary cirrhosis [120].

3.1.6 The mouse primary bile acid T-βMCA is an FXR antagonist

We found that T-βMCA is an antagonist of FXR in silico, in vitro, ex vivo and in vivo (Paper I Fig 6). By using computer modelling studies, we showed that T-βMCA can dock in the ligand binding pocket of FXR, where an agonist would normally bind (Paper I Fig 6A). We found that the presence of

T-βMCA decreased activity of the FXR protein in vitro in the presence of the FXR agonist GW4064 (Paper 1 Fig 6B, 6C). Since little is known about the mechanism by which antagonists act to decrease FXR activity, and T-βMCA is the first endogenous FXR antagonist discovered in mammals, it makes further studies of T-βMCA-FXR interactions crucial in elucidating mechanisms of gene regulation by antagonizing FXR ligands.

Studies have shown that T-βMCA mediated inhibition of FXR activity is associated with beneficial effects on lipid and glucose metabolism [110] as well as hepatic steatosis [145] in mice, but no studies on the effects of FXR antagonism have been done on humans since there are significant hurdles in treating patients with T-βMCA. We performed an *in vivo* study of FXR antagonism by oral treatment of T-βMCA on germ-free mice (Paper 1 Fig 6E, 6F) since we had seen in the in vitro studies that βMCA is not an FXR antagonist. Administration of T-βMCA orally to CONV-R mice is therefore ineffective in antagonizing FXR since the gut microbiota deconjugates the T-βMCA before it reaches the ileum, as BSH containing microbes are present already in the duodenum. The human gut microbiota can deconjugate T-βMCA (Wahlström et al 2015, submitted) even though T-βMCA is not endogenous to the human bile acid pool. Furthermore, these studies are done on humanized mice (GF mice that have been colonized with human microbiota) and studies on how βMCA would be further metabolized in the human system have not yet been done.

3.2 Paper II

In Paper I, we found that the gut microbiota activates FXR in the intestine and regulates its target genes Fgf15, Shp and Ibabp. However, we found that the FXR target gene Ibat/Asbt is regulated by the microbiota even in the absence of FXR, suggesting alternative pathways of microbial regulation of some FXR regulated genes. This suggested that while the microbiota regulates some genes through FXR, there are additional pathways of microbial regulation of FXR target genes. Even though a plethora of studies are underway targeting the microbiota and FXR to treat metabolic disease, we have very little overview on microbial regulation of gene expression and the role of FXR in it.

We and others have shown that the gut microbiota's effect on bile acid metabolism, liver steatosis, glycemic control and lipid metabolism can be mediated through intestinal FXR and regulation of its downstream genes[73, 110] . At the same time hepatic but not intestinal FXR is needed for lipid accumulation in mice on a cholesterol-rich diet [146] . Hence, many of the gut microbiota's effects on systemic metabolic features is mediated through regulation of genes in these two organs, but so far we only know about the regulation of genes that have been studied in a targeted way through quantitative real time PCR. In paper II, we map expression levels of genes regulated by the microbiota in the liver and the ileum of wild-type and FXR-deficient mice, in order to study the role of FXR in microbial regulation of genes in these organs.

3.2.1 Gut microbiota regulates unique sets of genes and biological processes in the liver and the intestine

Through sequencing of total RNA in intestinal and liver tissue from GF and CONV-R mice, we confirmed that the gut microbiota regulates largely unique sets of genes in the liver and the intestine (Paper II Fig 1A). This shows organ-specific gene regulation by the microbiota and suggests separate mechanisms through which the gut microbiota exerts its control on different host organs. Further, GO-term analysis confirms organ-specific gene regulation by the microbiota and its metabolites and underscores the importance of mapping microbial regulation of receptors such as FXR in each organ that it is expressed in. This is especially true for microbial regulation of FXR activity since whole-body FXR targeting studies have generated conflicting results and can probably be explained by the "FXR effect" being a

sum of all organ-specific effects throughout the system, which we have until now known little of.

3.2.2 Gut microbiota regulates more genes in an FXR dependent way in the liver than in the intestine

We know from our previous work [73] that much of the gut microbiota's regulation of metabolism in the host is mediated through bile acid modulation and altered FXR signaling. We therefore also sequenced the total RNA from GF and CONV-R mice that are FXR-deficient in order to comprehensively map the role of FXR in microbial regulation. As can be seen in the gene expression heat maps in Paper II Figure 2, microbial regulation of gene expression could be clearly divided into FXR dependent and independent groups in both organs.

Additionally, we found that significantly higher numbers of genes were regulated by the microbiota in an FXR dependent way in the liver (859 genes) than in the intestine (105 genes) with only 18 genes being common between the tissues. This finding is in accordance with previous findings that FXR binds to largely different sets of genes in the liver and the intestine and that there are more FXR binding sites in the liver than in the intestine [85].

Taken together our data imply that the mechanism of microbial regulation of FXR in the liver is different from the intestine. The mechanism behind this tissue specific regulatory profile of FXR target genes by the microbiota may be through differential recruitment of corepressors and coactivators and needs to be studied further.

3.2.3 Gut microbiota regulates more biological processes through FXR in the liver than the intestine

We find many more biological processes are regulated in an FXR dependent fashion by the microbiota in the liver than in the intestine (Paper II Fig 4C). In the intestine, FXR mediated regulation by the microbiota was exerted to the highest fold enrichment on "steroid metabolic process", a GO-term which has "bile acid metabolism" and "cholesterol metabolism" as its child processes. This is in complete agreement with our findings from Paper I that the gut microbiota strongly regulates bile acid metabolism genes in the intestine through FXR.

While studies have shown hepatic FXR to be important in other metabolic processes, such as lipid accumulation [146], we do not know if the microbially regulated biological processes in the liver are under the regulation of hepatic or intestinal FXR since we used mice that were whole body knockouts. Further studies on tissue-specific knockout mice lacking FXR in the intestine or the liver are needed to clarify which of these biological processes are regulated by FXR in each organ.

3.2.4 Gut microbiota regulates FXR downstream genes through direct FXR binding to genes in the intestine

In order to learn more about the mechanism behind how the microbiota regulates FXR-mediated gene expression, we investigated whether microbiota alters FXR binding to the genome. FXR binding sites that have been previously identified with chromatin immunoprecipitation sequencing in the liver and the intestine, and we looked for these binding sites in the microbiota regulated genes we identified here. Interestingly, we found that the FXR dependent genes in the intestine had higher number of FXR binding sites than FXR independent genes but this was not the case in the liver. This suggests that microbial regulation of FXR dependent genes in the intestine is through direct effects on FXR binding to DNA, while in the liver, FXR dependent genes are regulated in more complex ways and not only through direct binding. It is likely that microbial regulation of FXR dependent genes in the liver is through regulation of its corepressors and coactivators, as well as effects on protein-protein interactions between FXR and other transcription factors such as Hnf4a1 and Lrh-1, consequently regulating their downstream genes. Importantly, much of the effects in the liver may be mediated through microbial regulation of intestinal FXR signaling, as discussed above.

Mechanisms of FXR mediated regulation of gene expression by the microbiota need to be studied further. In this regard microbial regulation of genome-wide FXR binding studies with chromatin immunoprecipitation sequencing experiments on liver and ileum from GF and CONV-R mice can provide a better overview of which microbially regulated genes in each organ are modulated by direct FXR binding. Combining these results with the expression profile of GF and CONV-R mice from tissue-specific knockouts of FXR in the liver and the intestine would clarify the role of FXR in microbial regulation in these organs.

4 METHODOLOGICAL CONSIDERATIONS

Here, I will discuss some considerations that need to be made in interpreting results in this thesis with the methods that have been used in mind. For specific details on all methods used in the thesis, please refer to the Methods and Materials section of Paper I and Paper II.

4.1 The mouse as a model for studying human physiology and disease

The mouse is widely used in medical research as a model for studying human physiology and disease. Because we cannot perform invasive studies at the early stages of knowledge generation through medical research on humans, we have to rely on model organisms to execute experiments that are invasive and generate mechanistic knowledge of physiology and disease. The mouse is an excellent model, in that it is extremely similar to humans in anatomy, physiology and genetics. In fact, we share a 95% similarity with the mouse genome and orthologues of most proteins can be found between the two species, making genetic research on mice applicable to human genetics [147]. In addition, the adult mouse has a small body size, a short lifespan and large litters, making it economically possible to execute large scale experiments.

4.2 The mouse as a model for studying the gut microbiota and bile acid metabolism

In this thesis, we use the mouse as a model for studying the impact of the gut microbiota on host bile acid metabolism and signaling. Despite the large degree of similarities between humans and mice in spite of the phylogenetical distance between our species, when it comes to the gut microbiota as well as bile acid metabolism, there are significant differences between mice and humans [148]. First of all, even though the majority of the gut microbiota in humans and mice can be divided into the phyla Firmicutes and Bacteroidetes, there are considerable differences between the two species in their gut microbial profiles at the genera level [149]. This may cause intrinsic differences in microbial biotransformation of bile acids and lead to differences in bile acid profiles between mice and humans. In fact, it has recently been shown that bile acids are in fact metabolized in different ways

in GF mice colonized with human microbiota compared with those colonized with mouse microbiota (Wahlström et al 2015, submitted).

Secondly, there are key differences between the bile acid profile of mice and humans. MCAs, tauro-conjugated alpha and beta forms of which we have identified in Paper I as endogenous FXR antagonists, only exist in mice and are not found in humans. CDCA, which is the precursor for the primary α- and β-MCA is found in humans instead, but CDCA has very different chemical and signaling properties from MCAs. MCAs are much more hydrophilic than CDCA, and because both these species constitute approximately half of the bile acid pool in mice and humans respectively, the murine bile acid pool is more hydrophilic than humans. In addition, while α- and β-MCA are antagonists for FXR, CDCA is the strongest endogenous FXR agonist. In terms of FXR activity, this means that while in the mouse, FXR activation depends on a balance between agonists (CA) and antagonists (MCAs), in humans both primary bile acids CA and CDCA are FXR agonists. As discussed in the Results section, this has consequences for using T-βMCA for treating human disease, as well as for testing drugs in mice for targeting FXR.

4.3 The germ-free mouse model

The germ-free mouse model is used widely to conduct research on the gut microbiota [31, 150]. By definition, GF mice are born and raised in sterile environments free from any environmental microbes. They breathe filtered air and eat and drink sterilized food and water [151]. A complete lack of the microbiota in these animals has enabled us to study fundamental changes in physiology in the absence of our commensal microbes. In addition, colonization of GF mice with microbiota from hosts with different phenotypes as well as single bacterial colonies enables us to study causal relationships between the microbiota and disease states.

Studies on GF mice are designed with using their conventionally-raised counterparts as controls: age and gender matched mice that breathe filtered air and eat and drink sterile food and water like the GF mice but are exposed to the normal flora in the environment and thus have a normal microbiota. Compared with their CONV-R counterparts, GF mice are leaner, have less body fat and consume more food, have improved glucose tolerance and blood lipid profile and have an underdeveloped immune system. Due to these intrinsic differences from their controls, it may be speculated that changes in bile acid metabolism that we see in GF mice may be due to their unique condition and environment, rather than an isolated effect of the microbiota.

We have therefore also studied bile acid metabolism by treating CONV-R mice with antibiotic cocktails that remove a large part of the microbiota and render them similar to GF mice in their microbial profile, and replicated our findings from GF mice in the antibiotic treated mice.

4.4 Statistical analysis of RNA-sequencing data

Analysis of data generated by Next Generation Sequencing is subject to setting arbitrary cut-off points as well as choosing parameters to generate gene lists of biological significance. We have used conventional levels of stringency while setting cut-off points for all genes that have been taken into consideration in Paper II and only genes with a difference in expression between groups of p-value less than 0.05 were used in the analysis. Furthermore, for the GO-term analysis, we set the cut-off to $p<0.05$ after a Bonferroni correction for multiple testing, which for ontology terms is a conservative correction.

5 CONCLUSIONS

The conclusions from this thesis are:

- Bile acid metabolism is under tight microbial regulation.
- Through regulation of the bile acid profile, the gut microbiota regulates activity of the nuclear receptor FXR and its downstream genes.
- The mouse primary bile acid T-βMCA is an endogenous FXR antagonist and its levels are under microbial regulation.
- Gut microbiota regulates different sets of biological processes through FXR in the liver and the intestine.
- Gut microbiota likely regulates FXR target genes in the intestine by directly modulating FXR binding to promoter regions.
- FXR-mediated regulation by the gut microbiota in the liver is not through direct FXR binding to the genome and likely through modulation of protein-protein interactions between FXR and other regulators.

6 FUTURE DIRECTIONS

Given the complexity of the gut microbiota, the deeply intertwined relationship between the microbiota and bile acid metabolism in the host, and the therapeutic potential that lies in treating metabolic disorders through microbiota mediated modulation of bile acid signaling, much remains to be done in this field of research.

First, our finding that T-βMCA is an FXR antagonist and consequent findings that show potential of eliciting positive effects on diet induced obesity and NAFLD through increased T-βMCA levels in mice, warrant further studies on testing the potential of treating these conditions in humans with T-βMCA. We need to know more about how the human microbiota metabolizes T-βMCA in the intestinal lumen and whether the antagonistic effect of T-βMCA on intestinal FXR and its consequent positive effect on metabolic parameters translate in human physiology. It is, however, likely that the human gut microbiota can deconjugate T-βMCA and therefore abolish the antagonistic activity. Thus derivatives that cannot be deconjugated may have to be developed, and UDCA may be a suitable candidate, but more studies are needed to show its mechanism of FXR antagonism. Treating healthy individuals with oral T-βMCA and measuring whether Fgf19 levels decrease in the blood following this treatment would be a natural starting point. If this is the case, a next step would be to treat patients with obesity and NAFLD with T-βMCA in order to study whether the positive effects of T-βMCA in these disease conditions in mice can be translated to humans.

Also, identifying the enzyme in mice that synthesizes T-βMCA should be a priority. Once it is identified, knockout mice would provide a much better model for studying bile acid metabolism since the bile acid profile should be more human-like with CDCA instead of MCA. Furthermore, these mice can be rederived as GF and colonized with human microbiota to study the interactions between the human microbiome and CA/CDCA and thus provide a better model for studying FXR targets.

In addition, mapping of FXR target genes and its microbial regulation in humans should be studied in order to increase the translational aspect of the current thesis. Studies of chromatin immunoprecipitation with anti-FXR antibodies followed by sequencing under physiological and disease conditions, combined with studying protein-protein interactions between FXR and other regulators and analysis of the effect on the transcriptome,

would generate a better understanding of the complex regulatory profile of FXR and the discrepancies in targeting it for treating disease.

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