The interplay between bile acids and gut microbiota in metabolic and hepatobiliary disease

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Amor fati

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ABSTRACT

The gut microbiota, a metabolic regulator, orchestrates the production of bioactive metabolites such as secondary bile acids. Growing evidence suggests that shifts in the gut microbiota composition are linked to metabolic and hepatobiliary disease. Bile acids are involved in lipid digestion and metabolic signaling and undergo microbial transformation in the gut, producing secondary bile acids. This thesis examines the interplay between bile acids and the gut microbiota. *Clostridium scindens* accounts for a minor fraction of the gut microbiota but is one of the few known species capable of generating secondary bile acids by 7α-dehydroxylation. We showed that even at low abundance, *C. scindens* had a marked impact on metabolism in mice, and we established a link between deoxycholic acid and metabolic dysregulation in type 2 diabetes (T2D). Data suggests that modulation of the bile acid and gut microbiota composition may promote some of the benefits of bariatric surgery, and we examined postprandial bile acid kinetics pre- and post-surgery, revealing an altered post-surgery response. Bariatric surgery increased hyodeoxycholic acid, which was linked to T2D remission. The distinct bile acid profiles of mice and humans are due to CYP2C70, and *Cyp2c70*-deficient mice display manifestations of hepatobiliary disease. We showed that gut microbiota influences neonatal survival and liver disease in *Cyp2c70*$^{-/-}$ mice. Amelioration of the liver phenotype was associated with a more hydrophilic biliary bile acid profile, largely driven by microbially induced ursodeoxycholic acid production. In summary, we provide evidence of the crucial role of the interplay between bile acids and gut microbiota in health and disease.

Keywords: gut microbiota, bile acids, type 2 diabetes, hepatobiliary disease

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Sammanfattning

Gallsyror är hormonlika molekyler som tillverkas i levern. De är en del av gallan och utsöndras i tarmen efter måltid för att underlätta upptag av fett och fettlösliga vitaminer. Gallsyror fungerar även som ligander vid cellsignalering och kan därmed påverka metabola processer i kroppen. I framförallt tunn- och tjocktarmen kan bakterier i tarmmikrobiotan modifiera gallsyrorna och därmed förändra deras egenskaper. Mikrobiellt modifierade gallsyror kallas sekundära gallsyror. Tarmmikrobiotan är förändrad i många sjukdomar, därbland typ 2 diabetes, primär skleroserande kolangit och primär biliär kolangit och metaboliter producerade av tarmmikrobiotan kan påverka hälsan på många sätt.

Clostridium scindens utgör en relativt liten del av tarmmikrobiotan, men är en av få bakterier som kan producera de sekundära gallsyrorna deoxicholsyra och litocholsyra. Vi visar att *C. scindens* redan vid mycket låga nivåer, starkt påverkar metabola processer genom produktion av sekundära gallsyror. Vi visar även att deoxicholsyra är kopplat till försämrad glukosmetabolism och försämrad blodfettsammansättning hos individer med typ 2 diabetes.

Bariatrisk kirurgi är ett samlingsnamn för operationer som leder till viktnedgång hos patienter med fetma. En av de föreslagna mekanismerna är att operationen påverkar tarmmikrobiota och gallsyrasammansättningen. Vi undersöker hur gallsyror i blodet påverkas av måltidstester före och efter bariatrisk kirurgi och kan visa att operationen förändrade gallsyraresponsen. Vi visar även att en ökning av fastenivåer av hyodeoxicholsyra är kopplade till remission av typ 2 diabetes efter bariatrisk kirurgi.

Djurförsök, framförallt med möss är viktiga för förståelsen av hur sampelet mellan gallsyror och tarmmikrobiotan fungerar, men begränsas av att sammansättningen av gallsyror skiljer sig åt mellan möss och människor. Möss som saknar *Cyp2c70*-genen har en gallsyraprofil som liknar den hos människor. Dessa möss uppvisar tecken på leversjukdom och genom att göra möss som saknar *Cyp2c70* bakteriefria visar vi att tarmmikrobiotan är avgörande för överlevnad i tidig ålder och att mikrobiotan lindrar leversjukdom i möss som saknar *Cyp2c70*. Förbättringen av leversjukdom var kopplad till att tarmmikrobiotan ökade andelen vattenlösliga och således mindre levertoxiska gallsyror.

Sammanfattningsvis understrycker denna avhandling betydelsen av samspelet mellan gallsyror och tarmmikrobiotan i metabola-, och lever- och gallgångssjukdomar.

List of papers

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

I. Wahlström, A., Brumbaugh, A., Sjöland, W., Olsson, L., Wu, H., Henricsson, M., Lundqvist, A., Makki, K., Hazen, S. L., Bergström, G., Marschall, H.-U., Fischbach, M. A., Bäckhed, F. Production of deoxycholic acid by low-abundant microbial species is associated with impaired glucose metabolism.
In revision.

II. Wahlström, A., Aydin, Ö., Olsson, L., Sjöland, W., Henricsson, M., Lundqvist, A., Marschall, H.-U., Franken, R., van de Laar, A., Gerdes, V., Hofsø, D., Groen, A. K., Hjelmesæth, J., Nieuwdorp, M., Bäckhed, F. Alterations in bile acid kinetics after bariatric surgery in patients with obesity with or without type 2 diabetes.
Submitted manuscript.

III. Sjöland, W., Wahlström, A., Makki, K., Schöler, M., Molinaro, A., Olsson, L., Greiner, T. U., Caesar, R., de Boer, J. F., Kuipers, F., Bäckhed, F., Marschall, H.-U. Absence of gut microbiota reduces neonatal survival and exacerbates liver disease in *Cyp2c70*-deficient mice with a human-like bile acid composition.
Clin. Sci. **137**, 995-1011 (2023).

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Abbreviations

12-epiDCA	12β-deoxycholic acid
12-oxoDCA	12-oxo-deoxycholic acid
16S rRNA	16S ribosomal RNA
3,12-oxoDCA	3,12-dioxodeoxycholic acid
3oxo-DCA	3-oxodeoxycholic acid
3-oxoLCA	3-oxolithocholic acid
3β-HSD	3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase
7-oxoCA	7-oxo-cholic acid
7-oxoCDCA	7-oxo-chenodeoxycholic acid
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
bai	Bile-acid-inducible
Base	Base community
BMI	Body mass index
BSEP	Bile salt export pump
BSH	Bile salt hydrolase
C4	7α-hydroxy-4-cholestene-3-one
CA	Cholic acid
CA-7S	Cholic acid-7-sulfate
CD	Conventionalized

CDCA	Chenodeoxycholic acid
CK19	Cytokeratin 19
CONV-R	Conventionally raised
Cq	Quantitative cycle
Cyp2c70^{ako}	Acute hepatic Cyp2c70 knockout
DCA	Deoxycholic acid
ddPCR	Droplet digital PCR
ER	Endoplasmic reticulum
Fgf15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FMT	Fecal microbiota transplantation
FXR	Farnesoid X receptor
GCA	Glyco-cholic acid
GCDCA	Glyco-chenodeoxycholic acid
GF	Germ-free
GHCA	Glyco-hyocholic acid
GHDCA	Glyco-hyodeoxycholic acid
GLCA	Glyco-lithocholic acid
GLP-1	Glucagon-like peptide-1
G-MCA	Glyco-murocholic acid
GUDCA	Glyco-ursodeoxycholic acid
HbA1c	Glycated hemoglobin

HCA	Hyocholic acid
HDCA	Hyodeoxycholic acid
HOMA-IR	Homeostatic model assessment for insulin resistance
HSDH	Hydroxysteroid dehydrogenase
HUM	Humanized
IBABP	Ileal bile acid binding protein
IBAT	Ileal bile acid transporter
IBD	Inflammatory bowel disease
iso-alloLCA	3β,5α-lithocholic acid
isoDCA	3β-deoxycholic acid
isoLCA	3β-lithocholic acid
isoUDCA	3β-ursodeoxycholic acid
LCA	Lithocholic acid
MCA	Muricholic acid
MMT	Mixed-meal test
NGT	Normal glucose tolerance
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP	Organic-anion-transporting polypeptides
OCA	Obeticholic acid
PBC	Primary biliary cholangitis
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance

PSC	Primary sclerosing cholangitis
PXR	Pregnane X receptor
qPCR	Quantitative real-time PCR
RORγt	RAR-related orphan receptor gamma
RXR	Retinoid X receptor
RYGB	Roux-en-Y gastric bypass
S1PR2	Sphingosine-1-phosphate receptor 2
SCAPIS	Swedish Cardiopulmonary Bioimage Study
SCFA	Short-chain fatty acid
SG	Sleeve gastrectomy
SHP	Small heterodimer partner
SULT	Sulfotransferase
T2D	Type 2 diabetes
TCA	Tauro-cholic acid
TCDCA	Tauro-chenodeoxycholic acid
TDCA	Tauro-deoxycholic acid
TGR5	Takeda G-protein receptor 5
T_H17	T helper 17 cell
TLCA	Tauro-lithocholic acid
TMC	Timepoint for maximum concentration
TUDCA	Tauro-ursodeoxycholic acid
TαMCA	Tauro-alpha-muricholic acid

TβMCA	Tauro-beta-muricholic acid
TωMCA	Tauro-omega-muricholic acid
UDCA	Ursodeoxycholic acid
UPLC-MS/MS	Ultra-high-performance liquid chromatography-tandem mass spectrometry
VDR	Vitamin D receptor
WT	Wild type
αMCA	Alpha-muricholic acid
βMCA	Beta-muricholic acid
ωMCA	Omega-muricholic acid

Introduction

Interest in gut microbiota has soared due to its vital role in regulating host metabolism and physiology. The gut microbiota interacts with hormone-like molecules known as bile acids. However, our grasp of the interplay between bile acids and gut microbiota is far from complete. This thesis intends to enhance the current understanding of their relationship and discusses this intricate interplay and its profound effects on metabolic and hepatobiliary disease.

The microbial landscape

The fact that trillions of microbes[1] colonize us is a relatively new finding on the grand scale of history. It was not until the 17th century that Antonie van Leeuwenhoek discovered that human feces hosts bacteria and other microorganisms that he named animalcules[2]. Today, we know that microbes widely colonize the human body, with highly interindividual variations in microbial composition. Colonization occurs at all body sites, such as the mouth, the skin, the vagina, and the gastrointestinal tract[3]. However, the microbiota comprises a diverse ecosystem of microorganisms, with not only bacteria but also fungi, viruses, bacteriophages, archaea, and single-cell eukaryotes[4]. Although animalcule as a term has faded into the annals of history, the microbiota field thrives. Following up on van Leuwenhoek's original finding that feces contain bacteria, the gut microbiota has received attention as a metabolic organ, and research implicating the microbes of the gastrointestinal tract in human health has gained much traction. Thus, the body of knowledge on the microbial component of the microbiome is far more extensive compared to that of the other communities[5], and this thesis focuses on the interplay between gut microbial communities and bile acids.

Gut microbiota

The gut microbiota is composed of the microorganisms that inhabit the gastrointestinal tract, collectively impacting host metabolism[4]. It has evolved over time, resulting in a symbiotic relationship between the host and resident microorganisms. The host provides an excellent environment for bacteria to flourish while they assist with the metabolism of various nutrients and xenobiotics[6]. The gut microbiota is also involved in regulating[7–9] and maturing the immune system[10].

Our first exposure to microbes occurs during birth, and the *in utero* fetal environment is sterile[11,12]. Infants are exposed to microbes from their mothers during birth, with their microbial profiles influenced by the mode of delivery[13,14]. Microbes colonize infants, and the gut microbiota evolves into an adult-like microbiota over time[15,16]. The gut microbiota has an adult-like configuration at five years of age. However, it has not fully matured, and some bacterial taxa associated with health in adults are still low abundant at five years of age[17]. Moreover, bile acids have been shown to shape the small intestinal gut microbiota in neonatal mice, with bile acid supplementation driving the microbial composition to a more adult-like configuration[10].

Since the gut microbiota is involved in the catabolism and transformation of nutrients, it occupies a pivotal position in host metabolism. This metabolic feature of the gut microbiota is responsible for transforming dietary components into a number of bioactive metabolites that influence host health. Microbially produced metabolites that impact health include short-chain fatty acids (SCFAs), tryptophan metabolites, vitamin metabolites, and secondary bile acids[18]. As a result, an abundance of research has established associations between gut microbiota composition and health, particularly in individuals with specific health conditions. Examples of pathophysiological conditions with which gut microbiota has been associated include obesity[19–24], type 2 diabetes (T2D)[25–29], cholelithiasis[30], primary sclerosing cholangitis (PSC)[31–35], primary biliary cholangitis (PBC)[36], cardiovascular disease[37–39], asthma[40], inflammatory bowel disease (IBD)[41], and cancer[41,42].

Microbial diversity is a proxy of ecological community structure. In the prior cases, a reduced gut microbiota diversity or altered community composition has been associated with disease. Moreover, microbial diversity is subdivided into α-diversity and β-diversity where α-diversity measures the microbial diversity within the community, whereas β-diversity measures the dissimilarity between samples or communities[43]. Sustained changes in dietary habits and long-term dietary interventions can influence the configuration of the gut microbial community[44–46]. However, the gut microbiota responds both to long-term and short-term changes in dietary habits. In a human cohort of individuals consuming either a plant-based or an animal-based diet for five consecutive days, an increase in β-diversity was seen in the animal-based diet group after only two days. The animal-based diet decreased levels of the SCFAs acetate and butyrate and increased the abundance of the secondary bile acid deoxycholic acid (DCA) in feces[47].

The gut microbiota is a central metabolic regulator, has been associated with various diseases, and has the potential to considerably impact host metabolic health by producing microbiota-derived metabolites. Secondary bile acids, one of the most abundant microbiota-derived metabolites, have a pronounced impact on health and disease and will be reviewed extensively throughout this thesis.

A glance into the liver

As far back as ancient Mesopotamia, the significance of the liver was recognized. It was believed that communication with deities could be expedited by hepatoscopy, the practice of looking into the liver of a sacrificed animal and reading the messages contained within[48]. While the importance of the liver was understood, if divine communication via the liver is possible, it is beyond the scope of this thesis. With that said, the liver has essential functions beyond its supposed role in divine communication, including its production of bile.

Bile acids

Bile acids are one of the main constituents of bile, a green-yellowish aqueous solution produced by hepatocytes and stored within the gallbladder. Other components include phospholipids, cholesterol, amino acids, steroids, vitamins, and biliary proteins[49]. Bile acids contain a hydrophobic and a hydrophilic face, with a carboxyl acid side chain predominantly conjugated to taurine or glycine. Their polar and non-polar faces result in amphipathic properties. Their structure lends them detergent properties and, consequently, the functionality to form micelles and facilitate the emulsification and solubilization of dietary fats and fat-soluble vitamins[50]. Bile acids are commonly classified as either primary or secondary, where the primary bile acids in humans are cholic acid (CA) and chenodeoxycholic acid (CDCA), and are hepatically synthesized, whereas secondary bile acids result from metabolism by the gut microbiota[51].

Hepatocytes comprise the vast majority of the parenchymal tissue in the liver, and bile acid synthesis occurs in hepatocytes from metabolites produced in the catabolism of cholesterol. The synthesis occurs in a multistep reaction through the classical or neutral pathway and the alternative or acidic pathway[52]. The classical pathway accounts for the majority of bile acids synthesized, whereas the alternative pathway is a minor contributor to bile acid synthesis in

humans[53]. The classical pathway starts with a rate-limiting reaction where cholesterol is first converted to 7α-hydroxycholesterol by CYP7A1. Conversion to 7α-hydroxy-4-cholestene-3-one (C4) by 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase (3β-HSD) follows, and CYP8B1 controls the subsequent production of CA, influencing the ratio of CA: CDCA synthesis[54,55]. Importantly, C4 is used as a marker for bile acid synthesis in humans[56].

The first and second reaction in the alternative pathway is controlled by CYP27A1, producing 27-hydroxycholesterol with conversion to 3β-hydroxy-5-cholestenoic acid. In the next step, CYP7B1 converts 3β-hydroxy-5-cholestenoic acid to 3β, 7α-dihydroxy-5-cholestenoic acid, concluding with several steps, ultimately producing CDCA[54]. Figure 1 illustrates specific steps in the synthesis of bile acids.

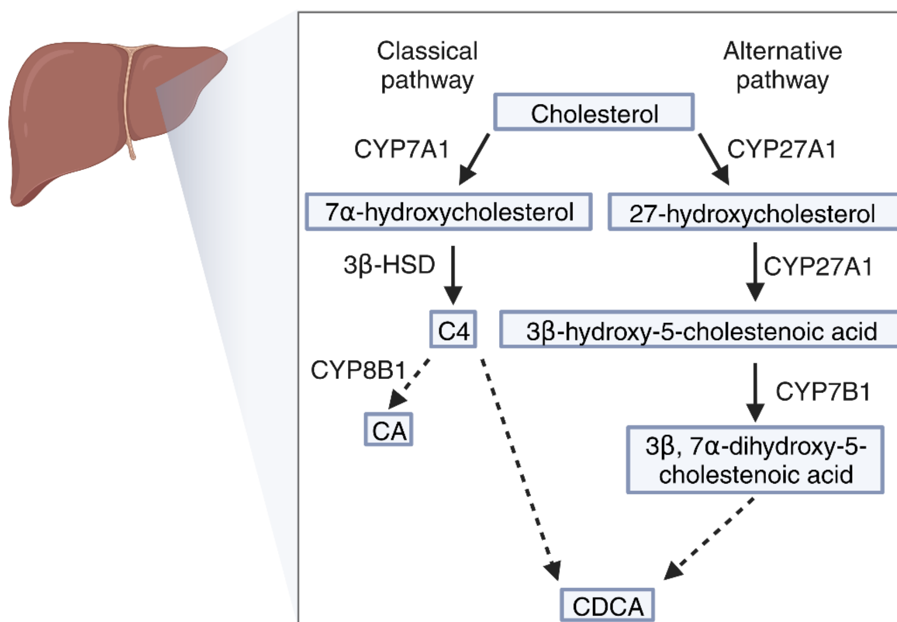


Figure 1 Select steps in the classical (neutral) and alternative (acidic) hepatic bile acid synthesis pathways. Dotted arrows denote that several intermediary transformations that occur are not shown. Created using BioRender.

Before bile acids are stored in the gallbladder, bile acid-CoA: amino acid N-acyltransferase conjugates bile acids in the liver at C24[57,58] with glycine or taurine at an approximate 3:1 ratio[59,60]. This ratio can be altered by dietary

intake of taurine but not by dietary glycine[61,62]. In early gestation, this ratio is skewed toward taurine in fetal bile[63], which persists in newborn infants, and shifts toward glycine with age[64]. The glycine: taurine ratio can also be altered in individuals with certain hepatobiliary or ileal conditions[65,66].

Enterohepatic circulation of bile acids

After synthesis, bile acids are transported from the hepatocytes to bile canaliculi and are emptied into the gallbladder, where they are concentrated and stored. Upon ingestion of a meal, lipids and proteins stimulate the release of cholecystokinin from the mucosa in the duodenum, which in turn binds the cholecystokinin receptor on the gallbladder. This leads to the contraction of the gallbladder and the release of mixed micelles of bile acids and phospholipids into the duodenum. Bile acids exert their solubilizing functions in the intestine, facilitating the transport and uptake of lipids[67,68].

As bile acids travel further down the gastrointestinal tract, conjugated bile acids are actively reabsorbed in the terminal ileum. Ileal bile acid transporter (IBAT) transports conjugated bile acids across the apical border of enterocytes lining the intestine. They are bound by cytosolic ileal bile acid binding protein (IBABP), regulating intracellular transport. Their transport across the basolateral end into the portal vein is primarily performed by the heterodimeric transporter organic solute transporter α/organic solute transporter β. Bile acids make their way back to the liver through the portal venous blood and are actively transported back into the liver via organic-anion-transporting polypeptides (OATP) and Na^+-taurocholate cotransporting polypeptide (NTCP)[69,70]. Thus, bile acids are re-conjugated to glycine or taurine and reenter the cycle. This recirculation process is referred to as enterohepatic circulation[69], which occurs approximately 6-10 times daily[50]. Primarily in the ileum, bile acids are deconjugated by bacteria, leading to an escape of the active intestinal transport of conjugated bile acids. This minor fraction (~5%) travels along the gastrointestinal tract into the colon, where they are metabolized by gut microbiota, modifying their structure and hydrophobicity. However, to a limited degree, bile acids are reabsorbed into the enterohepatic circulation via passive diffusion in the colon[71]. Humans' total bile acid pool comprises an estimated 2-4 g, of which approximately 0.2-0.6 g is expelled daily through fecal excretion[50]. Figure 2 depicts the enterohepatic circulation of bile acids.

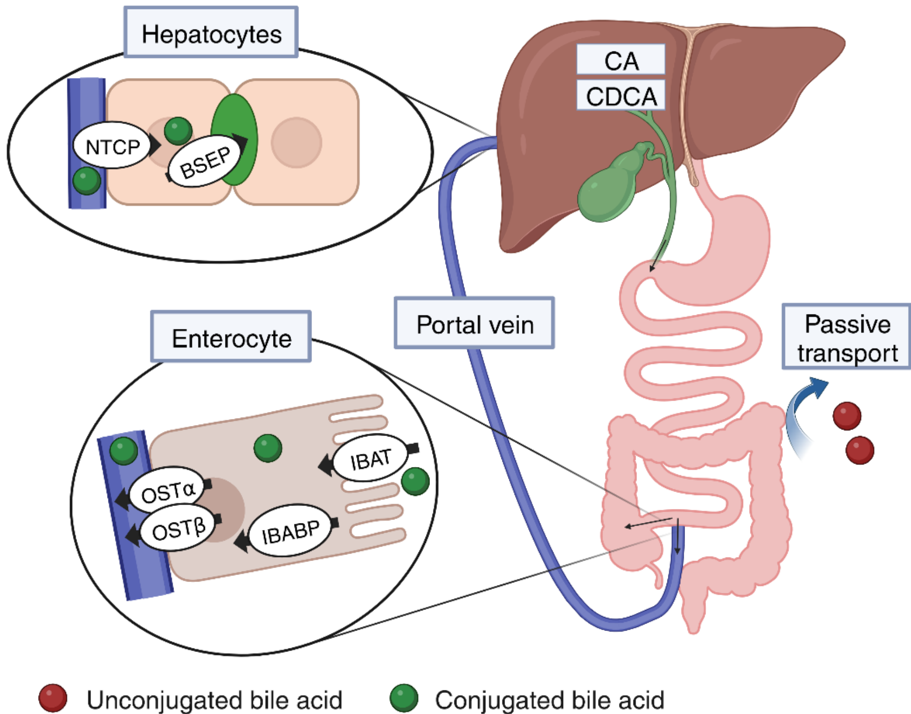


Figure 2 The enterohepatic circulation in humans. Created using BioRender.

The excretion of bile acids can further be facilitated by sulfonation, granting it a negative charge and thus increasing the water solubility, impeding the passive absorption over the intestinal epithelium. The sulfonation process is carried out by sulfotransferases (SULTs) in the liver, small intestine, and adrenal glands. SULT2A1 is responsible for bile acid sulfonation but also exhibits selectivity for androgens, estrogens, and glucocorticoids. While the most common bile acid sulfonation site in humans is the C3 hydroxyl-group, SULT2A1 can also sulfonate hydroxyl groups in other positions[72]. In addition, SULT2A8 has been identified to be capable of sulfonation, with a higher specificity for 7α-OH bile acids than non-7α-OH bile acids, including DCA and lithocholic acid (LCA). SULT2A8 expression is also generally higher in male mice compared to female mice[73,74].

Bile acid and gut microbiota crosstalk

The gut microbiota and bile acids individually affect host metabolism. However, bile acids and gut microbiota also act in synergy.

Bacterial metabolism of bile acids

The gallbladder secretes bile acids into the intestine post-prandially, which are then deconjugated by bacterial bile salt hydrolase (BSH), particularly in the small intestine but also in the colon. BSH is the enzyme responsible for the hydrolysis of bile acids at C24 and is found in several bacterial genera, specifically *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, and *Lactobacillus*[51]. Moreover, BSH is also found in some archaea, which are prevalent in humans[51]. In the colon, bile acids that manage to escape the enterohepatic circulation undergo bacterial biotransformation by the expulsion of their 7-hydroxyl group[51], and 7α-dehydroxylation in the human gut seems limited to unconjugated bile acids[75]. The pathway for 7α-dehydroxylation has recently been elucidated, and all eight necessary genes are present in the bile-acid-inducible (*bai)* operon[76]. This pathway has been observed in a select few bacterial species in the human gut[51,77]. However, probably not all bacteria capable of 7α-dehydroxylation have to date been identified. Computational analysis has identified several uncultured bacteria in the *Oscillospiraceae* family that may perform 7α-dehydroxylation[78].

CA and CDCA are the main substrates for bacterial 7α-dehydroxylation, converting CA to DCA and CDCA to LCA[79]. Secondary bile acids reach high micromolar concentrations in the human colon[80], but the abundance of known 7α-dehydroxylating bacteria in the gut microbiota is relatively low in humans[81]. Despite this, DCA, LCA, and their related epi-, iso-, and oxo-metabolites are highly abundant in the human gut. Since bacteria known to contain the *bai* operon are relatively low abundant, these few species' metabolic output appears extraordinary. *Clostridium scindens* contains the *bai* operon and is a well-characterized 7α-dehydroxylating bacteria[82,83], and despite its low abundance, it varies little over time and retains a stable presence in the gut microbiota[84]. Therefore, only focusing on changes in the abundance of a bacteria such as *C. scindens* might not shed light on its full impact on host health.

In addition to dehydroxylation, bile acids can also undergo reduction[85,86] and oxidation[87] by gut bacteria generating epi-, iso-, and oxo-bile acids such as 12-epi-deoxycholic acid (12-epiDCA), 3β-iso-deoxycholic acid (isoDCA), 12-oxo-deoxycholic acid (12-oxoDCA), and 3β-lithocholic acid (isoLCA), diversifying the bile acid pool. A depiction of metabolically healthy individuals' fecal bile acid profiles can be found in Figure 3.

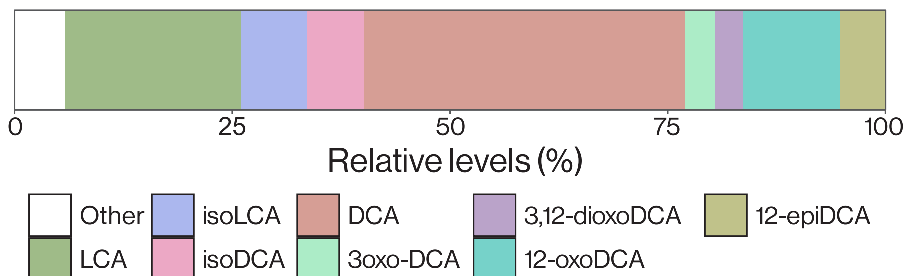
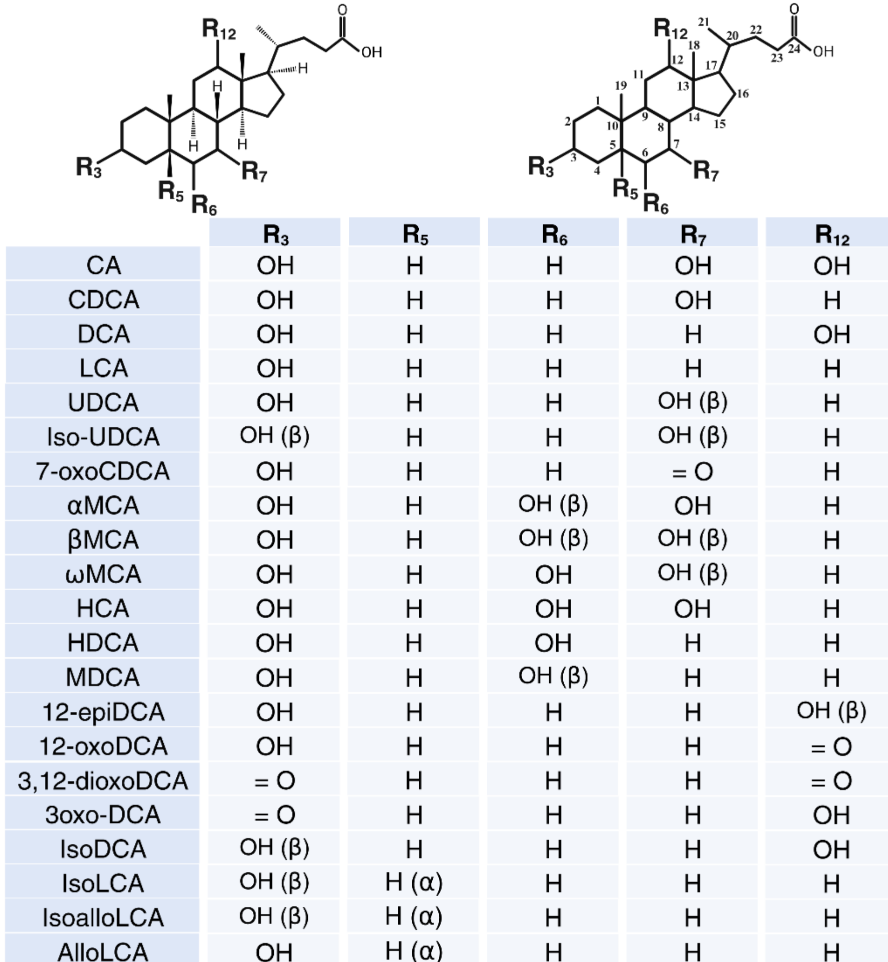


Figure 3 Proportions of individual bile acids in feces of metabolically healthy individuals with normal glucose tolerance from the IGT cohort. The cutoff for "Other" is < 2% of the total bile acids. n = 105.

Certain bacteria in the gut harbor hydroxysteroid dehydrogenases (HSDHs) responsible for the toggling between hydroxyl and keto groups. As follows, microbial HSDHs generate epi-, iso-, and oxo-bile acids and are found in different forms, including the 3α, 3β, 7α, 7β, 12α, or the 12β-form. They are named after the position they modify and are specific to the α- or β-orientation. The epimerization, or shift from, e.g., 3α- to 3β-position is performed by an α-HSDH and β-HSDH duo with an intermediate generation of an oxo-bile acid[88]. Additionally, the production of 5α-hydrogenated bile acids, known as allo-bile acids, has been suggested to be an arm of the 7α-dehydroxylation pathway[89], with one group identifying that the *baiP* gene in *C. scindens* ATCC 35704[90], and the *baiJ* gene in *C. scindens* VPI 12708 and *C. hylemonae* DSM 15053[79], encode 5α-reductases implicating them in the formation of allo-bile acids. Phylogenetic analyses of human metagenomic datasets from fecal samples only found the *baiJ* and *baiP* genes in the Firmicutes phyla[91]. Although allo-bile acids seem relatively uncommon in the general population, one study found them elevated in centenarians and identified bacteria capable of their production, raising the question of whether allo-bile acids could be involved in human longevity[92]. A figure showcasing structures of different bile acids can be found in Figure 4.



	R_3	R_5	R_6	R_7	R_{12}
CA	OH	H	H	OH	OH
CDCA	OH	H	H	OH	H
DCA	OH	H	H	H	OH
LCA	OH	H	H	H	H
UDCA	OH	H	H	OH (β)	H
Iso-UDCA	OH (β)	H	H	OH (β)	H
7-oxoCDCA	OH	H	H	= O	H
αMCA	OH	H	OH (β)	OH	H
βMCA	OH	H	OH (β)	OH (β)	H
ωMCA	OH	H	OH	OH (β)	H
HCA	OH	H	OH	OH	H
HDCA	OH	H	OH	H	H
MDCA	OH	H	OH (β)	H	H
12-epiDCA	OH	H	H	H	OH (β)
12-oxoDCA	OH	H	H	H	= O
3,12-dioxoDCA	= O	H	H	H	= O
3oxo-DCA	= O	H	H	H	OH
IsoDCA	OH (β)	H	H	H	OH
IsoLCA	OH (β)	H (α)	H	H	H
IsoalloLCA	OH (β)	H (α)	H	H	H
AlloLCA	OH	H (α)	H	H	H

Figure 4 Bile acid structures. All R_5 hydrogens are in β-orientation, and all R_3, R_6, R_7, and R_{12} hydroxyl groups are in α-orientation if otherwise not specified. Bile acids in the table are largely named after suggested nomenclature in literature[93]. Created using BioRender.

Furthermore, a newly discovered function of the gut microbiota is that some microbes can conjugate bile acids. The microbial conjugation of three amino acids, phenylalanine, tyrosine, and leucine, has recently been discovered. Certain strains of *Clostridium bolteae* have been shown to conjugate CA to phenylalanine and tyrosine, generating phenylalano-CA and tyroso-CA. The newly discovered bile acid conjugates were also elevated in individuals with IBD and cystic fibrosis. However, much remains unknown about these conjugates' impact and whether they are resultant or causative of disease[94].

Bile acids as signaling molecules

The involvement of bile acids in human metabolism extends beyond their well-established role in facilitating the digestion and absorption of lipids, as they also play a role as signaling molecules in metabolic processes. Bile acids act as signaling molecules by interacting with bile acid-sensitive receptors. These receptors serve as metabolic regulators, modulating a wide array of metabolic processes.

Farnesoid X receptor

Farnesoid X receptor (FXR) is a transcription factor encoded by the *NR1H4* gene in humans and is part of the nuclear receptor superfamily[95,96]. Originally, FXR was found to be activated by farnesol and its metabolites, precursors for synthesizing, e.g., steroids[97]. However, bile acids serve as the primary endogenous ligands of FXR, and their activation degree depends on the bile acid species. CDCA is the most potent endogenous agonist of FXR, followed by DCA and LCA[95,96]. Notably, the bile acid tauro-beta-muricholic acid (TβMCA), highly abundant in germ-free (GF) mice, is a potent FXR antagonist, whereas the deconjugated form beta-muricholic acid (βMCA) is not[98]. The expression of FXR is especially prominent in the small intestine and the liver, both locations exposed to high concentrations of bile acids. FXR is also expressed in other tissues, including the adrenal gland, the kidney, the bladder, and the ovary[99]. However, expression in close spatial proximity to high bile acid concentrations enables sensing, control, and regulation of bile acid synthesis[100]. Thus, FXR is an essential transcription factor in orchestrating bile acid metabolism and governs bile acid synthesis through a negative feedback loop by inhibiting *CYP7A1*[101], *CYP8B1*[102,103], and *CYP27A1*[104].

Bile acids in the liver bind hepatic FXR as a heterodimer-complex with retinoid X receptor (RXR) binding to the FXR response element on the promoter for the nuclear receptor small heterodimer partner (SHP), inducing the expression of *SHP*. SHP inactivates liver receptor homolog-1, thereby inhibiting *CYP7A1* expression[105,106]. Moreover, SHP represses *CYP8B1* through interaction with hepatocyte nuclear factor 4 alpha[102,103].

Bile acids located in the intestine bind intestinal FXR, inducing the expression of fibroblast growth factor 15 (*Fgf15)* in mice and fibroblast growth factor 19 (*FGF19)* in humans, and activates fibroblast growth factor receptor 4[107,108]. FGF19 signaling has been shown to require β-klotho[109] and may function as a co-receptor[110], leading to inhibition of *CYP7A1* expression and, as a result,

suppression of bile acid synthesis[107,111]. Moreover, several transporters involved in the transport of bile acids across cells are under the control of FXR. FXR, as a heterodimer complex with RXRα, binds to the promoter of *ABCB11,* encoding the bile salt export pump (BSEP), and regulates its transcription[112]. In humans and mice, FXR also regulates IBAT[113,114], responsible for transporting bile acids into the intestinal epithelium, and influences IBABP[115,116], responsible for intracellular transport.

Takeda G-protein receptor 5

Bile acids can also bind Takeda G-protein receptor 5 (TGR5), a G-coupled receptor localized to the cell membrane, inducing the internalization of the receptor[117]. *TGR5* is expressed in various physiological sites, including muscle cells and brown adipocytes[118], and its activation by CA has been noted to increase energy expenditure in brown adipocytes[119]. Furthermore, the observation that TGR5 induced glucagon-like peptide-1 (GLP-1) secretion in mouse enteroendocrine L cells[120] spurred the investigation of its role in glucose homeostasis. Indeed, TGR5 has been observed to improve glucose homeostasis in mice by bile acid-induced release of GLP-1 from enteroendocrine L cells[121].

Other bile acid-binding receptors

Apart from the well-studied bile acid receptors FXR and TGR5, there are a number of known bile acid-sensitive receptors, including vitamin D receptor (VDR), retinoic acid receptor-related orphan nuclear receptor (RORγt), sphingosine-1-phosphate receptor 2 (S1PR2), and pregnane X Receptor (PXR). The effects of the active form of vitamin D, calcitriol, are mediated through its target VDR and are involved in immune system modulation[122]. LCA and 3-oxolithocholic acid (3-oxoLCA) are ligands for VDR, suggesting a role for these bile acids in regulating the immune system[123]. VDR has also been shown to regulate bile acid synthesis by inhibiting *CYP7A1*[124].

RORγt is involved in promoting T_H17-cell differentiation[125] and binds 3-oxoLCA, which has thus been suggested to be an immuno-modulating bile acid. Furthermore, 3β,5α-lithocholic acid (iso-alloLCA) was in the same study shown to be involved in modulating the immune system by increasing regulatory T-cell differentiation[8]. Another study established human gut bacteria that produce 3-oxoLCA and isoLCA, which similarly can suppress T_H17 differentiation by inhibiting RORγt[7]. In the same study, levels of 3-oxoLCA, isoLCA, and 3α-HSDH enzymes responsible for their generation were decreased in individuals with IBD[7]. Interestingly, isoDCA has also been

shown to be involved in the induction of peripheral regulatory T cells[9], further implicating secondary bile acids in immune-regulation.

Along the same lines, LCA and 3-oxoLCA have also been shown to activate the orphan nuclear receptor PXR, regulating bile acid synthesis by repressing *Cyp7a1*[126]. Pregnenolone 16α-carbonitrile has been shown to alleviate hepatotoxicity mediated by LCA in female rats[127]. One study showed that feeding wild type (WT) and PXR-deficient mice LCA (0.125 mg/g) leads to hepatotoxic effects with elevated alanine aminotransferase (ALT), whereas feeding PXR-deficient and WT mice both LCA and pregnenolone 16α-carbonitrile (0.4 mg/g) leads to reduced hepatotoxic effects only in WT mice, implicating PXR in protection against the hepatotoxicity of LCA[126]. The hepatoprotective effect of PXR against bile acid-mediated toxicity is supported by another study that noted that CDCA and DCA activate PXR[128]. PXR thereby serves to detoxify bile acids, xenobiotics, toxic compounds, and harmful metabolites and promote detoxification[129]. Expression of *PXR* is prominent in both the liver and small bowel[130]. PXR binding to the SULT2A1-promoter induces expression of *SULT2A1*, prompting sulfonation of bile acids and detoxification[129]. Additionally, sulfonation primes the bile acid for excretion through the urine, and 40-70% of bile acids excreted through the urine are sulfated[72].

Conjugated bile acids have also been reported to bind S1PR2 and may be involved in the development of cholestasis and cholangiocarcinoma[131,132].

Hence, depending on the bacteria present in the community, the composition of bile acid profiles may shift. Since bile acids function as signaling molecules, microbial transformation of bile acids influences the composition of the bile acid profile, and the resultant bile acid pool may possess varying hydrophobicity and activation properties of bile acid-sensitive receptors.

To recapitulate, bile acids have been demonstrated to bind a multitude of different receptors with many different putative physiological effects. Delineating the effect of binding to individual receptors remains a challenge, and disentangling their combined effects will likely remain a challenge for many years to come.

Bile acid toxicity

Bile acids can be toxic at high concentrations, particularly hydrophobic bile acids, as their detergency may disrupt cell membranes[133]. Their hydrophobicity is affected by conjugation, ionization, and functional groups present. Bile acid ionization is determined by amino acid amidation and the pH of the surrounding environment. Consequently, the hydrophobicity of a bile acid shifts with its ionization in a pH-dependent manner. The hydrophobicity of taurine-conjugated CA, DCA, CDCA, and ursodeoxycholic acid (UDCA) is insensitive to changes in pH between at least ~2-9, remaining ionized across a wide range of pH. On the other hand, the ionization of the glycine-conjugated and unconjugated forms of these bile acids is sensitive to pH changes[134].

When measuring bile acids with ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), functional groups (e.g., hydroxyl groups) are utilized for chromatographic separation, and the mass of the analyte is accounted for, allowing differentiation between different unconjugated and conjugated bile acids. Table 1 shows published indices of Heuman's method of calculating bile acid hydrophobicity.

Table 1 Previously published values for Heuman's bile acid hydrophobicity index. is estimated[135], ** as measured by Poša M[136], and the remaining values are Heuman DM's original observations[134]. Created using BioRender.*

	Taurine conjugate	Glycine conjugate		Unconjugated	
Bile acid	**Ionized**	**Ionized**	**Unionized**	**Ionized**	**Unionized**
ωMCA	- 0.98*				
UCA	- 0.94				
αMCA	- 0.84				
βMCA	- 0.78				
UDCA	- 0.47	- 0.43	- 0.15	- 0.31	+ 0.49
HCA	- 0.45				
HDCA	- 0.35	- 0.30	+ 0.01		
CA	0	+ 0.07	+ 0.3	+ 0.13	+ 0.83
CDCA	+ 0.46	+ 0.51	+ 0.77	+ 0.59	+ 1.37
DCA	+ 0.59	+ 0.65	+ 0.93	+ 0.72	+ 1.46
LCA	+ 1.00	+ 1.05	+ 1.34		
6-oxoLCA**				- 0.35	+ 0.41
7-oxoCA**				- 0.32	+ 0.37
12-oxoCA**				- 0.30	+ 0.40
7-oxoCDCA**				- 0.24	+ 0.50
12-oxoDCA**				- 0.22	+ 0.52

Deoxycholic acid

The hydrophobic bile acid DCA has been shown to induce inflammation in the ileum and colon and alter enterohepatic circulation in mice fed a 0.2% DCA-supplemented diet for 24 weeks[137]. Female mice fed the same 0.2% DCA-containing diet for 24 weeks had an altered gut microbiota, accompanied by intestinal inflammation, altered expression of FXR-controlled genes, and increased total fecal bile acids. Daily gavage for six weeks with the intestine-specific FXR agonist fexaramine normalized the DCA-induced phenotype, and antibiotic treatment for 24 weeks partially abrogated the DCA-induced intestinal inflammation[138]. Another study showed that DCA prevents wound healing *in vitro* and that the prevention may be mediated through FXR. Restoration of biopsy-induced wounds in the colon of mice was inhibited by topical administration of DCA and promoted by topical administration of UDCA[139].

DCA has also been posited to modulate glucose homeostasis in mice, with one study showing that male mice fed a 0.1% DCA-containing high-fat diet for

three weeks led to impaired glucose homeostasis[140]. Regarding the influence of DCA on the liver, it should be considered hepatotoxic at high concentrations. A study in mice showed that DCA-feeding for seven days induces elevated ALT already at 0.01% and that a concentration of 1% is lethal[141].

Lithocholic acid

Already in the 1960s, the hepatotoxicity of the hydrophobic LCA was recognized, and chronic administration of LCA was observed to induce liver damage in rabbits[142], and infusion of tauro-lithocholic acid (TLCA) induced cholestasis in rats[143]. Feeding LCA (1%) to mice induces necrosis of hepatocytes, creating areas known as bile infarcts, destruction of bile ducts in smaller portal areas accompanied by portal inflammation, and periductular fibrosis. Moreover, LCA feeding for four days leads to increased neutrophil infiltration, elevated serum bile acids, bilirubin, and ALT[144–146]. A study in mice evaluating the hepatotoxicity of different concentrations of diet-supplemented bile acids showed that even feeding of low levels of LCA (0.03%) for seven days was hepatotoxic as measured by elevated serum ALT[141].

Interestingly, in contrast to the observed hepatotoxicity, intraperitoneal LCA administration (30 mg/kg) has been reported to reduce intestinal permeability and apoptosis in the intestinal epithelium of a dextran sulfate sodium-induced murine model of intestinal inflammation. Administration of the hydrophilic UDCA (30 mg/kg) also conferred the same protection[147,148]. On the other side of the coin, one study used the PSC *Mdr2*$^{-/-}$ mouse model with severe cholestatic liver disease to evaluate how colitis affects liver disease and demonstrated that dextran sulfate sodium-induced colitis has a protective effect on liver disease by reducing bile acid synthesis[149].

Ursodeoxycholic acid

In contrast to the hydrophobic bile acids DCA and LCA, UDCA has shown hepatoprotective effects[150]. Tauro-ursodeoxycholic acid (TUDCA) has been observed to have anti-apoptotic effects in rat hepatocytes and protects against GCDCA-induced apoptosis, whereas neither tauro-chenodeoxycholic acid (TCDCA) nor glyco-ursodeoxycholic acid (GUDCA) conferred the same protection[151]. Another study evaluating apoptosis suggested that UDCA may protect against DCA-induced apoptosis in rat hepatocytes[152]. The choleretic effect of UDCA (the stimulation of bile secretion from hepatocytes) has been suggested to be by regulation of transporters in the bile canaliculi[153]. Moreover,

since UDCA is a hydrophilic bile acid[134], its role in decreasing the hydrophobicity and cytotoxicity of biliary bile acids could play another vital role in mediating its beneficial effects[153]. It has also been posited that UDCA could promote the secretion of bicarbonate anions from cholangiocytes, cultivating an alkaline pH outside their apical cell membrane and creating an "HCO_3^- umbrella", leading to deprotonation of bile acids, and thus protecting against cytotoxic effects of bile acids[154]. TUDCA has also been studied in genetically induced mouse models of obesity. Specifically, intraperitoneal injections of TUDCA (500 mg/kg/day) to leptin-deficient mice improved glycemic control, steatosis, and insulin and glucose tolerance compared to vehicle-treated leptin-deficient mice. Furthermore, TUDCA improved insulin sensitivity as measured by hyperinsulinemic-euglymic clamps and reduced endoplasmic reticulum (ER) stress[155].

Unlike feeding high concentrations of LCA and CDCA to mice, feeding even 3% UDCA to mice for seven days was not lethal and did not lead to elevated ALT levels[141]. UDCA is also generally safe and well-tolerated in various pathophysiological conditions in humans. Indeed, there is clinical and real-world evidence for the beneficial effects of UDCA as it is used as a pharmacological treatment in PBC[156,157], cholethiasis[158], intrahepatic cholestasis of pregnancy[159], and in PSC[160], albeit with uncertain benefits.

Bile acid hydrophobicity

Despite individual bile acids' detrimental or beneficial effects, the bile acid pool rarely comprises a single or a select few bile acids. It is rather multifaceted, with continuous transformations and excretion of bile acids when passing through the enterohepatic circulation. As a rule of thumb, the hepatotoxicity of bile acids positively correlates with bile acid hydrophobicity, but the feeding of more hydrophobic bile acids may not directly translate to a more hydrophobic bile acid profile due to bacterial and hepatic transformations[141]. Still, the hydrophobicity of bile acid profiles might find use as a proxy for hepatotoxicity. Here, the bile acid hydrophobicity index, as developed by Heuman, may prove useful[30,161,162]. Nevertheless, the lack of published indices limits its use in complex bile acid profiles, such as those found in serum, the small bowel, and the colon[134,136].

Bile acids & gut microbiota: human vs mouse

Bile acids exist in all vertebrates. However, they come in many flavors, and there is a broad diversity with discernible variations in the bile acid pool

between species[163]. Differences in bile acid pools across species challenge human bile acid metabolism studies. Despite the widespread use of murine models in bile acid metabolism research, discrepancies exist between humans and mice concerning bile acid profiles. These differences can likely, at least in part, be attributed to species differences in the gut microbiota composition and, thus, the biotransformation of bile acids. The microbial community in humans and mice is compositionally different, and there are specific evident anatomical and physiological differences, such as the presence of a much larger cecum in mice compared to humans, differences in gastric emptying, and intestinal transit time[164].

One study using 16S ribosomal RNA (16S rRNA) sequencing compared the microbial profile of fecal samples in humans and mice and found 89% shared genera. Therefore, the gut microbiota profiles of humans and mice might seem to be at least qualitatively similar. However, principal coordinate analysis (PCoA) analysis using either the unweighted or weighted UniFrac distance matrix showed that mice and humans cluster separately. Furthermore, the relative abundances of genera largely differed between humans and mice[165]. Another study used whole-metagenome shotgun sequencing to construct a bacterial gene catalog of mouse gut microbiota and compared it to human reference genome databases. They constructed sets of core microbiota found in all samples, and of the top 20 genera, only 13/20 genera were shared between humans and mice. Functional analyses of human and mouse gut microbiota showed a 95.2% functional homogeneity between the two groups. However, there was a mere 4% overlap between mouse and human microbial genes[166], and human and mouse gut microbial profiles differed greatly.

Although gut microbiota undeniably influences bile acid biotransformation, differences in the primary bile acids substantially influence the composition of the bile acid pool of the host. In addition to the primary bile acids CA and CDCA present in humans and mice, mice possess an additional set of primary bile acids: alpha-muricholic acid (αMCA) and βMCA[51,98]. Furthermore, UDCA is categorized as a secondary bile acid in humans but is synthesized by the liver in mice[167,168], thereby classifying it as a primary bile acid in mice[98]. For this reason, considering species-dependent differences, one bile acid may be considered a primary bile acid in one species and a secondary one in another. Moreover, the mouse primary bile acids, αMCA, βMCA, and UDCA, are more hydrophilic than human primary bile acids[98,134]. Another obstacle in extrapolating findings to humans arises from the species-dependent variations in conjugation patterns. In contrast to the human liver, the murine liver

predominantly conjugates bile acids with taurine[71]. Additionally, unlike humans, where the classical pathway is the dominant bile acid synthesis pathway, the classical and alternative pathways produce bile acids in relatively equal amounts in mice[53].

The total sum of the discrepancies between human-to-mouse bile acid metabolism and gut microbiota composition are pitfalls necessary to consider in translational research and probe the need for improved models.

Modeling human bile acid metabolism

Mouse models have been pivotal in advancing the understanding of the interplay between bile acids and gut microbiota, leading to many important insights. Despite their usefulness, owing to human-to-mouse differences in the interplay between gut microbiota and bile acid metabolism, the mouse has limitations as a model of human bile acid metabolism.

Considerable efforts have been made to identify the enzyme responsible for the transformation of CDCA to murine primary bile acids. Recent studies have revealed that CYP2C70 is responsible for the species-specific differences in bile acid metabolism between mice and humans[167]. CDCA is usually not abundant in mice as it is rapidly transformed into muricholic acids (MCAs) by CYP2C70. One study generated hepatic acute *Cyp2c70* knockout mice (*Cyp2c70*^{ako}) by CRISPR/Cas9 genome editing. *Cyp2c70*^{ako} mice exhibited a more human-like serum bile acid profile with decreased proportions of βMCA and omega-muricholic acid (ωMCA) but with increased αMCA. To evaluate whether *Cyp2c70* transforms CDCA to MCAs *in vivo,* an intravenous infusion of d4-CDCA or d5-αMCA was given to *Cyp2c70*^{ako} and WT mice. They only allowed for hepatic first-pass metabolism and collected bile to evaluate the hepatic transformation of the deuterated bile acid species. When given d4-CDCA, most d4-CDCA was unchanged in *Cyp2c70*^{ako} and WT mice. They also observed increased relative levels of d4-αMCA (~17%), d4-βMCA (~2%), and d4-UDCA (~0.3%) in WT mice, whereas *Cyp2c7*0^{ako} had d4-αMCA (~1%), no d4-βMCA, nor d4-UDCA, indicating that CYP2C70 has 6β-hydroxylation activity, and may have weak 7-epimerization activity. Administration of d5-αMCA, in the same manner, netted most of the d5-αMCA unchanged, with increased proportions of d5-βMCA (~2.5%) in WT mice but none in *Cyp2c70*^{ako} mice. No d5-CDCA nor d5-UDCA was identified in WT or *Cyp2c70*^{ako} mice, reinforcing the notion that CYP2C70 has 7-epimerization activity. CYP2C70 thus seems to be able to perform 6β-hydroxylation

producing αMCA from CDCA, and βMCA from UDCA, and to perform 7-epimerization producing βMCA from αMCA, and albeit at a meagre rate produce UDCA from CDCA[168]. Figure 5 illustrates differences in the metabolism of CDCA in humans and mice.

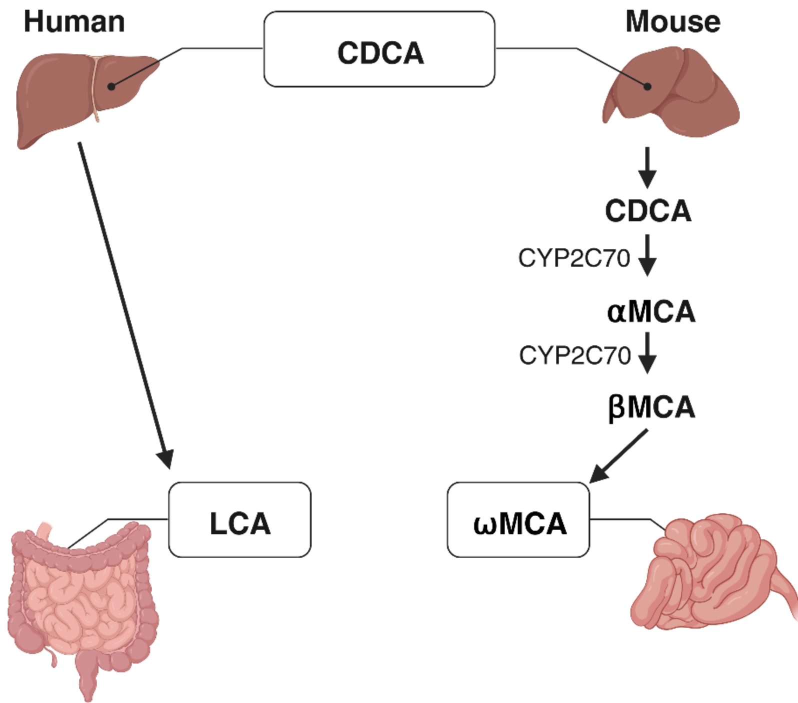


Figure 5 Differences in the fate of CDCA in humans and mice. Left side: human; right side: mouse. Created using BioRender.

The discovery that ablation of *Cyp2c70* in mice leads to a more human-like bile acid profile paved the way for creating constitutive knockout models of *Cyp2c70*. In tandem, several mouse models were generated to capitalize on the newfound understanding that CYP2C70 transforms CDCA into MCAs[161,162,169]. One study generated *Cyp2c70*$^{-/-}$ mice and found that it led to the elimination of the production of MCAs *in vivo*[161]. In some species, bile acids undergo hepatic re-hydroxylation at C7, observable in mice but absent in humans[170]. Another study found that *Cyp2a12* is responsible for 7α-rehydroxylation in the mouse hepatocyte and generated a *Cyp2c70*$^{-/-}$ mouse model and a *Cyp2c70*$^{-/-}$ and *Cyp2a12*$^{-/-}$ dual knockout mouse model. As expected, they found that MCA levels were depleted in both models[162].

Another study found that *Cyp2c70*$^{-/-}$ mice lacked MCAs and had increased levels of TCDCA and TUDCA in bile and serum. Interestingly, 12-week-old and 32-34-week-old female *Cyp2c70*$^{-/-}$ mice had higher levels of total serum bile acids than male *Cyp2c70*$^{-/-}$ mice. Moreover, *Cyp2c70*$^{-/-}$ mice exhibit an age-dependent hepatobiliary phenotype. 3-week-old *Cyp2c70*$^{-/-}$ mice had elevated ALT and aspartate aminotransferase (AST) levels, accompanied by increased hepatic fibrosis and cholangiocyte proliferation, indicating neonatal cholestasis. However, the cholestasis diminished with time, but 12-week-old *Cyp2c70*$^{-/-}$ mice still displayed portal fibrosis and cholangiocyte proliferation, with elevated AST and ALT levels. Moreover, 12-week-old male *Cyp2c70*$^{-/-}$ mice had an altered gut microbiota compared to *Cyp2c70*$^{+/+}$ mice. Interestingly, 32-34 week-old female *Cyp2c70*$^{-/-}$ mice showed increased relative liver weight, spleen weight, total serum bile acid concentrations, and considerable cholangiocyte proliferation and hepatic fibrosis, while male *Cyp2c70*$^{-/-}$ mice did not. UDCA (0.1%) feeding was able to reverse cholangiopathy, elevated hepatobiliary enzyme levels, hepatic gene expression patterns, intestinal barrier dysfunction, and ER stress of *Cyp2c70*$^{-/-}$ females at 5-12 weeks of age[161]. In addition, several studies have reported that *Cyp2c70*$^{-/-}$ mice have hepatic injuries, cholangiopathy, and elevated AST and ALT levels, with a more severe phenotype in *Cyp2c70*$^{-/-}$ females[161,171,172].

PBC is a rare chronic cholestatic liver disease predominant in women where UDCA is used as a first-line treatment[157,173,174]. In fact, *Cyp2c70*$^{-/-}$ mice have been proposed to serve as a model for PBC[161], and the female-predominant phenotype has been suggested to be due to sex-specific discrepancies in *Cyp8b1* expression[175]. The hydrophobic FXR-antagonist obeticholic acid (OCA) has been approved as a pharmacological treatment for PBC in individuals where UDCA proves insufficient[176,177]. This probed one study to feed female *Cyp2c70*$^{-/-}$ mice with OCA (62.5 mg/kg) for four weeks. Although bile acid synthesis decreased, OCA proved insufficient in alleviating the hepatobiliary phenotype of *Cyp2c70*$^{-/-}$ mice[171].

Another strategy to ameliorate the hepatobiliary phenotype of *Cyp2c70*$^{-/-}$ mice may be administering an IBAT inhibitor, as it has shown benefit in other manifestations of cholestasis. *Mdr2*$^{-/-}$ mice are a model of cholestatic liver disease lacking *Mdr2*(*Abcb4*) encoding the canalicular phospholipid flippase, causing a lack of phospholipids in bile[178]. *Mdr2*$^{-/-}$ mice exhibit severe liver disease with periductular fibrosis and cholangiopathy and serve as a model for PSC[179]. Feeding *Mdr2*$^{-/-}$ mice the IBAT-inhibitor A4250 (0.01%) ameliorated sclerosing cholangitis[180]. Another example where IBAT-inhibition has shown

potential is Alagille syndrome, a rare disorder most commonly originating from mutations in *JAG1* and less commonly in *NOTCH2*[181]. Alagille syndrome primarily affects infants and young children who often suffer from severe cholestasis. Administration of the IBAT-inhibitor maralixibat has been shown to decrease serum bile acids and pruritis in Alagille syndrome[182].

It is tempting to postulate that the hepatobiliary phenotype of *Cyp2c70*$^{-/-}$ mice may result from the human-like and vastly more hydrophobic bile acid profile compared to *Cyp2c70*$^{+/+}$ mice. However, one study showed that feeding *Cyp2c70*$^{-/-}$ mice the IBAT-inhibitor SC-435 (0.006%) alleviated hepatobiliary injury and decreased total liver bile acids but did not alter the hepatic bile acid hydrophobicity index when compared to *Cyp2c70*$^{-/-}$ mice on chow[175]. This suggests that the increased hepatic bile acid hydrophobicity index is not solely responsible for the hepatobiliary phenotype but may need to be accompanied by increased levels of total hepatic or circulating bile acids.

To recapitulate, *Cyp2c70*$^{-/-}$ mice have a more human-like bile acid composition devoid of MCAs, display a female-predominant hepatobiliary phenotype, and may be a more physiological-relevant model for understanding bile acid-gut microbiota interplay in the context of human hepatobiliary disease.

Bile acids & gut microbiota in health and disease

At this point, I hope I have been able to convey how vital the gut microbiota and bile acids are to host metabolism. Since the gut microbiota and bile acids are so intertwined in metabolic processes, their interplay profoundly affects health and disease, and their implications in metabolic and hepatobiliary diseases invite a great deal of scrutiny.

Metabolic disease

Metabolic disease is a major public health concern, and obesity has been on the rise since the 1970s[183] and as of 2020, 14% of the world population was estimated to be obese. In 2030, over 1.5 billion individuals are projected to be affected by obesity[184]. Obesity is linked to the development of T2D, and approximately 529 million individuals had diabetes in 2021, with T2D accounting for 96% of cases. By 2050, over 1.31 billion individuals are projected to have diabetes[185].

T2D is characterized by hyperglycemia and insulin resistance[186]. There is a strong correlation between obesity and insulin resistance, and insulin resistance is marked by a blunting of the effects of insulin. Thus, insulin cannot sufficiently suppress hepatic glucose production and has insufficient effects on glucose disposal in skeletal muscle. In the context of insulin resistance, the pancreas compensates for the elevated insulin burden with increased insulin secretion, and β-cell dysfunction occurs when the burden of insulin production is too high[186].

Obesity

Initial reports of the gut microbiota's role in obesity appeared in the early 2000s. Early studies linking the gut microbiota to obesity stem from the observation that GF mice gavaged with cecal content from conventionally raised (CONV-R) mice increase in adiposity despite consuming less food[19]. Several observations connecting the gut microbiota to obesity followed, with one study utilizing a small cohort of individuals who were either obese or lean. Obese individuals had an increased relative abundance of Bacteroidetes and decreased relative abundance of Firmicutes compared to lean individuals[187], supported by a study in genetically obese mice associating obesity with similar relative changes in Bacteroidetes and Firmicutes[20]. However, later research noted inconsistent changes in the relative abundances of Bacteroidetes and Firmicutes in obesity, and the Bacteroidetes: Firmicutes microbial signature does not seem reproducible in larger cohorts[188–191].

However, the gut microbiota has been linked to obesity in other respects. In a large Danish cohort, individuals were stratified by microbial gene richness into either a low- or high-gene-richness group. A lower gut microbial diversity was associated with increased adipose tissue, low-grade inflammation, and metabolic dysregulation, and individuals with a lower microbial richness were inclined to gain more weight[22]. Another study evaluated dietary intervention on the gut microbiota in obese or overweight individuals, similarly stratified into a low- or high-gene richness group. Correspondingly, individuals in the low gene richness group also exhibited metabolic dysregulation that could be improved by dietary intervention. However, the dietary intervention was less effective in improving low-grade inflammation in the low gene richness group[45].

A meta-analysis later re-analyzed ten studies that used 16S rRNA sequencing to investigate the gut microbiota in obesity. Although α-diversity was overall

lower in the pooled dataset, the effect size of the reduction of α-diversity in obesity was low[191].

Another study delved into the relationship between host genetics and the gut microbiota in a large cohort of monozygotic and dizygotic twin pairs using 16S rRNA sequencing[192]. They found that monozygotic twins have more similar gut microbial communities than dizygotic twins as measured by the unweighted UniFrac distance. *Christensenellaceae* was also the most heritable taxon and was more abundant in individuals with lower body weight. Colonizing GF mice with human feces from an obese individual supplemented with *Christensenella minuta* led to a lower weight than without *C. minuta*[192]. Multiple subsequent studies support the heritability of *Christensenellaceae* and have associated it with host metabolic health[193].

However, another study using whole-metagenome shotgun sequencing reported no correlation between ancestry and gut microbial diversity or composition in a large Israeli cohort, replicated in a large Dutch cohort[194]. They also found that relatives who did not share a household did not have similar gut microbial composition. However, genetically unrelated individuals living together had compositionally similar gut microbial communities, pointing to the environment's influence on gut microbiota. Importantly, they report that environmental factors have a much greater impact than genetics on the gut microbiota and that the gut microbiota composition is correlated with host phenotypes, including body mass index (BMI), high-density lipoprotein, cholesterol, and fasting glucose levels[194]. Although the influence of environment factors in molding gut microbial communities overrides genetics, other studies have examined associations between genetics and gut microbiota composition. Researchers examined 24 cohorts differentiated by age, ancestry, processing of samples, and the female: male ratio using 16S rRNA sequencing[195]. They found 31 loci associated with different taxa, although, after strict multiple testing correction, only the lactase gene loci remained, which was associated with Bifidobacterium. Although heritability was concluded to be limited, larger study sizes, coupled with whole-metagenome shotgun sequencing and consistent sample processing across cohorts, might provide greater insight into the heritability of gut microbes[195].

The role of bile acid metabolism in obesity is still unclear, with varying reports of changes in bile acid metabolism in obesity. Consequently, reports of whether conjugated or unconjugated total fasting levels of bile acids are increased or not in obese individuals are mixed, and there are several reports

of associations to insulin resistance[196–199]. However, reports regarding a reduction in the postprandial excursion of serum bile acids in obesity seem more consistent[200,201]. In addition, data supports the involvement of FXR in obesity. In fact, several studies report that lean $FXR^{-/-}$ mice have higher cholesterol levels and poorer glycemic control[202–204].

On the contrary, there are reports that high-fat diet-fed or genetically-induced obese $FXR^{-/-}$ mice are resistant to obesity[205–207]. One study showed that gut microbiota differs between high-fat diet fed CONV-R $FXR^{-/-}$ and CONV-R WT mice, demonstrating that gut microbiota influences adiposity through FXR[205]. Furthermore, another study observed congruent changes in fasting glucose and insulin in high-fat diet-fed $FXR^{-/-}$ mice. However, there was no difference in weight gain between high-fat-fed WT and $FXR^{-/-}$ mice[208]. Gut microbiota from different facilities and breeders differs[209], and the different gut microbial compositions can influence the bile acid profile dissimilarly, which could serve as a potential explanation for the discrepancies between studies.

TGR5 activation improves glucose homeostasis in mice through GLP-1[121], while the FXR-agonist GW4064 has been reported to decrease glycolysis and inhibit GLP-1 secretion in L cells. The same study found that $FXR^{-/-}$ mice have higher GLP-1 secretion after a glucose bolus[210]. On the contrary, some research reports that the activation of both FXR and TGR5 with the dual FXR- and TGR5-agonist INT-767 enhances insulin sensitivity and glucose and lipid metabolism in high-fat diet fed-mice, suggesting interactions between FXR and TGR5 in host metabolism. In fact, activation of FXR induces the expression of *Tgr5*[211]. Expanding on the role of FXR in obesity, studies in mice have shown that deletion of intestinal FXR[212–214], but not hepatic FXR[206], improves insulin resistance and leads to a reduction in weight gain, attenuating diet-induced or genetically-induced obesity.

Moreover, high-fat diet-fed mice administered the FXR antagonist glyco-muricholic acid (G-MCA) demonstrate a reduction in weight gain, suggesting a role for intestinal FXR in mediating adiposity[213]. In addition, activation of FXR with the intestine-specific FXR-agonist fexaramine has been reported to induce TGR5 signaling through changes in gut microbiota. This led to increased GLP-1 secretion and improved glucose and insulin tolerance, while antibiotic treatment abrogated the beneficial effects[215].

Taken together, the link between gut microbiota and obesity is marked by a slightly reduced microbial diversity, and there is a robust link between gut microbiota and metabolic health through *Christensenellaceae*. Data supports the involvement of the bile acid receptors FXR and TGR5 in regulating obesity. However, gaps remain in our understanding of the mechanisms underlying their regulation.

Type 2 diabetes

The established relationship between the gut microbiota and obesity provoked further examination of gut microbes in metabolic disease. A milestone within the field was the establishment of a link between gut microbiota and T2D. A study on a small cohort employing 16S rRNA sequencing revealed that the gut microbiota differs between individuals with T2D and healthy controls[216]. Shifts in gut microbiota were also observed in a sizeable Chinese cohort of individuals with T2D and healthy controls using whole-metagenome shotgun sequencing[26]. Individuals with T2D had an altered gut microbiota, increased abundance of opportunistic pathogens, increased capacity for sulfate reduction, and a decreased abundance of some butyrate-producing bacteria. However, the cohort's characteristics differed in, *e.g.*, sex and age between healthy individuals and individuals with T2D[26]. A well-controlled study encompassing 70-year-old European women with T2D, impaired glucose tolerance, or normal glucose tolerance focused on metagenomic characterization[27]. Functional analyses revealed potential functional alterations in individuals with T2D, including pathways involved in starch and sucrose metabolism and fructose and mannose metabolism. Notably, metformin and the degree of glucose control were associated with some species' gene functions[27], an observation later supported by several studies evaluating the impact of metformin on the gut microbiota in humans and mice[29,217–219]. In treatment-naïve individuals with T2D, metformin was later shown to induce shifts in the gut microbiota, partly mediating some of its beneficial effects[220]. Furthermore, plasma bile acids, but not fecal bile acids levels, had significantly larger increases after four months of metformin treatment compared to placebo. The same study observed an increase in the abundance of the bacterial gene *bsh* after two months of metformin treatment, potentially leading to an increase in unconjugated bile acids. Along the same lines, unconjugated bile acid levels were negatively correlated with a change in glycated hemoglobin (HbA1c) in metformin-treated individuals[220].

A subsequent study implicated bile acids in mediating the effects of metformin, showing that metformin treatment in individuals with T2D decreased the

abundance of *Bacteroides fragilis* and increased levels of unconjugated and FXR-antagonizing taurine- and glycine- UDCA. Intestinal FXR was required for the beneficial effects of metformin in mice, and administration of *B. fragilis* to mice counteracted the effects of metformin. Interestingly, gavage of GUDCA improved glucose tolerance in mice with diet-induced obesity[221].

Since modulation of the gut microbiota and bile acid profiles are implicated in the beneficial effects of metformin, its use should be considered a confounder in metagenomic studies of the gut microbiota in T2D[29]. However, a study considered prediabetic individuals that were not ordinated metformin. They characterized the gut microbiota by 16S rRNA sequencing and found that treatment-naïve prediabetic individuals had a lower abundance of *Clostridium*, *Akkermansia muciniphila*, and some butyrate-producing bacteria[28]. To resolve the issue of metformin confounding results, another study using whole-metagenome shotgun sequencing investigated whether the gut microbiota is altered in treatment-naïve individuals with impaired glucose tolerance, a combination of impaired glucose tolerance and impaired fasting glucose, or T2D[25]. Changes in the gut microbiota were associated with insulin resistance. Moreover, the gut microbiota was altered in individuals with impaired glucose tolerance, impaired glucose tolerance and fasting glucose, and T2D, but not in individuals with impaired fasting glucose. Potential butyrate producers included *Faecalibacterium*, *Roseburia*, and *Alistipes,* which harbor genes for butyrate synthesis. The relative abundance of microbial genes involved in butyrate synthesis was altered in individuals with prediabetes and T2D but not with normal glucose tolerance[25].

On the flip side, changes in bile acids profiles in T2D are unclear. Whether conjugated or non-conjugated total fasting levels of serum bile acids are increased in individuals with T2D is still unsettled[196–199]. The postprandial excursion of serum bile acids in individuals with T2D has less consistent reports than those in obese individuals. When comparing individuals with T2D versus obese individuals, there are reports of increased postprandial bile acid excursions[222,223], but also reports of unchanged postprandial bile acids[224] in individuals with T2D. The varying and somewhat contradictory reports of changes in bile acid profiles could, equivalently to the gut microbiota, be impacted by the degree of insulin intolerance. Adding to that, in a cohort of non-diabetic individuals, the insulin resistance as measured by a euglycemic insulin clamp correlated with increased plasma 12α-OH bile acids. Moreover, the 12α-OH: non-12α-OH bile acid ratio was linked to several insulin resistance metrics, including greater insulin levels, glucose, glucagon, and

triglycerides. When they examined plasma from individuals with T2D, they observed higher total bile acids levels but no increase in 12α-OH bile acids. However, a constraint to consider is that the individuals with T2D were not treatment-naïve and used anti-diabetic agents[199].

Since the gut microbiota is altered in T2D and generates secondary bile acids, it is conceivable that bile acids could be used to project the risk of developing T2D. A five-year longitudinal investigation into the matter showed that although the serum hyocholic acid (HCA): CDCA ratio was associated with BMI, homeostatic model assessment for insulin resistance (HOMA-IR) and HbA1c in prediabetic individuals, it was not useful for predicting the risk of developing T2D. Neither did they observe any correlation between insulin resistance and 12α-OH bile acids, but a potential explanation might be that insulin resistance was measured by HOMA-IR and not by euglycemic insulin clamps[225].

Remarkably, HCA, a primary bile acid in pigs[163], was shown to have anti-diabetic effects by promoting improved glucose homeostasis. Specifically, HCA improved glycemic control through GLP-1 release in mouse models of genetically-induced obesity and in high-fat diet-fed $TGR5^{+/+}$, but not $TGR5^{-/-}$ mice[226]. In a small cohort of healthy-, prediabetic-, or drug-naïve diabetic individuals, levels of some 6α-OH bile acids were associated with improved glycemic control[226]. Modulation of the gut microbiota to increase 6α-OH HCA-species, including HCA, hyodeoxycholic acid (HDCA), glyco-hyocholic acid (GHCA), glyco-hyodeoxycholic acid (GHDCA) is thus an attractive prospect. One study in mice showed that supplementation of the fiber oligofructose in a Western-style diet increased levels of 6α-OH bile acids and that the 6α-OH HDCA improves glycemic control through TGR5[227]. Supporting the role of 6α-OH HCA-species in glycemic control, one study examined four separate cohorts and identified that poorer metabolic outcomes were associated with lower levels of 6α-OH HCA-species[228]. More specifically, in a cohort of healthy lean-, healthy overweight/obese-, or drug-naïve overweight/obese individuals with T2D, the 6α-OH HCA-species were decreased in serum in both overweight groups compared to healthy lean individuals. Furthermore, in a separate cohort, HCA-species were decreased in serum and feces of prediabetic- and drug naïve diabetic individuals compared to healthy controls[228], strengthening the link between HCA-species and glycemic status.

Bariatric surgery as a treatment

Bariatric surgery offers a potential solution to two global health concerns: obesity and T2D. Bariatric surgery refers to surgeries that lead to weight loss in obese individuals, and they induce remission in over 75% of individuals with T2D[229]. The most common bariatric surgery type worldwide is sleeve gastrectomy (SG), followed by Roux-en-Y gastric bypass (RYGB)[230,231], which constitute very different procedures. SG is achieved by removing a large portion of the stomach (~80%) while leaving the intestine intact. In contrast, RYGB is carried out by reducing the stomach area accessible to food and connecting the intestine to the stomach. This leads to a bypass of 95% of the stomach, the duodenum, and part of the jejunum[232].

GLP-1 agonists, such as semaglutide, or dual agonists of GLP-1 and glucose-dependent insulinotropic polypeptide, such as tirzepatide, have recently shown great promise in treating obesity[233,234] and T2D[235,236], and the dual agonist tirzepatide may approach a weight reduction similar to bariatric surgery. However, they are not without drawbacks, and treatment with tirzepatide[234] or semaglutide[233] is reported to decrease lean mass. In addition, a cause for concern is that in individuals diagnosed with T2D, GLP-1 agonist treatment has been associated with an increased risk of experiencing major cardiovascular adverse events compared to those treated with bariatric surgery[237].

Since bariatric surgery is an umbrella term for different surgical procedures, it is important to consider that different mechanisms may induce weight reduction. With that said, there are discrepancies in reports on how bile acid signaling is involved depending on the surgery. RYGB has been shown to increase fasting serum levels of total bile acids and FGF19[238] and has been reported to increase fasting serum bile acids[239] and FGF19 levels, and changes have been associated with T2D remission[240]. RYGB has also been reported to increase postprandial bile acid levels and FGF19, which is not seen in medical management[241]. However, there are also opposing reports of changes in bile acid and FGF19 levels[224]. In addition, there are reports that bile acid excursions are reduced in obese individuals compared to lean controls[200,201], which increase after RYGB[201]. Since bariatric surgery alters the bile acid composition and increases total bile acid levels, bile acid binding receptors may partly mediate the beneficial effects of bariatric surgery.

Indeed, in mice, FXR has been shown to contribute to the beneficial effects of SG[242]. Although SG and RYGB fall under the bariatric surgery umbrella, they

are distinctly different procedures. Consequently, in mice, FXR has been reported not to be required for body weight reduction after RYGB. However, FXR signaling was required to mediate improvements in glycemic control that were independent of body weight[243]. Both RYGB and SG reduce body weight and improve glycemic control in murine models of GLP-1 signaling deficiency[244,245]. Moreover, it has been reported that TGR5 is not required for body weight reduction after SG[246]. However, TGR5-deficiency led to an altered cecal microbiota profile and altered fasting serum bile acid levels. SG-operated TGR5-deficient mice had poorer glycemic control than SG-operated WT mice, suggesting that TGR5 may still mediate improvements in glycemic control, possibly through shifts in the bile acid profile[246]. However, another study in mice reported that TGR5 is involved in weight reduction after SG[247]. Furthermore, SG led to similar alterations in serum bile acids in both WT and TGR5-deficient mice[247], leaving the role of TGR5 in SG uncertain. Shifting focus to RYGB instead, one study found similar weight reduction after RYGB in WT and TGR5-deficient mice but with no effect of TGR5 on glycemic control. Their interpretation was that TGR5 is redundant for the beneficial effects of RYGB[248]. However, bile acids were not measured in this study. In contrast, other studies indicate that TGR5 may mediate beneficial effects on glycemic control in humans[228], and consistent with the link between decreased HCA-species and T2D, HCA-species have been reported to be increased in a small cohort of individuals that underwent RYGB. Notably, levels of HCA-species were shown to vary with remission of T2D[228].

RYGB has also been implicated in modulating gut microbiota in humans, with one study showing long-term changes in gut microbiota compared to pre-surgery BMI-matched, severely obese individuals[249]. The RYGB group exhibited increased postprandial total bile acid levels and FGF19. Transplantation of human feces to GF mice decreased fat gain in mice receiving microbiota from individuals that underwent RYGB, compared to mice that received microbiota from pre-surgery BMI-matched severely obese individuals[249]. Another study examined the gut microbiota of severely obese individuals applying whole-metagenome shotgun sequencing. They revealed that individuals had a decreased microbial gene richness prior to RYGB, which increased after surgery and was accompanied by changes in gut ecology and the serum metabolite profile. However, although microbial richness increased, it remained low one year after RYGB[250].

To recapitulate, bariatric surgery induces weight loss and T2D remission and modulates bile acid metabolism and gut ecology. The metabolic benefits of

bariatric surgery have been linked to 6α-OH bile acids. However, underlying mechanisms are poorly understood, and gaining mechanistic insight may open the door to non-surgical treatments that mimic the beneficial effects of bariatric surgery.

Hepatobiliary disease

Apart from metabolic disease, changes in the gut microbiota have also been linked to hepatobiliary disease. This section is restricted to describing the hepatobiliary diseases PSC and PBC.

Primary sclerosing cholangitis

PSC is an uncommon, chronic liver disease commonly presenting between 30 and 40 years of age and is often accompanied by jaundice, pruritis, and cholestasis[251,252]. Characteristics of PSC are inflammation and fibrosis within the extrahepatic and intrahepatic bile ducts. As time progresses, PSC can culminate in the development of end-stage liver disease and liver malignancies[251,252]. Development of PSC has been considered male-predominant. However, the male predominance of the disease has been questioned, and one study found that 2/3 of newly diagnosed individuals were female. However, PSC in females is associated with a better prognosis and may more often be subclinical[253].

UDCA has been proposed as a treatment for PSC[160]. High doses of peroral UDCA (28-30 mg/kg/day) have been shown to decrease liver injury enzymes but increase the risks for cirrhosis, varices, cholangiocarcinoma, liver transplantation, and death[254]. Hence, the arguments for using high doses of UDCA in PSC seem dubious. Lower doses of UDCA (13-15 mg/kg/day) have also been evaluated, and one study observed a decrease in AST and alkaline phosphatase but with no improvement in time to treatment failure[255]. However, data is inconsistent between studies, and some studies report a decrease in liver injury enzymes and improvement in clinical outcomes, while others observe no improvements[160]. Thus, the benefit of UDCA in PSC is questionable, and there is currently no available pharmacological treatment that alters the trajectory of the disease. As a result, the only treatment with curative potential is liver transplantation[256]. Nonetheless, liver transplantation is not without limitation, and post-transplantation, ~20% of individuals have a recurrence of PSC[253,257].

PSC is tightly coupled to changes in the gut microbiota, and depending on geographical location, 20-80% of individuals suffering from PSC also have IBD, most commonly in the form of ulcerative colitis[256,258]. Delineating cause and effect in the link between gut microbiota and PSC has proved difficult, in part due to comorbidities, stage of liver disease, dietary habits, and use of therapeutic drugs and antibiotics[256]. However, individuals with PSC have an altered gut microbiota compared to healthy controls, particularly marked by decreased α-diversity. Across several studies, the relative abundance of *Veillonella*, *Streptococcus*, and *Enterococcus* has been seen to be increased, and data supports an association of *Veillonella* with the disease stage[256].

With gut microbes responsible for generating secondary bile acids, it is conceivable that bile acids play a role in PSC. Studies have shown that the lack of microbiota in the *Mdr2*$^{-/-}$ PSC mouse model aggravates liver damage[32] and increases hepatic bile acids, which could be improved by administration of the FXR-agonist GW4064, inhibiting CYP7A1 and bile acid synthesis[259]. However, in a human cohort of PSC, individuals with PSC with C4 levels under 6.05 nmol l^{-1} had reduced survival and a 7.9 times higher risk of death or liver transplantation. The differences in C4 and survival might be explained by disease staging and depend on the hepatic capacity for bile acid production[259].

The NOD.c3c4 mouse is a biliary inflammation model used to model PBC but has been suggested to be a workable model of PSC due to their biliary inflammation phenotype. Notably, one study found that CONV-R NOD.c3c4 mice had increased relative liver weight, increased portal inflammation, and increased T cell, neutrophil, and macrophage-positive cells around bile ducts compared to GF NOD.c3c4 mice, indicating a role of the gut microbiota in mediating biliary inflammation in NOD.c3c4 mice[260]. Moreover, data indicates that biliary bile acids are altered in individuals with PSC compared to healthy controls[261], but fecal bile acids are not altered between individuals with PSC-IBD and individuals with IBD[262].

The etiology of PSC is still unknown, and the link between the gut microbiota and PSC remains poorly understood, with a potential role of bile acids. Whether there is a causal relationship between alterations in gut microbiota and the onset of PSC is elusive. The lack of treatment options and the severity of the disease emphasize the urgency for new therapeutic strategies and a deeper understanding of the pathophysiology of PSC.

Primary biliary cholangitis

PBC is a chronic cholestatic autoimmune liver disease. It affects the intrahepatic bile ducts, leading to the progressive destruction of bile ducts and cholestasis[263]. The prevalence of PBC is considerably higher than PSC and is the most prevalent autoimmune liver disease worldwide. PBC has a female predominance and typically presents at ~ 40-60 years of age[264]. While the role of UDCA in PSC is debated, UDCA (13-15 mg/kg/day) substantially improves transplant-free survival in individuals with PBC[157].

The gut microbiota is altered in PBC, and one study using 16S rRNA sequencing compared gut microbiota between UDCA-naïve individuals with PBC and healthy controls. The α-diversity was lower in individuals with PBC, and the microbial composition differed from healthy controls. The relative abundance of twelve genera, including *Veillonella* and *Streptococcus,* differed between groups, and UDCA treatment restored six of these genera to levels of healthy controls[36].

Another study evaluated the gut microbial communities in UDCA-treated individuals with PBC according to response to treatment, assessed by a reduction of gamma-glutamyl transpeptidase ≥ 69% after one year of UDCA treatment. Responders and non-responders differed by relative abundance of the *Faecalibacterium* genus[265]. Use of the bile acid sequestrant colesevelam in PBC was also linked to changes in the relative abundance of two *Lachnospiraceae* species, and *Klebsiella pneumoniae and Roseburia intestinalis*. Moreover, individuals with greater remission (as measured by a greater change in the proportion of serum bilirubin than the median) of PBC exhibited increases of the two *Lachnospiraceae* species and SCFAs levels[266].

In-depth profiling of bile acids has been done in UDCA-naïve PBC, observing increased serum total bile acid levels without changes in fecal bile acid excretion. that total bile acid levels were increased in serum without changes in fecal bile acid excretion. Moreover, individuals with PBC displayed increased proportions of primary and glycine-conjugated bile acids in serum. Total serum bile acids were increased with the disease stage, with an increased proportion of primary bile acids and an increased conjugated: unconjugated ratio. Likewise, serum FGF19 was increased in individuals with PBC and increased with disease staging[267].

Taken together, bile acids and the gut microbiota have been shown to be altered in PBC and potentially play a role in its pathogenesis.

Summary

Microbiota widely colonize our body, and the gut microbiota profoundly affects host physiology and plays a pivotal role in many metabolic processes. The gut microbiota produces secondary bile acids, and an altered microbial composition may shift the composition of the bile acid pool. Hence, there is a reciprocal relationship between gut microbiota and bile acids. Both gut microbiota and bile acids have been implicated in health and disease, although we are still in the dark regarding underlying mechanisms and potential causal relationships in humans.

A deeper understanding of the interplay between bile acids and gut microbiota in health and disease might open new avenues for treating metabolic and hepatobiliary diseases.

Aim

This thesis aims to clarify the interplay between gut microbiota and bile acid metabolism in metabolic and hepatobiliary disease. The specific aims of the thesis are outlined below.

Specific aims of the thesis:

1. Elucidate the effect of the low-abundant bacterial species *Clostridium scindens* on DCA production and the effects of DCA on host glucose metabolism (**Paper I).**
2. Clarify how bariatric surgery alters postprandial bile acid kinetics (**Paper II)**.
3. Investigate how the human and mouse gut microbiota affects bile acid metabolism and liver pathology in a mouse model with a human-like bile acid profile. (**Paper III)**.

Methodological considerations

Studies of the interplay of bile acids and gut microbiota require a set of diverse techniques. This chapter outlines methodological considerations and offers a brief explanation of methods for the papers in this thesis. Details about specific materials and methods of each paper can be found in the Appendix.

Profiling of gut microbiota

The development of next-generation sequencing has opened up a completely different approach to studying gut microbiota, generating a large amount of data and providing information on a scale that was previously inaccessible[268]. We used two methods based on high-throughput sequencing to profile the gut microbiota, and microbial communities were analyzed by 16S rRNA sequencing or whole-metagenome shotgun sequencing. Profiling of the gut microbiota by 16S rRNA sequencing is more cost-effective than shotgun metagenomics but does not allow for as great resolution[269]. However, 16S rRNA sequencing still allows for the analysis of complex bacterial communities in the gut metagenome. It is based on the utilization of the 16S rRNA gene of ribosomes in bacteria and archaea for identification[270].

The 16S gene comprises nine hypervariable regions (V1-V9)[271]. One or several subregions are sequenced, such as V4, V6, or V1-V3. Using the 16S rRNA for metagenomic studies still has pitfalls, and accurate taxonomic resolution is generally reserved for the genera level. Sequencing the whole 16S gene provides better resolution than using a select region[272]. In paper III, we used the V4 region for identification. Although the V4 region provides lower resolution than the full 16S gene[272], one study reported V4 having better sensitivity for taxonomic assignment than other single hypervariable regions[271]. Moreover, we utilized DADA2's assignTaxonomy()[273,274], which uses the ribosomal database project classifier for the taxonomic assignment and exhibits superior classification accuracy in the V2 and V4 region[275].

16S rRNA sequences are often clustered into operational taxonomic units based on a 97% sequence similarity[276]. However, recent development has led to the formulation of amplicon sequence variants based on an exact sequence match instead. In part due to their improved resolution and ability to merge datasets from different experiments, it has been suggested that amplicon sequence variants should replace operational taxonomic units as the

convention for 16S rRNA profiling[277]. For paper III, we used amplicon sequence variants. It is important to keep in mind that the entire genomes are not sequenced with 16S rRNA sequencing, and therefore, it does not provide potential function profiling of the metagenome. Although there are tools for inferring functional profiling[278,279], predicting potential functions may produce deceptive results.

In contrast, whole-metagenome shotgun sequencing is based on sequencing all DNA present in the sample, allowing for functional profiling of the metagenome[280]. In paper I, we introduced a well-defined bacterial community and used whole-metagenome shotgun sequencing to resolve gut microbes at the species level. Although whole-metagenome shotgun sequencing allows for better resolution, it is more time-consuming and less cost-efficient. To evaluate the role of the gut microbiota in, *e.g.*, mouse phenotypical characteristics, the resolution obtained from 16S rRNA sequencing allows for adequate gut microbial profiling, including identifying differences between groups by microbial diversity. For these reasons, we used 16S rRNA sequencing in paper III.

A plethora of different metrics exist for the measurement of microbial diversity. As a reminder, α-diversity measures the richness of the microbial community, while β-diversity can be used to compare diversities between gut microbial communities and measure dissimilarities between communities[43]. Standard metrics for measuring the α-diversity include Richness, Phylogenetic diversity, Chao1, ACE, Shannon's index, and Simpson's index, with the latter two accounting for both microbial richness and evenness. Hence, Shannon's and Simpson's index include information on the number of times each taxon was detected in the community[43]. Differences in β-diversity can be tested with Permutational multivariate analysis of variance (PERMANOVA). PERMANOVA partitions variation in fractions according to a defined dissimilarity measure such as Bray-Curtis or UniFrac. It can deal with zero-inflated data and does not make assumptions based on the distribution of variables or the defined dissimilarity metric[281]. Due to these qualities, it is suitable for use in studies of gut microbiota where many taxa might not be present in all samples, and we used PERMANOVA to test differences in β-diversity in gut microbial communities.

Mouse experimentation

One of the most common ways of modeling human bile acid metabolism is with inbred strains of *Mus musculus*, also known as the house mouse. This preference can be ascribed to their short gestation time, extensive phenotypic characterization and genetic similarity to humans, the complete sequencing of the mouse genome, and the availability of genetically modified mouse strains[282].

GF mouse models have been employed to investigate the role of gut microbiota in bile acid metabolism and have been a significant asset in this respect, enabling the uncovering of details in the interplay between bile acids and gut microbiota. GF mice are born and raised in a germ-free environment, completely devoid of microorganisms[283]. Their lack of bacteria means they cannot deconjugate nor metabolize primary bile acids into secondary ones. Consequently, GF mice allow for comparative studies between mice devoid of microbiota and those either mono-colonized or colonized with a community of microbes, e.g., a fecal microbiota transplantation (FMT) from a human donor.

Studies have assessed how the microbial composition and bile acid profile differ following colonization of GF mice with a human- or mouse-derived microbiota. One study transplanted human fecal or mouse cecal samples to GF mice and utilized 16S rRNA sequencing. They noted a difference in β-diversity as a function of the weighted UniFrac distance with a greater dissimilarity between human donor samples and recipient mice, compared to mouse donor samples and corresponding recipient mice. Moreover, mice that received a transplant of human fecal samples generated fewer secondary bile acids than those that received mouse-derived microbiota[284]. Of note, 6β-epimerization of βMCA is the typical route for the production of the normally abundant ωMCA[285], and two experiments colonizing mice with human gut microbiota from separate donors only found low cecal levels of ωMCA. This suggests that mice colonized with human gut microbiota may have a universally reduced capacity to perform 6-epimerization and produce ωMCA[284].

Another study gavaged human feces to GF mice fed a low-fat diet rich in polysaccharides. At seven weeks of age, mice were either switched to a Western-style diet or maintained on the low-fat diet for eight weeks. They sampled feces for analysis with 16S rRNA sequencing. The first generation of mice transplanted with human feces shared 88% similarity to the original donor sample regardless of diet, with a loss of eight genera present at low abundance

in the human fecal sample. Generational transfer of the microbial profile was possible without a loss of α-diversity measured by the Shannon and Chao1 index, although the succeeding generation only shared a 73% similarity of genera with the first generation[286]. These studies suggest that gut microbiota can successfully be transplanted into GF mice, albeit with reduced microbial diversity.

In paper I, we performed bacterial transplantations of a simplified microbial community with or without the 7α-dehydroxylating bacteria *C. scindens* to GF mice to evaluate its effect on metabolic output. Similarly, in paper III, we performed bacterial transplantations of human feces or cecal content from a CONV-R mouse to evaluate the role of the gut microbiota in the *Cyp2c70*$^{-/-}$ mouse model with a human-like bile acid profile.

In spite of efforts to produce an improved human bile acid metabolism model, there are still limitations with the *Cyp2c70*$^{-/-}$ mouse model. For one, *Cyp2c70*$^{-/-}$ mice do not have glycine-conjugated bile acids. The anatomical differences and the discrepancy between mouse and human gut microbial profiles remain a challenge and need to be accounted for when translating results into a human context. Moreover, although *Cyp2c70*$^{-/-}$ mice have a human-like bile acid profile, they suffer from transient cholestasis and cholangiopathy, which might make them more suitable as a model of cholangiopathy rather than providing insight into human bile acid signaling dynamics.

Quantification and analysis of bile acids

Bile acid quantification can be performed with untargeted metabolomics as a hypothesis-generating experiment to detect and identify all metabolites in a sample, not just bile acids. Quantification is commonly done using liquid chromatography-mass spectrometry-based methods. However, untargeted metabolomics does not provide absolute quantification[287], and another option is targeted quantification of bile acids. This is possible through a number of different methods, including commercially available kits, enzyme-linked immunosorbent assay, and nuclear magnetic resonance. These methods suffer shortcomings concerning either sensitivity or specificity. For targeted bile acid quantification, UPLC-MS/MS is, albeit expensive, a very sensitive and specific method. Given these attributes, its popularity is unsurprising[288]. Targeted quantification is limited by the requirement of prespecified molecular weights, e.g., by selecting 514.2 Da in Q1 and 79.8 Da in Q3, only ions that apply to our criteria reach the detector. Hence, we miss potentially interesting

metabolites with different masses. That the gut microbiota was able to produce the microbially conjugated bile acids tyroso-CA, phenylalano-CA, and leuco-CA was not discovered until 2018[94], and there are likely additional important bile acid species and bile acid conjugations that we are not aware of at present. Future studies would benefit from including as many bile acid standards as possible. However, an upside is that, unlike untargeted approaches, we detect absolute concentrations and not merely the relative peak intensity between samples[287].

Apart from liquid chromatography-based methods, gas chromatography-mass spectrometry methods for detecting bile acids have been in use since 1960[289]. However, the throughput of gas chromatography-mass spectrometry-based methods for quantification is lower since methods involve derivatization. Moreover, the difficulty in identifying stereoisomers hampers the use of gas chromatography-mass spectrometry for bile acid quantification[288].

In paper I, we quantified bile acids in the cecum and vena cava from mice and plasma and feces from individuals with normal glucose tolerance or with T2D with UPLC-MS/MS. We quantified bile acids in CA-supplemented bacterial cultures with gas chromatography-mass spectrometry.

In paper II, we measured bile acids with UPLC-MS/MS in the plasma and feces of individuals before and after bariatric surgery and examined the response of bile acids after a mixed-meal test (MMT). We included measurements from seven different time points over a span of 120 minutes, and we examined the kinetical response and changes in bile acids. Since the response in plasma bile acid levels may differ between individuals, with respect to peak concentrations, timepoint of peak concentrations, and differences in fasting concentrations, we adopted a few core metrics to facilitate bile acid analysis. Still, similar metrics are common, and analogies can be made to pharmacokinetic drug administration studies, where C_{max} is analogous to the maximum concentration, and the timepoint for maximum concentration is analogous to t_{max}[290].

In paper III, we compared the composition of bile acid profiles between GF, humanized (HUM), and conventionalized (CD) *Cyp2c70*$^{-/-}$ mice and examined the proportion of individual bile acids. The production of UDCA might occur by bacterial 7-oxidation of CDCA generating the intermediate 7-oxo-chenodeoxycholic acid (7-oxoCDCA), with subsequent reduction to UDCA[291]. Thus, increasing proportions of UDCA likely reflect a decrease in the

proportion of CDCA. Furthermore, the proportions of bile acids are compositional and not independent, and bile acid proportions covary with the abundance of taxa. It follows that we cannot distinguish between the effect of a decrease in TCDCA and an increase in TUDCA or tauro-cholic acid (TCA). Figure 6 shows an example of the obstacle when examining how the proportions of bile acids vary with the abundance of a bacterial taxon.

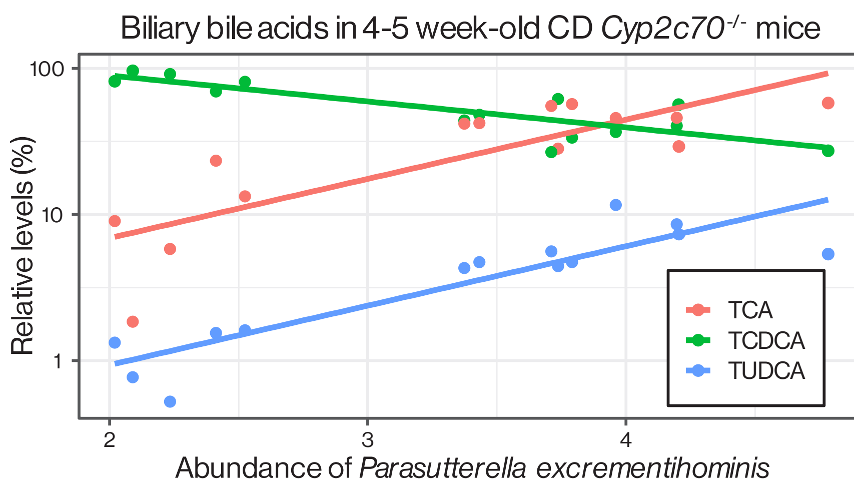


Figure 6 Change in biliary TCA, TCDCA, and TUDCA proportions with varying centered log-ratio abundance of Parasutterella excrementihominis in 4–5-week-old conventionalized Cyp2c70$^{-/-}$ mice.

To circumvent this issue, we calculated and examined the biliary hydrophobicity index as it accounts for the composition of the biliary bile acid profile. A drawback of treating bile acids as an index is that delineating how a specific bile acids covaries with the abundance of a bacterial taxon is not possible. Instead, we identify how the bacterial taxon varies with the hydrophobicity. The bile acid hydrophobicity index was calculated using Heuman's method by summing every bile acid's mole fraction multiplied by its respective hydrophobicity index[134].

Bile acid quantification by UPLC-MS/MS

Samples were extracted and diluted to avoid detector saturation and to fit in the mass spectrometer's dynamic range. Bile acids were separated chromatographically on a reverse-phase C18 column at a flow rate of 0.4 L/min at 60°C over 20 minutes. Mobile phase A consisted of water with 7.5 mM ammonium acetate and 0.019% formic acid, and mobile phase B consisted of

acetonitrile with 0.1% formic acid. The chromatographic separation is initiated with isocratic elution, and the portion of the mobile phases is then shifted over time. Since elution depends on the polarity of the analyte, the gradient is altered over time with increasing B phase to elute less polar analytes. Table 2 demonstrates the portion of each mobile phase under the 20-minute program.

Table 2 Gradient for separation of bile acids by chromatography for quantification of bile acids. Created using BioRender.

Time (min)	A phase (%)	A phase (%)
0	80	20
1	80	20
5	65	35
14.5	5	95
14.6	0	100
18.5	0	100
18.6	80	20
20	80	20

During chromatographic separation, bile acid analytes are eluted from the column, ionized by electrospray ionization, and analyzed with multiple reaction monitoring covering expected retention times in negative mode on a QTRAP 5500 System (SCIEX).

Samples were spiked with deuterated bile acids and measured, and internal standards were chosen to be as similar as possible to the measured analyte. As an example, d4-CDCA or d4-UDCA are appropriate as an internal standard for DCA as they both have two hydroxyl groups. Analytes were quantified against internal standards using an external calibration curve for adjustments.

After data acquisition, the area of bile acids was integrated with MultiQuant in chromatograms using the MQ4 (Paper I & II) or the SignalFinder[292] (Paper III) algorithm with manual integration adjustments when necessary. The SignalFinder algorithm was implemented to broaden the linear range by adjusting for detector saturation. Measured values were then multiplied by the dilution factor and adjusted for sample weight when applicable (*e.g.,* feces, gallbladder, liver).

Imaging

In paper III, we performed histology and immunohistochemistry to evaluate cholangiocyte proliferation and hepatic fibrosis. Although these analyses can be done biochemically, we decided to adopt a microscopy-based approach to assess the extent and distribution of fibrosis and cholangiocyte proliferation in liver tissue.

All analysis and quantification of images were done with Python and Napari[293]. ImageJ and Fiji are powerful tools for image analysis. However, Napari is integrated into the Python ecosystem and also allows for the use of well-matured libraries for image analysis, such as scikit-image and SimpleITK. A conda environment with Python (3.9.15) was set up for package management. The napari (0.4.17)[293] and the devbio-napari (0.8.1)[294] packages were installed. Images were obtained as .tiff files and analyzed using Napari headlessly. In order to avoid subjectivity associated with manual segmentation, analysis was done using in-house developed automated methods.

Sirius red

Increased collagen deposition functions as a marker for fibrosis and Sirius Red is used to stain collagen red and other structures yellow, enabling qualitative and quantitative investigations into the degree of hepatic fibrosis[295]. We stained liver sections for collagen deposition with Sirius Red (Direct Red 80), and images were taken with a 10x objective with bright-field microscopy on an Apotome Axioplan 2 (Zeizz). Sirius Red also stains basement membranes, and in some cases, using polarized light increases specificity as stained basement membranes do not exhibit birefringence. However, if using plane-polarized light, it might be necessary to rotate the microscope stage and capture several images to visualize all collagen fibers, hampering subsequent quantification[295]. This might be solved by using circularly polarized light, and using polarized light may be crucial in certain tissues such as skin[296]. However, bright field microscopy without polarized light of liver sections is common[259,297], but choosing the correct dye for collagen staining is crucial, as other Sirius Red dyes may differ in specificity and stain differently.

Images were acquired, loaded, and represented as NumPy arrays. Analysis of images was performed by first training a pixel classification model, and random forest decision trees were trained on a subtracted image for semantic segmentation with three classes, and the trained model was applied to all images obtained. The three classes corresponded to collagen fibers, white

space and vessels, and yellow-stained tissue. A map of the segmented area corresponding to the collagen class was obtained, and segmented pixels were counted. All segmentation maps were manually controlled to ensure the quality of segmentation.

Cytokeratin 19

Cholangiocyte proliferation, also called ductular reaction, occurs after liver injury and entails the formation of small bile ducts[298]. Cytokeratin-19 (CK19) is expressed in cholangiocytes and functions as a marker[299]. Sections of livers from mice were stained for CK19, and images were obtained with a 10x objective on an Eclipse Ni-E (Nikon) using fluorescence microscopy.

Images of CK19 stained sections were acquired, loaded, and represented as NumPy arrays. However, compared to the analysis of Sirius Red staining, the anti-CK19 antibody produced appreciably significant contrasts between stained and unstained areas. The sequence of image processing steps was optimized to label CK19-stained areas accurately. To quantify stained areas, light intensity was inverted, and a top hat box filter was applied to remove background and noise. A median filter was applied to smooth the remaining noise, and auto thresholding using Renyi entropy was used to label stained areas.

Analysis of gene expression

While gene expression can be determined by bulk- or single-cell RNA sequencing, quantitative real-time PCR (qPCR) is a cost-efficient targeted gene expression analysis technique. The stepwise outline of a qPCR experiment comprises several steps: (i) RNA extraction, (ii) cDNA synthesis, (iii) and the preparation and running of reactions. In paper I, we examined the hepatic expression of *Cyp7a1* and *Cyp8b1* involved in regulating bile acid synthesis. In paper III, we examined the expression of markers of inflammation and infiltration by qPCR. We used the RNeasy Kit (Qiagen) to extract RNA from mouse liver, followed by cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reactions for qPCR were prepared using the fluorescent SYBR Green Supermix (Bio-Rad) dye, which intercalates dsDNA during amplification. SYBR Green binds to all dsDNA and may bind to unspecific products and primer dimers. The melt curve of every reaction was assessed for unspecific products to prevent unspecific binding from influencing results. Moreover, primers were validated

for the absence of unspecific products by gel electrophoresis. Quantification cycle values (Cq) were analyzed by the ΔΔCq-method using an in-house developed data pipeline.

Statistical considerations

Parametric tests assume the data distribution but can still be trusted when testing non-normal distributions, provided the sample size is sufficient[300]. However, since mouse studies often have low sample sizes, non-parametric testing is often preferable. For this reason, we generally used non-parametric testing for data derived from mice but opted for parametric tests for larger sample sizes.

In paper I, we used Mann-Whitney U to analyze mouse physiology data, and to test differences in the insulin tolerance test, we performed a repeated measures 2-way ANOVA with Šídák's multiple comparisons test. Moreover, since bile acid data is rarely normally distributed, we used Mann-Whitney U with Benjamini-Hochberg correction to adjust for multiple testing. The same holds true for the correlation analyses, where we used Spearman's correlation with Benjamini-Hochberg correction.

In paper II, we opted for non-parametric tests to analyze bile acids as they were not normally distributed, but since the design was paired, we used Wilcoxon signed-rank tests. However, for clinical categorical variables, we used McNemar's test. For continuous clinical variables that were normally distributed, we used two-tailed paired Student's t-tests.

In paper III, apart from mouse physiology data, we almost exclusively used non-parametric tests as, e.g., bile acids concentrations were not normally distributed. However, we focused on comparing groups against littermate *Cyp2c70*$^{+/+}$ controls and not across colonization, as GF *Cyp2c70*$^{-/-}$ mice had high lethality, and the low numbers could lead to issues with powering the tests.

Results and discussion

This section concisely summarizes the findings of the papers included in this thesis and includes an accompanying discussion. Further details can be found in the Appendix.

Paper I

The gut microbiota is altered in T2D[25–28]. However, the full impact on metabolism is still elusive, and changes in the microbial community may affect the metabolic capacity of the gut microbiota. Consequently, it is a challenge to understand and elucidate how community composition and function affect metabolism. The gut microbiota produces bioactive metabolites, among which secondary bile acids are one of the most abundant. The secondary bile acids DCA and LCA are produced by bacterial 7α-dehydroxylation of CA and CDCA[71]. However, 7α-dehydroxylating bacteria account for a minor partition of the gut microbiota[81], and only a select few bacteria are capable of 7α-dehydroxylation. *Clostridium scindens* is one such capable species[82], and we investigated its generation of DCA *in vivo* and *in vitro* and linked DCA levels to metabolic dysregulation in T2D[77].

Results

Assessing bacterial DCA production in a simplified community

We constructed a bacterial base community (base) representing a simplified rendition of human gut ecology, composed of nine bacterial species lacking the *bai* operon responsible for bacterial 7α-dehydroxylation (Table 3). Individual cultures of bacteria supplemented with CA did not produce DCA, and only individual cultures with *Bacteroides caccae, Bacteroides thetaiotaomicron*, and *Bacteroides ovatus* produced 7-oxo-cholic acid (7-oxoCA).

Table 3 Base community composition and whether monocultures produce 7-oxoCA when supplemented with 100 µM CA in vitro. Created using BioRender.

Bacteria	Substantial 7-oxoCA production?
Bacteroides caccae	Yes
Bacteroides vulgatus	
Eubacterium rectale	
Bacteroides thetaiotaomicron	Yes
Bacteroides uniformis	
Parabacteroides distasonis	
Bacteroides ovatus	Yes
Ruminococcus torques	
Dorea longicatena	

Co-culturing all nine bacteria did not lead to DCA production. However, an individual culture of the well-characterized *C. scindens* containing the *bai* genes could 7α-dehydroxylate CA to produce DCA. Cultures of base community supplemented with *C. scindens* produced DCA. In addition, supplementing the base community with serially diluted *C. scindens* produced DCA at dilutions of $1{:}10^4$, $1{:}10^5$, and even $1{:}10^6$ in 24 h cultures.

Clostridium scindens produces DCA and alters glycemic control

We introduced the base community with or without *C. scindens* to GF mice by gavage to evaluate its production of DCA and effects on metabolism in vivo. In GF mice, CA is only found in low quantities[98]. Hence, the mice were fed a CA-supplemented chow diet (1%) for the duration of the experiment (2 weeks) to increase the substrate concentration for the formation of DCA. We could not identify *C. scindens* in the cecum of mice colonized with base + *C. scindens* by whole-metagenome shotgun sequencing, but using digital droplet PCR (ddPCR), we could quantify *C. scindens*. The microbial genome copies of *C. scindens* were very sparse (1:14397 compared to combined genome copies of the remainder of the community). However, despite the low abundance of *C. scindens* in base + *C. scindens* mice, they had substantial amounts of tauro-deoxycholic acid (TDCA) and DCA in the cecum and vena cava serum, whereas base – *C. scindens* did not. To compare to physiological levels of DCA in mice, we quantified serum and cecal DCA in CONV-R mice fed either a CA diet (1%) or a chow diet. CONV-R mice had higher cecal DCA levels and similar serum DCA levels. Although the CA diet was hepatotoxic as measured by increased serum AST and ALT levels, base + *C. scindens* and base - *C. scindens* mice had similar levels. Mice with base + *C. scindens* had reduced

hepatic expression of *Cyp7a1* compared to base - *C. scindens*, while *Cyp8b1* expression was similar.

Furthermore, we examined whether *C. scindens* affects metabolic parameters, and the body weights were homogenous across both groups. Fat percentage and relative liver weights were higher in base + *C. scindens* mice, while the relative cecum weight was lower than in base – *C. scindens* mice. As adiposity increased in base + *C. scindens* mice, we examined the levels of triglycerides and observed an increase in liver triglycerides, which correlated with the relative liver weight. Cecal SCFAs and branched-chain fatty acids did not differ between base + *C. scindens* mice and base - *C. scindens* mice. To evaluate the impact of *C. scindens* on glycemic control, we performed an intraperitoneal insulin tolerance test where base + *C. scindens* mice exhibited impaired insulin tolerance. However, fasting blood glucose and insulin levels were not significantly different.

We conclude that *C. scindens* can affect metabolism even when sparsely populating the gastrointestinal tract.

Elevated levels of DCA in individuals with T2D are associated with poor metabolic traits

We then assessed whether DCA could be linked to T2D in the IGT Microbiota and Swedish Cardiopulmonary Bioimage Study (SCAPIS) cohorts. Women and men aged 50-64 from the Gothenburg region in Sweden were recruited from the census register to be part of the IGT Microbiota and SCAPIS cohorts. We selected 200 individuals with treatment-naïve T2D or with normal glucose tolerance (NGT) from the IGT Microbiota cohort (45 treatment-naïve T2D, 45 BMI-matched controls with NGT) and the SCAPIS cohort (55 treatment-naïve T2D, 55 BMI matched controls with NGT). We matched them according to age, sex, weight, BMI, and statin and proton pump inhibitor use, and individuals did not use metformin or weight reduction agents. Exclusion criteria were antibiotic usage within the last three months, cancer (unless relapse-free the last five years), cognitive dysfunction, diabetes, infectious disease treatment, inflammatory disease, lack of understanding of Swedish, born outside of Sweden, or steroid or immunomodulatory drug treatment. The Ethics Review Board in Gothenburg approved the study, and all cohort participants gave informed consent. Further specifics of the cohorts have previously been detailed[25,301].

Individuals with T2D had increased total bile acids, and specific bile acids that were increased included DCA, isoDCA, and 12-epiDCA in plasma. DCA was the most abundant bile acid in feces, and total bile acids and DCA trended toward an increase in feces, but no bile acids differed between individuals with NGT and individuals with T2D. However, DCA levels in feces correlated well with plasma DCA levels. DCA in plasma correlated with HOMA-IR in individuals with T2D but not in NGT individuals. Interestingly, DCA, isoDCA, and 12-epiDCA in feces correlated positively with triglycerides, the triglyceride-glucose index, HOMA-IR, fasting blood glucose, HbA1c, insulin, and C-reactive protein, and correlated negatively with high-density lipoprotein in individuals with T2D but not in NGT-individuals. These findings align with dysregulation of glycemic control and lipid metabolism.

Discussion

This paper shows how a low-abundance 7α-dehydroxylating bacteria can significantly impact metabolic output. *C. scindens* produces DCA even in a very low abundance *in vitro* and when added to a base community and given to GF mice. Moreover, we linked DCA to metabolic variables in a human cohort of individuals with NGT or T2D.

In vitro bacterial monocultures supplemented with CA produced 7-oxoCA in 3/12 monocultures: *Bacteroides caccae*, *Bacteroides thetaiotaomicron*, and *Bacteroides ovatus*. In mice colonized with base + *C. scindens*, we could not detect *C. scindens* by whole-metagenome shotgun sequencing, which was below the detection limit. However, we were able to detect it by ddPCR. Even a minuscule amount of *C. scinden*s produced DCA to a considerable degree. Additionally, mice colonized with base + *C. scindens* had reduced insulin tolerance. In line with this, a previous study feeding mice a high-fat diet supplemented with DCA (0.1%) for three weeks reported that DCA-feeding negatively influences glycemic control in mice[140].

Mice colonized with base + *C. scindens* exhibited an increase in liver weight. We fed the mice CA (1%) to increase the substrate concentration for *C. scindens* since GF mice have low levels of CA. Feeding mice with CA (1%) has previously been shown to elevate serum AST levels[141], raising the question of whether the hepatotoxicity affected glycemic control. However, mice with base + *C. scindens* had an altered insulin tolerance compared to mice with base - *C. scindens*, indicating an effect mediated by *C. scindens*, possibly by the production of DCA. Moreover, it is well established that *C. scindens* produces

the secondary bile acids DCA and LCA from CA and CDCA[71]. However, additional gut bacteria may contribute to the formation of DCA and LCA, and DCA levels of base + *C. scindens* mice were lower than CONV-R mice. Expression of *Cyp7a1* was downregulated in base + *C. scindens* mice, which likely reflects a response to the dietary CA.

Total bile acids were increased in plasma in individuals with T2D compared to individuals with NGT. However, total bile acid levels were not increased in feces in individuals with T2D. One study showed that the gut microbiota was altered in treatment-naïve individuals with T2D, and changes in gut microbiota were associated with insulin resistance[25]. Interestingly, the relative abundance of microbial genes involved in butyrate synthesis was found to be decreased in treatment-naïve individuals with T2D[25]. Butyrate is vital for epithelial function and regulates the gut mucosal barrier[302], and data suggests that obesity and insulin resistance are linked to chronic low-grade inflammation, which in turn is associated with increased intestinal permeability[303]. Another reason could be increased bile acid absorption in T2D. Therefore, possible causes could be changes in intestinal permeability or absorption, which might explain the elevation of total plasma bile acids but the lack of change in total fecal bile acids. However, we could not explore whether intestinal permeability or absorption of bile acids is altered in T2D as the study was not designed for this purpose. In summary, we identified a link between DCA and dysmetabolism in individuals with T2D, and DCA correlated with poorer glycemic control and lipid metabolism.

Paper II

In paper I, we provided evidence that the 7α-dehydroxylating bacteria *C. scindens*, even at low abundance, produces substantial amounts of DCA and can greatly affect host output. Moreover, we linked DCA levels to dysmetabolism in T2D. Obesity and T2D constitute major public health concerns with limited curative treatments. Bariatric surgery, however, remarkably induces remission of T2D in over 75% of individuals. It also induces an average excess body weight loss of 55.9% and constitutes an excellent option for treating obesity and T2D[229]. The mechanism of how bariatric surgery mediates its beneficial effects is not yet defined. However, bile acids and FGF19 have been reported to be altered after bariatric surgery[238–241], and FXR[242,243] and TGR5[246–248] are implicated in mediating the beneficial effects of bariatric surgery. In paper II, we clarified how post-prandial bile acid kinetics are altered after bariatric surgery.

Results

Individuals scheduled to undergo bariatric surgery were included in the BARIA cohort[304]. In total, 38 individuals (22 without T2D and 16 with T2D) underwent a mixed-meal test prior to and after bariatric surgery. The majority underwent RYGB (32/38). In those with T2D, 6 underwent mini gastric bypass. Individuals between 18-65 years with obesity-related health conditions and a BMI ≥35 kg/m^2 or a BMI ≥40 kg/m^2 qualified for inclusion. Criteria for exclusion were coagulation disorders, excessive alcohol intake (>14 units/week), a known genetic predisposition for insulin resistance or glucose intolerance, pregnancy or breastfeeding, primary lipid disorders, psychiatric conditions, renal insufficiency (creatinine >150 mol/L), uncontrolled hypertension (blood pressure >150/95 mmHg). The Ethical Review Board of the Academic Medical Center, Amsterdam, approved the study, and all cohort participants gave informed consent.

Bariatric surgery increased fasting plasma bile acids and decreased fecal bile acids

Bariatric surgery increased total fasting bile acid levels in plasma and increased glyco-chenodeoxycholic acid (GCDCA), glyco-cholic acid (GCA), CA, HDCA, HCA, tauro-alpha-muricholic acid (TαMCA), and tauro-omega-muricholic acid (TωMCA). However, 3β-ursodeoxycholic acid (isoUDCA), isoDCA, and isoLCA decreased after bariatric surgery. In contrast, total bile acid levels in fecal samples decreased after surgery, particularly LCA and iso-LCA. Likewise, TCDCA, TDCA, GCA, GUDCA, and glyco-lithocholic acid (GLCA) decreased. Bariatric surgery did not induce a change in fecal weight. Fasting plasma C4 was reduced after surgery and correlated with bile acid concentrations in feces.

Bariatric surgery-induced postprandial bile acids and FGF19 excursions

MMTs were performed prior to and one year following bariatric surgery to assess changes in the postprandial bile acid response. We adopted specific core metrics to analyze bile acid kinetics, the maximum concentration, the maximum rise from fasting, and the time point for the maximum concentration (TMC).

Bile acids responsiveness was sorted into two categories: bile acids with a median Maximum rise / Fasting concentration > 1.0 were considered

"responsive", implying a twofold increased fasting concentration in over 50% of individuals, while those under 1.0 were considered "non-responsive".

Notably, prior to surgery, unconjugated bile acids, C4 and FGF19, were non-responsive, and total bile acids and all taurine- and glycine-conjugated bile acids apart from GHDCA were responsive. Bariatric surgery induced changes in the kinetics of bile acids, and consequently, some bile acids had different postprandial responses after surgery and were classified differently in the post-surgery group. Moreover, bariatric surgery induced a higher and early postprandial excursion of total bile acids and a late postprandial excursion of FGF19 levels. Interestingly, the fasting levels of some typically low-abundance 6α-OH bile acids, GHDCA, GHCA, HDCA, and HCA increased after surgery.

The impact of bariatric surgery on metabolic variables in T2D

Bariatric surgery led to weight loss in all individuals (median 25.8%). However, individuals with T2D lost less weight compared to non-T2D individuals. Levels of fasting blood glucose and insulin, postprandial glucose, HbA1c, and C-peptide were decreased after surgery, but individuals with T2D had higher levels than individuals without T2D. Postprandial insulin levels were similar before surgery. After surgery, both groups exhibited a sharp early excursion. Importantly, 75% of individuals with T2D could discontinue anti-diabetic drugs after bariatric surgery.

Bile acid response is attenuated in individuals with T2D after bariatric surgery

Early responsive bile acids were designated with a TMC at 30 minutes or earlier in more than 70% of individuals. The bile acid response was attenuated in individuals with T2D after bariatric surgery, with altered maximum rise of specific early responding bile acids (GCA, TCA, TDCA) only in the non-T2D group. Correspondingly, the maximum rise of some late-responsive bile acids (CA, CDCA, and DCA) was altered only in the non-T2D group. The TMC of early responding bile acids GCA, TCA, and TDCA were only increased in non-T2D individuals. In contrast, bariatric surgery increased both groups' maximal concentration of the early-responding GCA and late-responding CA, and CDCA.

Bariatric surgery normalizes postprandial FGF19 and fasting C4 in individuals with T2D

Although bile acid responsiveness was attenuated in individuals with T2D, bariatric surgery induced changes in FGF19 and C4. The post-prandial maximal concentration of FGF19 increased after surgery only in individuals with T2D, and the concentration of fasting C4 was decreased in individuals with T2D but not in non-T2D individuals.

T2D remission is associated with fasting HDCA levels

We next set out to understand whether changes in bile acid levels after bariatric surgery could be connected to remission of T2D. Notably, fasting levels of the non-responsive HDCA and GHCA were increased after bariatric surgery. In order to evaluate whether there was an association between bile acid levels after bariatric surgery and remission of T2D, we used a separate bariatric surgery cohort of solely individuals with T2D who underwent bariatric surgery. For the validation cohort, we included 39 individuals with T2D who underwent RYGB. Individuals over 18 years with a BMI >33kg/m^2 or a formerly confirmed BMI >35kg/m^2 with T2D as measured by HbA1c ≥6.5% (48 mmol/mol) or ≥6.5% (48 mmol/mol) using anti-diabetic agents, qualified for inclusion. Remission of T2D was considered as HbA1c ≤6.0% (42mmol/mol) without using anti-glycemic agents. The Regional Committees for Medical and Health Research Ethics in Norway approved the study, and all cohort participants gave informed consent.

In the validation cohort, levels of HDCA were increased following bariatric surgery. Covariance analysis revealed that HDCA co-varied with remission of T2D in the validation cohort. Moreover, bariatric surgery induced the greatest changes in HDCA levels in individuals with remission of T2D.

Discussion

In this paper, we characterized bile acid kinetics after MMTs prior to and following bariatric surgery. Most individuals (32/38) underwent RYGB, and six individuals underwent mini gastric bypass instead. However, the alternate procedure leads to a relatively similar anatomical configuration, and like RYGB, bile acids do not interact with food until around the jejunum.

We measured bile acids in peripheral plasma and feces. Bariatric surgery decreased fasting plasma C4 and fecal bile acids, reflecting a lower excretion of bile acids and decreased bile acid synthesis. However, plasma bile acids

were increased after bariatric surgery, suggesting an increased absorption of bile acids. Particularly, primary bile acids were responsible for the increase in plasma bile acids after bariatric surgery. IBAT reabsorbs conjugated bile acids in the intestine[69]. The increase in fasting plasma bile acids after bariatric surgery might thus be a product of more efficient reabsorption of bile acids from the intestine to the portal venous blood. Hepatic bile acid transporters efficiently reabsorb bile acids from portal venous blood. However, a small proportion escapes into the peripheral blood due to portosystemic spillover[305]. While portal venous plasma would more accurately reflect absorptive processes, we were constrained to measurements of bile acid levels in peripheral plasma, as portal venous plasma sampling requires more invasive sampling procedures.

All glycine- and taurine-conjugated bile acids, apart from GHDCA, exhibited a post-prandial response prior to surgery. Unconjugated bile acids, however, did not. Notably, bariatric surgery induced an early post-prandial response in several conjugated bile acids and a late response in several glycine- and unconjugated-bile acids. Since food does not reach the duodenum with RYBG or mini gastric bypass, primarily conjugated bile acids may have a more rapid transit through the intestine after bariatric surgery. Indeed, bariatric surgery induced an early response and decreased the TMC of several conjugated bile acids. Interestingly, some unconjugated bile acids showed a late response, while most unconjugated bile acids showed no response. The gut microbiota may thus have a greater capacity to quickly and readily transform these late-responsive bile acids. Possibly, e.g., microbial production of the unresponsive 7-oxoCDCA may stem from several different precursor bile acids depending on the individual's gut microbial composition, and it may not have a well-defined response. Since gut microbial production of secondary bile acids primarily occurs in the colon, the late responsive unconjugated bile acids are subject to passive absorption rather than active transport by IBAT in the small intestine.

Furthermore, FXR has been implicated in mediating the beneficial effects of bariatric surgery[243,249]. We observed that bariatric surgery induced post-prandial bile acid and FGF19 excursions. Individuals with pre-surgery T2D had a blunted post-prandial FGF19 response prior to surgery, with a particularly increased response after surgery. The observed increase in post-prandial FGF19 likely reflects increased FXR activation supporting the role of FXR in bariatric surgery. We also found that bariatric surgery decreased postprandial C4. However, C4 lacked a post-prandial response with relatively

static levels, and thus, the increased post-prandial FGF19 levels after surgery do not seem to reflect an acute decrease in bile acid synthesis. Finally, we observed that the 6α-OH HDCA was linked to remission of T2D and that the greatest changes in HDCA levels were found in T2D-remitters. This is in line with a previous study, showing that 6α-OH HCA-species were increased after RYGB and that HCA-species covary with T2D remission[228], further supporting the involvement of 6α-OH bile acids in conferring metabolic benefits in bariatric surgery.

In this paper, we clarified how bile acids respond after bariatric surgery. Bariatric surgery induced changes in the post-prandial kinetics of certain bile acids, while other bile acids had altered fasting levels, and the fasting levels of 6α-OH HDCA were linked to the remission of T2D.

Paper III

In paper I, we investigated DCA production by a low-abundant microbial species and linked DCA to human dysmetabolism. In paper II, we examine bariatric surgery as a treatment for obesity and T2D and characterize post-prandial bile acid kinetics. However, the gut microbiota and bile acids have also been implicated in hepatobiliary diseases, and the gut microbiota is altered in both PBC[36] and PSC[256].

A challenge in studies of the interplay between bile acids and gut microbiota is that mice have an additional set of primary bile acids, αMCA and βMCA. In addition, UDCA, categorized as a secondary bile acid in humans, can be found in GF mice and is thus a primary bile acid[98]. However, CYP2C70 was recently discovered as the enzyme responsible for converting CDCA to the murine primary bile acids[167]. Several *Cyp2c70*-deficient mouse models were generated[161,162,168,169,172,175] as a more human-like bile acid metabolism model. Since gut microbiota has been linked to PBC and PSC, we hypothesized that the gut microbiota plays a role in the hepatobiliary phenotype of *Cyp2c70*$^{-/-}$ mice.

In paper III, we examine the impact of gut microbiota on the hepatobiliary phenotype of *Cyp2c70*$^{-/-}$ mice and show that gut microbiota improves survival and protects against liver disease in *Cyp2c70*$^{-/-}$ mice.

Results

Since CONV-R *Cyp2c70*$^{-/-}$ mice have liver fibrosis and cholangiopathy, we rederived *Cyp2c70*$^{-/-}$ as GF to evaluate whether gut microbial ecology plays a role in the pathogenesis of their hepatobiliary phenotype. We re-derived *Cyp2c70*$^{+/-}$ as GF by cesarean section and housed them in sterile plastic isolators.

A lack of microbiota decreases survival in germ-free *Cyp2c70*$^{-/-}$ mice

Initially, we identified that the lack of microbiota led to decreased survival in *Cyp2c70*$^{-/-}$ mice at weaning (3 weeks old) and by the genotype ratio deviating from the expected Mendelian ratio. We genotyped mice at birth to determine if the decreased survival was due to intrauterine mortality or whether GF *Cyp2c70*$^{-/-}$ mice had increased postnatal mortality. Indeed, genotypes corresponded to a Mendelian ratio at birth, suggesting that natal survival was not altered. Moreover, we monitored survival in GF *Cyp2c70*$^{-/-}$ mice longitudinally and identified a considerable lethality over time. To understand the physiological consequence of *Cyp2c70*-deletion in a GF setting, we divided mice into two groups and euthanized them either at 4-5 weeks of age (early time point) or 6-10 weeks of age (late time point). GF males had a decreased body weight at the early time point, whereas this was normalized at the late time point. The relative liver weight was increased in both sexes at both time points.

Microbial colonization improves survival of germ-free *Cyp2c70*$^{-/-}$ mice

To investigate whether colonization can improve survival, we colonized GF *Cyp2c70*$^{+/-}$ breeding pairs with either feces from a 47-year-old healthy male (HUM group) or cecal content from a CONV-R mouse (CD group) and investigated the genotype ratio of their litters. Both HUM and CD *Cyp2c70* mice followed the expected Mendelian ratio at weaning. However, the longitudinal survival decreased, and lethality was not eliminated post-colonization in HUM nor CD *Cyp2c70*$^{-/-}$ mice.

Conventionalization alleviates features of cholangiopathy and fibrosis

To evaluate the physiological consequence of microbial colonization, we euthanized HUM and CD mice according to the prior early- and late- grouping. There were no differences in body weight apart from a decrease in CD

Cyp2c70$^{-/-}$ males at the early time point, which was normalized at the late time point. At the late time point, there was a slight decrease in body weight in HUM *Cyp2c70*$^{-/-}$ mice. At the early time point, HUM and CD *Cyp2c70*$^{-/-}$ mice had an increased relative liver weight, persisting in HUM *Cyp2c70*$^{-/-}$ mice at the late time point but normalized in CD *Cyp2c70*$^{-/-}$ mice.

To determine if the differences in relative liver weight corresponded to hepatic injury, we evaluated hepatic collagen deposition with Sirius Red and cholangiocyte proliferation by CK19 staining. At the early time point, GF, HUM, and CD *Cyp2c70*$^{-/-}$ mice had increased fibrosis and cholangiocyte proliferation. However, at the late time point, GF and HUM *Cyp2c70*$^{-/-}$ mice had apparent fibrosis, whereas in CD *Cyp2c70*$^{-/-}$ mice, we only observed a mild increase in fibrosis. GF and HUM *Cyp2c70*$^{-/-}$ mice showed apparent features of cholangiopathy, whereas increased cholangiocyte proliferation was not present in CD *Cyp2c70*$^{-/-}$ mice.

Finally, *Cd177*, *Adgre1* (F4/80), *Tnfα*, and *Ccl2* expression increased in GF, HUM, and CD *Cyp2c70*$^{-/-}$ mice at the early time point. However, CD *Cyp2c70*$^{-/-}$ mice had similar expression to their *Cyp2c70*$^{+/+}$ controls at the late time point. Serum AST and ALT levels increased in GF, HUM, and CD *Cyp2c70*$^{-/-}$ mice at the early time point. At the late time point, GF and HUM *Cyp2c70*$^{-/-}$ mice had increased levels, while levels were normalized in the CD *Cyp2c70*$^{-/-}$ mice, particularly in the males.

Conventionalization increased the proportion of UDCA and decreased the biliary bile acid hydrophobicity

To evaluate whether bile acid profiles were involved in the hepatobiliary phenotype, we measured hepatic-, biliary-, serum, and cecal-bile acids. The bulk of GF *Cyp2c70*$^{-/-}$ bile acid profiles consisted of TCDCA and TCA, accompanied by minor amounts of other conjugated bile acids. HUM *Cyp2c70*$^{-/-}$ mice had similar hepatic, biliary, and serum bile acid profiles as GF *Cyp2c70*$^{-/-}$ mice. However, their cecal bile acid profile consisted of unconjugated and secondary bile acids, such as CDCA and DCA, with high LCA and cholic acid-7-sulfate (CA-7S) proportions. Interestingly, CD *Cyp2c70*$^{-/-}$ mice had increased proportions of TUDCA and UDCA in hepatic-, biliary- and serum bile acids profiles, particularly at the late time point, and the biliary bile acid hydrophobicity index was decreased at the early timepoint in CD *Cyp2c70*$^{-/-}$ females. However, cecal bile profiles in CD *Cyp2c70*$^{-/-}$ mice at the early and late time points included CDCA, DCA, LCA, isoLCA, and CA-7S, with only minute proportions of TUDCA and UDCA.

At the late time point, both CD *Cyp2c70*$^{-/-}$ males and females had a decreased biliary bile acid hydrophobicity index compared to GF and HUM *Cyp2c70*$^{-/-}$ mice, and the bile acid hydrophobicity index correlated with relative liver weight. Furthermore, the bile acid hydrophobicity index of CD *Cyp2c70*$^{-/-}$ mice correlated with hepatic expression of *Cd177*, *Adgre1*, *Tnfα*, and *Ccl2* and with serum AST and ALT levels.

Microbial ecology is linked to biliary bile acid hydrophilicity and liver weight

We evaluated the difference in cecal microbial diversity between the CD and HUM *Cyp2c70* mice. The engraftment in CD mice was more efficient than that in HUM mice. Furthermore, the difference in α-diversity between the human fecal inoculum and cecal microbiota in HUM mice was larger than between the mouse cecal inoculum and cecal microbiota in CD mice. In addition, PCoA using the weighted UniFrac distance matrix showed that the human inoculum clustered separately from the HUM mice, while the mouse inoculum clustered with CD mice.

To determine if the improved hepatobiliary phenotype was connected to the gut microbiota, we examined the cecal microbiota of CD *Cyp2c70*$^{-/-}$ mice. We performed a PCoA using the weighted UniFrac distance matrix, observing that the gut microbiota predominantly clustered according to time point. Gut microbiota differed between CD *Cyp2c70*$^{+/+}$ and CD *Cyp2c70*$^{-/-}$ mice at the early time point, between CD *Cyp2c70*$^{+/+}$ mice at the early and late time point, and between CD *Cyp2c70*$^{-/-}$ mice at the early and late time point, but not between CD *Cyp2c70*$^{+/+}$ and CD *Cyp2c70*$^{-/-}$ at the late time point, as tested by PERMANOVA. Moreover, the biliary hydrophobicity index explained a large portion of the ecological variation in the gut microbiota of CD *Cyp2c70*$^{-/-}$ mice at the early time point. Consequently, we performed Spearman's correlation analysis between the hydrophobicity index and bacterial taxa and found negative correlations with *Desulfovibrio* and *Parasutterella excrementihominis*. Furthermore, the abundance of these bacterial taxa increased at the late time point, and both the relative liver weight and the biliary hydrophobicity index correlated with the abundance of *Desulfovibrio* and *Parasutterella excrementihominis*.

Discussion

In this study, we report a vital role of gut microbiota in the survival and hepatobiliary phenotype of *Cyp2c70*$^{-/-}$ mice with a human-like bile acid profile.

GF *Cyp2c70*$^{-/-}$ mice had reduced neonatal survival, whereas HUM and CD *Cyp2c70*$^{-/-}$ mice did not. Interestingly, bile acids have been shown to drive the maturation of the gut microbiota in neonatal mice[10]. CYP2C70 regulates the production of MCAs from CDCA in mice[161,167,168], and from postnatal days 7-14, expression of *Cyp2c70* increases in neonatal mice[10]. The mouse liver normally handles CDCA by transforming it into MCAs, and *Cyp2c70*$^{-/-}$ mice thus accumulate high levels of CDCA, which might be responsible for hepatic injury.

Interestingly, both a human-derived and a mouse-derived microbiota protected *Cyp2c70*$^{-/-}$ mice from neonatal mortality, indicating protection against the hepatobiliary phenotype. HUM and CD *Cyp2c70*$^{-/-}$ mice had substantial proportions of CA-7S in the cecum. Sulfonation of bile acids facilitates fecal excretion[72] and may be one of the reasons for the discrepancy in survival between colonized and GF *Cyp2c70*$^{-/-}$ mice. However, the hepatic and biliary bile acid profiles of HUM and GF *Cyp2c70*$^{-/-}$ mice were relatively similar, and thus, another conceivable reason could be that the gut microbiota is crucial to ensuring normal hepatic or gastrointestinal development. The lack of the gut microbiota might then induce dysregulation of such processes, aggravated by a bile acid pool with a high proportion of the hydrophobic TCDCA.

At 6-10 weeks of age, hepatic injury only improved in mice colonized with a mouse microbiota as measured by cholangiocyte proliferation, fibrosis, relative liver weight, markers of inflammation, and infiltration. CD *Cyp2c70*$^{-/-}$ mice had increased proportions of (T)UDCA compared to CD *Cyp2c70*$^{+/+}$ mice. Importantly, UDCA feeding has previously been shown to relieve cholangiopathy and hepatic fibrosis in *Cyp2c70*$^{-/-}$ mice[161]. Furthermore, CD *Cyp2c70*$^{-/-}$ mice had increased proportions of UDCA compared to HUM *Cyp2c70*$^{-/-}$ mice. One hypothetical explanation for the discrepancy in bile acid profiles between CD and HUM *Cyp2c70*$^{-/-}$ mice is that the α-diversity in the HUM mice was relatively low compared to CD mice. The increased proportions of the hydrophilic UDCA in CD *Cyp2c70*$^{-/-}$ mice might be from the transformation of the hydrophobic CDCA. Microbial production of UDCA from CDCA has been shown to occur stepwise with intermediate oxidation, producing 7-oxoCDCA, generated by bacteria with 7α-HSDH, followed by reduction to UDCA by bacteria with 7β-HSDH[291]. Since HUM *Cyp2c70*$^{-/-}$ mice had a lower complexity gut microbiota, the lack of UDCA production might be attributed to the loss of specific bacteria with 7α- or 7β-HSDHs and, thus, a diminished capacity to produce UDCA.

Furthermore, at 6-10 weeks of age, the biliary bile acid hydrophobicity index was lower in CD *Cyp2c70*$^{-/-}$ mice than in GF and HUM *Cyp2c70*$^{-/-}$ mice. Although higher in GF and HUM *Cyp2c70*$^{-/-}$ mice, it is probably not solely responsible for the induction of liver injury. Administration of IBAT inhibitor to *Cyp2c70*$^{-/-}$ mice has been reported to alleviate the hepatobiliary phenotype and reduce total bile acid levels in the liver[175]. Hence, the more hydrophobic bile acid profile may need to be accompanied by elevated hepatic or circulating bile acids to induce liver injury.

An increase in abundance of *Desulfovibrio* and *P. excrementihominis* was noted in CD *Cyp2c70*$^{-/-}$ mice at 6-10 weeks compared to 4-5 weeks of age, and the abundance of both taxa correlated negatively with the biliary bile acid hydrophobicity index and the relative liver weight. In spite of the association between *Desulfovibrio* and *P. excrementihominis,* they are not necessarily involved in UDCA production and may play completely different roles in host metabolism. However, one study has implicated the *Parasutterella* genus in bile acid metabolism, as one study showed that colonization of mice with *Parasutterella* increased *Cyp7a1* expression, suggesting a role in controlling bile acid synthesis[306]. Furthermore, untargeted metabolomics revealed that *Parasutterella* colonization decreased the relative levels of several bile acids, including CA, TCA, and TDCA, indicating a role in influencing bile acid metabolism[306]. The *Cyp2c70*$^{-/-}$ mouse has been suggested as a model for PBC[161], and gut microbiota has been implicated in both PBC[36] and PSC[256]. Our study shows that gut microbiota is of utmost importance for the survival of *Cyp2c70*$^{-/-}$ mice. More in-depth phenotyping of the liver injury needs to be undertaken before we can confidently proclaim what disease they more closely reflect. This could be solved by histological evaluation to clarify whether extrahepatic biliary injury occurs and by investigating the histological characteristics of bile ducts and liver injury in the model.

In summary, we provide evidence for the gut microbiota as a determinant of survival and liver disease in *Cyp2c70*$^{-/-}$ mice with a human-like bile acid composition. Microbially induced production of UDCA mediated a less hydrophobic bile acid profile, which was associated with improved liver disease in *Cyp2c70*$^{-/-}$ mice.

Conclusion

- There is a complex reciprocal interplay between bile acids and the gut microbiota.
- Even when low-abundant, 7α-dehydroxylating bacteria such as *Clostridium scindens* are able to profoundly impact host metabolism by producing secondary bile acids.
- DCA is associated with glycemic dysregulation and an impaired lipid profile in individuals with T2D.
- Postprandial bile acid kinetics are altered after bariatric surgery and may mediate some of its beneficial effects.
- Fasting HDCA levels are associated with remission of T2D after gastric bypass.
- The gut microbiota influences neonatal survival and liver disease in *Cyp2c70*$^{-/-}$ mice, and a mouse-derived microbiota was associated with a more hydrophilic bile acid profile.
- Microbially induced production of UDCA may alleviate liver disease in *Cyp2c70*$^{-/-}$ mice.

Future perspectives

Paper I linked DCA production to impaired glucose metabolism and a worsened lipid profile in individuals with T2D. We showed that *C. scindens*, one of the few known bacteria capable of 7α-dehydroxylation, could substantially affect host metabolic features, even at a low abundance. Thus, low-abundant bacteria can considerably influence host metabolism, and their impact may not be fully captured by evaluating their abundance. Furthermore, studies have predicted that further species may be capable of 7α-dehydroxylation. To elucidate the full impact of 7α-dehydroxylation on host metabolism, culturing and characterizing these bacteria *in vitro* and *in vivo* will likely be necessary.

In individuals with T2D, fecal DCA and isoDCA correlated with C-reactive protein and white blood cells. DCA, isoDCA, and 12-epiDCA were the most elevated bile acids in plasma in individuals with T2D. Interestingly, isoDCA has recently been shown to induce peripheral regulatory T cells[9]. Moreover, the bile acids 3-oxoLCA, iso-alloLCA, and isoLCA have been proposed to have immunomodulatory effects by binding RORγt[7,8]. Further studies evaluating the effect of bile acids in immunomodulation may provide insight into the pathogenesis of T2D. Microbial biotransformation is responsible for the generation of iso-bile acids and may occur through epimerization by bacteria with 3α- and 3β-HSDHs[88]. Identifying and characterizing bacteria capable of these transformations may allow for rational design of potential probiotics aiming to prevent and treat diseases with inflammatory components.

In paper II, we investigated bile acids kinetics after bariatric surgery, showing that post-prandial bile acids kinetics are altered post-surgery. FXR and TGR5 have been implicated in mediating some of the beneficial effects of bariatric surgery[242,243,246,247,249]. However, understanding the role of the gut microbiota in modulating the change in bile acid kinetics is still unknown and is of vital importance. Evaluating the gut microbial composition and linking changes to alterations in bile acid kinetics might provide information about potential mechanisms of how bariatric surgery mediates metabolic benefits. Furthermore, we found that levels of the 6α-OH HDCA were associated with remission of T2D after bariatric surgery, and a previous study implicated HCA-species in T2D remission after RYGB[228]. Therefore, determining how bariatric surgery increases 6α-OH bile acids and if there are shifts in the gut microbiota that lead to the generation of 6α-OH bile acids is of future

importance. Identifying the mechanisms for the induction of 6α-OH bile acids and if bacteria are responsible could potentially uncover strategies for improving glycemic control in obesity and T2D.

The Heuman index was proposed in 1989 as a measure of bile acid hydrophobicity and has been diligently used to evaluate the hydrophobicity of bile acid profiles[30,161]. Although the index was determined for a select few bile acids (Table 1)[134,136], there is a need for updated published hydrophobicity indices for bile acids, of which standards were not yet available when the original study was performed. Since the biliary bile acid hydrophobicity index explained a large portion of the microbial variation in CD *Cyp2c70*$^{-/-}$ mice, it would be interesting to evaluate how bile acid hydrophobicity affects the gut microbiota in cholestatic liver diseases such as PSC and PBC, both diseases marked by altered gut microbiota. However, since there are few published hydrophobicity indices, they currently lack use in the intestinal environment. Investigating how the bile acid hydrophobicity index varies in different locations, how well it explains variation in the gut microbiota, and how this affects host phenotype, *e.g.,* in liver disease, may provide valuable information and allow for partly distinguishing their biochemical effects from those mediated by signaling through bile acid binding receptors.

In addition, there are pitfalls in the targeted quantification of bile acids with UPLC-MS/MS, and we only find what we are looking for. I firmly believe that we need to develop more all-encompassing methods for quantifying bile acids and expand our methods to quantify further bile acid species. Untargeted quantification of bile acids might lend a hand in this endeavor and serve as a hypothesis-generating compliment to targeted quantification.

Although we now know that the gut microbiota is an essential determinant of liver disease in *Cyp2c70*$^{-/-}$ mice, much still remains unknown about the development of liver disease. One study showed that colitis ameliorates liver disease by decreasing bile acid synthesis in the *Mdr2*$^{-/-}$ PSC mouse model[149]. To better understand the *Cyp2c70*$^{-/-}$ model, it is important to clarify how the intestinal environment is altered in *Cyp2c70*$^{-/-}$ mice and how intestinal permeability and enterohepatic circulation are affected. One study showed that bile acids mature the gut microbiota in neonatal mice[10]. Since *Cyp2c70*$^{-/-}$ mice have transient neonatal cholestasis, characterizing neonatal cytochrome enzymes and liver development may reveal valuable insight into the development of liver disease and how the interplay between bile acids and the gut microbiota is involved.

Alteration of the gut microbiota by probiotic supplementation might affect bile acid metabolism and serve as a future strategy to ameliorate the progression or symptoms of these diseases. *Lactobacillus rhamnosus* has been shown to suppress bile acid synthesis, increase fecal bile acid excretion, and improve fibrosis in both bile duct ligated and *Mdr2*$^{-/-}$ mice[307]. Similar studies of bacterial supplementation extended to *Cyp2c70*$^{-/-}$ mice may provide a better understanding of probiotic supplementation by virtue of their human-like bile acid composition.

Finally, understanding how bile acids bind and activate different receptors and how total bile acid levels and composition affect binding is still challenging to disentangle. An abundance of research has established FXR and TGR5 as important metabolic regulators. Alternatively, focusing on whether there is a reciprocal interplay between these and other bile acid binding receptors, *e.g.,* S1PR2, RORγt, and PXR, may provide a deeper understanding of bile acid signaling in health and disease. To give an example, cholestatic liver diseases lead to decreased bile flow and might lead to an accumulation of conjugated bile acids in the liver and gallbladder, possibly implicating S1PR2 in cholestatic liver disease. Hence, understanding the role of the total sum of bile acid binding receptors in disease may provide valuable information about their involvement in its pathogenesis.

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