

**ALTERATIONS IN BILE ACID HOMEOSTASIS AND DRUG METABOLISM IN  
GERM-FREE MICE**

BY

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## ABSTRACT

“We may be born 100% human but will die 90% bacterial—a truly complex organism!” (Goodacre, 2007). This statement reflects the fact that there are 10 times more bacterial cells in the human body compared to the number of human cells, and there are 100 times more genes in the human microbiome compared to the number of genes in the human genome. Gut bacteria and host communicate with each other and collectively determine many aspects of host physiology such as bile acid (BA) and drug metabolism. Gut bacteria varies significantly between individuals and therefore, may be responsible for the inter-individual differences in BA concentrations and drug responses. There are a number of diseases such as obesity, inflammatory bowel disorder, and autism, that have been associated with an abnormal bloom in certain gut bacteria or a decrease in the diversity of gut bacteria. Therefore, modulating gut bacteria by probiotics, prebiotics, and by fecal transplantation have become viable therapeutic strategies.

Alterations of gut bacteria in diseases or the therapeutic modulation of gut bacteria has the potential to alter host BA signaling and drug responses. Germ-free (GF) mice provide an excellent model system for understanding the functions of gut bacteria. The overall goal of this dissertation is to expand the understanding of the role of gut bacteria in regulating host BA homeostasis and hepatic drug metabolism.

In **Specific Aim 1**, I determined the changes in BA homeostasis and BA signaling in GF mice. BAs are amphipathic cholesterol metabolites that are synthesized in liver and secreted into bile. Gut bacteria metabolize primary BAs to secondary BAs. The majority of BAs is reabsorbed from the intestine, effluxed into the portal vein and

return to the liver. Therefore, the BA profile in the host is the result of the host hepatic enzyme activity and the gut bacterial enzyme activity. The BA profile is important because, BAs act like hormones and regulate host physiology by activating the BA receptors, namely the farnesoid X receptor (FXR) and transmembrane G-protein-coupled receptor (TGR5).

The BA profiles of both male and female GF mice are markedly altered compared to conventional (CV) mice. GF mice have an increase in total BAs in all the tissue compartments analyzed and decreased total fecal excretion of BAs compared to CV mice. This could be due to slower intestinal propulsion rates and increased BA reabsorption from the intestines. The dominant BAs in GF mice are taurine conjugated  $\alpha$  and  $\beta$  muricholic acids ( $T\alpha+\beta$  MCA). There is an increase in both ursodeoxycholic acid (UDCA) and MCAs and in the proportion of taurine conjugated BAs and these BAs result in a more hydrophilic BA pool in GF mice.

UDCA which was previously considered to be a secondary BA that is synthesized by gut bacteria increases in GF mice. Biotransformation experiments *in vitro* demonstrated that UDCA can be synthesized from CDCA by enzymes present in hepatic microsomes isolated from both GF and CV mice. This explains why UDCA is increased in GF mice, and is evidence that UDCA is a primary BA synthesized by hepatic enzymes in mice.

The altered BA profile in GF mice results in the activation of TGR5 signaling. Therefore, GF mice display all the characteristics of TGR5 activation, such as increased gallbladder size, increased serum GLP-1 levels, and increased mRNA of type 2

iodothyronine deiodinase (D2), the enzyme which increases energy expenditure as heat in brown adipose tissue.

In **Specific aim 2**, I determined alterations in the mRNA of drug metabolizing enzymes in livers of GF mice by RNA-Seq. Gene expression of a many hepatic Phase-1 and Phase-2 enzymes was altered in the absence of gut bacteria. Based on this study, I was able to generate of a list of genes in the liver that are altered by gut bacteria. Among these genes are Cyp2b10 and Cyp3a11 as their mRNAs were decreased in GF mice. In order to test the functional consequences, GF and CV mice were treated with the anesthetic pentobarbital and I observed that GF mice sleep longer than CV mice suggesting that pentobarbital metabolism is slower in GF mice. These observations strongly suggest that gut bacteria play an important role in regulating drug metabolizing enzymes in the liver.

Drug metabolizing enzymes with decreased mRNA levels in GF mice are probably increased in CV mice to help biotransform chemicals formed by gut bacteria. In contrast, drug metabolizing enzymes with increased mRNA levels in GF mice are likely enzymes whose function normally can be performed by the gut bacterial enzymes, and therefore in the absence of gut bacteria, are induced in the liver.

In conclusion, this dissertation work has provided a detailed roadmap of alterations in BA composition, BA signaling, and expression of hepatic drug metabolizing enzymes in the absence of gut bacteria and will help to understand how to alter gut bacteria in a beneficial way.

## **Dedication**

**This dissertation is dedicated to the three women who helped shape my life:  
my grandmother Lalitha, my mother Hamsa and my aunt Geetha.**

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I grew up with my grandparents, uncles and aunt. My uncle Prathap used to demonstrate small science experiments at home and got me interested in science at a

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## List of Abbreviations

### Abbreviation Full name

Abc	ATP-binding cassette transporters
Abcg 5/8	ATP-binding cassette sub-family G member 5/8
ACOX2	acyl-coenzyme A oxidase 2
Ahr	Aryl hydrocarbon receptor
AKR	Aldo-keto reductase
AKT	Protein kinase B
Aldh	Aldehyde dehydrogease
Aox	Aldehyde oxidase
Apo	Apolipoprotein
Asbt	Apical sodium dependent bile acid transporter
BA	Bile acid
BAAT	Bile acid-CoA:amino acid <i>N</i> -acyltransferase
BAL	Bile acid-CoA ligase
BSEP	Bile salt export pump
C/EBP $\alpha$	CCAAT/enhancer-binding protein $\alpha$
CA	Cholic acid
cAMP	cyclic AMP
CAR	Constitutive androstane receptor
CDCA	Chenodeoxyxholic acid
Ces	Carboxylesterases
CFTR	Cystic fibrosis transmembrane conductance regulator

CO	Carbon monoxide
CoA	Coenzyme A
CV	Conventional
Cyp	Cytochrome P450
D2	Type-2 iodothyronine deiodinase
DCA	Deoxycholic acid
DPP4	Dipeptidyl peptidase 4
ECDCA	6 $\alpha$ -ethyl-chenodeoxycholic acid
Ephx	Epoxide hydrolase
ERK	Extracellular-signal-regulated kinase
FAD	Flavin adenine dinucleotide
Fgf	Fibroblast growth factor
FMN	Flavin mononucleotide
Fmo	flavin monooxygenase
Foxo1	Forkhead transcription factor o1
FPKM	Fragments per kilobase of exon per million reads mapped
FXR	Farnesoid X receptor
G6Pase	Glucose-6-phosphatase
Gclc	Glutamate-cysteine ligase catalytic subunit
GF	Germ-free
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
GPCR	G protein-coupled receptor

Gst	Glutathione-S-transferase
HDCA	Hyodeoxycholic acid
HDL	High density-lipoprotein
HNF4 $\alpha$	Hepatocyte nuclear receptor 4 $\alpha$
HSD	Hydroxysteroid dehydrogenase
JNK	C-jun N-terminal kinase
KLF11	Kruppel-like factor 11
LCA	Lithocholic acid
LDL	Low-density lipoprotein
LPS	Lipopolysacharide
LRH-1	Liver receptor homolog-1
LXR	Liver X receptor
MCA	Muricholic acid
MDCA	Murideoxycholic acid
MRP	Multidrug resistance-associated protein
MTP	Microsomal triglyceride transfer protein
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide
NO	Nitric oxide
Nqo	Quinone reductase
Nrf2	Nuclear factor erythroid 2-related factor
NTCP	Sodium taurocholate cotransporting polypeptide
Oatp	Organic anion transporting polypeptide

OH	Hydroxy
Osta/ $\beta$	Organic solute transporter $\alpha/\beta$
PC	Phosphatidyl choline
PC1/3	Prohormone convertase
PEPCK	Phosphoenol pyruvate carboxykinase
PGC1 $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$
PKA	Protein kinase A
PLTP	Phospholipid transfer protein
POR	NADPH-cytochrome P450 reductase
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
RXR	Retinoid X receptor
SCFA	Short chain fatty acid
SHP	Small heterodimer protein
Slc	Solute carrier
SREBP	Sterol regulatory element-binding protein
Sult	Sulfotransferase
TGR5	Transmembrane G protein-coupled receptor 5
UCP	Uncoupling protein
UDCA	Ursodeoxycholic acid
UDP	Uridine dinucleotide phosphate
UDPGA	Uridine dinucleotide phosphate-glucuronic acid
Ugt	UDP-glucuronosyltransferase

VDR	Vitamin D receptor
VLDL	Very low-density lipoprotein

**CHAPTER 1**  
**INTRODUCTION**

### **1.1.1 Our microbial friends**

Human beings are merely an association of human and microbial cells and are thus called “supraorganisms” or “holobionts” (Backhed et al., 2004; Turnbaugh et al., 2007; Gordon et al., 2013). These microbes act as a “virtual organ”, and perform functions that benefit the host (O'Hara and Shanahan, 2006). There are many ecological habitats in the human body and each of these habitats allows for only a certain group of microbes to thrive. The microbial community size and composition vary between individuals and within an individual between the different body sites. The microbiota residing on each individual is unique and distinct just like an individual's fingerprint. (Costello et al., 2009). Diet, environmental exposure, host genetics, and early microbial exposure are some of the factors that affect these microbial communities (Human Microbiome Project, 2012).

Ever since the microscope was invented to study microbes, there has been an interest in learning about microbes that call the human body their home (Relman, 2009). Most of these organisms are anaerobic and therefore cannot be studied by traditional culturing methods (Salzman et al., 2002). Several new tools have been developed to study these microbes that have been difficult to culture, by using its genetic material. There is more microbial genetic material in the human body than human genetic material (Backhed et al., 2004; Turnbaugh et al., 2006; Turnbaugh et al., 2007). Metagenomics is the culture-independent study of the microbial DNA, RNA, and proteins to elucidate the structure and function of microbial communities in a sample (Gordon, 2012). These new tools help to find answers to questions such as, what is the

number of microorganisms present in the human body, what is their diversity and how do they influence the human body.

One of the shocking revelations of the human genome project was that there are about 200 proteins encoded by the human genome that only have homologues in bacteria. This observation opened up the discussion about a possible horizontal transfer of these genes from bacteria to humans or even a possible gene transfer that occurs in the opposite direction, that is a horizontal transfer from vertebrates to bacteria (Lander et al., 2001). This observation resulted in a second human genome project to sequence the genetic material of all the endogenous microbiota (Relman and Falkow, 2001; Sears, 2005).

The term 'microbiome' denotes the collective genome of all the indigenous microbes. There have been many projects initiated worldwide in the last decade to study the human microbiome. They include the Human Microbiome Project (HMP) of the US, the Metagenomics of the Human Gut Tract (MetaHIT) project of the European commission, the Canadian Microbiome Initiative and the Australian Jumpstart Human microbiome project. These microbiome projects have provided a huge database with a wealth of information that has answered some questions and raised many more.

Information from these studies have now demonstrated that among all other body sites, the intestinal tract has the largest number and biggest diversity of microbial species, and that bacteria constitute the major part of gut microbiota, however, viruses and yeast are also key residents (Group et al., 2009; Relman, 2012).

### **1.1.2 Establishment of gut bacteria**

The long standing belief is that human babies are born sterile and are later inoculated with the bacteria from the mother's vagina if born vaginally or with the bacteria from the mother's skin if born by caesarean section. However, recent studies illustrate the presence of bacteria in cord blood, fetal membranes, amniotic fluid, and baby's first stool sample, questioning the dogma that the fetus is sterile (Jimenez et al., 2005; Jimenez et al., 2008). Labelled gut bacteria administered to pregnant mice are detected in the gut contents of pups delivered by caesarean section, suggesting a vertical mother to baby transmission of gut bacteria (Jimenez et al., 2008). Breastfeeding is an additional route of transmission of maternal microbiota. Human breast milk has the appropriate nutrients to promote the growth of beneficial gut bacterial strains in the baby's gut. In addition, breast milk also has anywhere between 100 to 600 bacterial species, that populate the baby's gut. Thus, by both the internal and external mother to baby transmission, the baby's initial gut bacterial composition is shaped (Jimenez et al., 2008; Funkhouser and Bordenstein, 2013; Jost et al., 2013).

The baby however, does not have a replica of the mother's gut bacteria; instead babies have a signature gut bacterial composition that is very different from their parent's gut bacteria (Palmer et al., 2007). Studies following changes in baby's gut bacteria composition over time have illustrated that they are dynamic, but stabilize and resemble an adult's gut bacteria composition by 3 years of age (Yatsunenکو et al., 2012).

### 1.1.3 Gut bacterial composition and number

There are about  $10^{14}$  microbial cells in the intestinal tract of human. This number is significant when one realizes that the whole human body has only about  $10^{13}$  cells (Luckey, 1972). Sequencing the bacterial 16S rRNA gene has demonstrated that although there are significant inter-individual differences in the number of bacterial species, the gut bacteria are primarily composed of members belonging to the phyla *Bacteroidetes* and *Firmicutes*. Members of the phyla *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* are also seen in the gut bacterial community but in smaller numbers (Eckburg et al., 2005). The ratio of the members of *Firmicutes* to *Bacteroidetes* is often quantified because of its association to increased energy extraction from food (Turnbaugh et al., 2006). Although the gut bacterial community composition of an individual displays some resilience, it can be perturbed by changes in age, diet and use of medications, such as antibiotics.

Infant gut bacteria are dominated by the genus *Bifidobacterium* but its number decreases with age. More interestingly, a decreased number of *Bifidobacterium* in the gut of infants correlates with developing obesity in adults (Kalliomaki et al., 2008). The *Firmicutes* to *Bacteroidetes* ratio is low in infants and in elderly and high in adults (Mariat et al., 2009). Moreover, the diversity of the gut bacterial community decreases with age, which may lead to increased susceptibility to infections and decreased digestive capacity (Woodmansey, 2007).

The gut bacterial community changes rapidly due to changes in the diet. Fat, carbohydrates, plant-based food that is high in fiber, and animal-based food that is high in amino acids are all factors that influence the bacterial population of the gut

(Turnbaugh et al., 2009; Faith et al., 2011). Changing the diet from plant-based to an animal-based food for as little as four days modifies the gut bacteria from a predominant butyrate producing population to a predominant bile acid metabolizing population (David et al., 2014).

The use of antibiotics can also alter the gut bacteria rapidly. After an antibiotic regimen, the gut bacterial community reverts back to its initial state in 3 weeks, although, the reversion process is incomplete and some bacterial species are lost in the process (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011). Sub-therapeutic doses of oral antibiotics have been administered to farm animals to promote growth. Similarly, antibiotic use in humans is associated with obesity (Thuny et al., 2010). Recently, the growth promotion effects of oral sub-therapeutic antibiotics were demonstrated to be due to changes in gut bacteria (Cho et al., 2012).

Dysbiosis or the imbalance in the proportion of beneficial and nonbeneficial bacteria in the gut is associated with a wide variety of human disorders, such as inflammatory bowel disorder (Frank et al., 2007), obesity (Ley et al., 2006) and autism (de Theije et al., 2013; Hsiao et al., 2013). Although, it is clear that the gut bacterial diversity is altered in these disorders, the question that still needs to be answered is whether this change in gut bacteria is the cause or effect of the disease. Furthermore, which group of the bacteria changes in disease states is controversial. For example, Ley et al. demonstrated that the proportion of *Bacteroidetes* ratio decreases and the proportion of *Firmicutes* to *Bacteroidetes* increases in the gut of obese individuals compared to lean individuals (Ley et al., 2006). However, other studies contradict this observation (Duncan et al., 2008; Murphy et al., 2012) and associate obesity with an

increase in certain other groups such as *Lactobacillus* (Armougom et al., 2009; Million et al., 2011) and *Staphylococcus* (Kalliomaki et al., 2008). Another example, is the observation that there is a reduction in the members of the *Firmicutes* and *Bacteroidetes* phyla in the intestinal tract leading in some individuals with Crohn's disease and ulcerative colitis (Frank et al., 2007). However, another study demonstrated an increase in members of the *Bacteroidetes* phyla (the genera *Bacteroides* and *Prevotella*) in inflammation-driven colon cancer (Sobhani et al., 2011; Ahn et al., 2013; Zackular et al., 2013). This emphasizes the fact that although the gut bacterial community is viewed as a pathogen, how gut bacteria may cause the disease in the host is often not clear.

#### **1.1.4 Benefits of gut bacteria**

The benefits of gut bacteria are multifold, ranging from extracting more energy from food to protecting against pathogens. Moreover, there is evidence to show that gut bacteria are important for host nutrition. Gut bacteria have enzymes to synthesize vitamins that the host cannot, especially vitamin K and vitamin B (LeBlanc et al., 2013). Gut bacteria also have enzymes to digest plant fibers and extract energy as short chain fatty acids (SCFAs), such as acetate, butyrate, and propionate from components of food that otherwise cannot be digested by the host (Backhed et al., 2004; Velagapudi et al., 2010). Gut bacteria metabolize primary BAs to secondary BAs. In addition, gut microbes also influence the process of digestion by regulating the intraluminal levels of the neurotransmitters, such as norepinephrine, dopamine, and nitric oxide, thereby

regulating gut motility and gastric emptying (Sobko et al., 2005; Asano et al., 2012; Evans et al., 2013).

Gut bacteria regulate intestinal immune and inflammatory responses. The communication between the gut bacteria-derived products and the toll-like receptors present on the intestinal epithelial cells and immune cells is important for protection against infection and inflammation (Rakoff-Nahoum et al., 2004; Arpaia et al., 2013; Furusawa et al., 2013). Incidentally, exposure to gut bacteria in early life is critical for this protection (Olszak et al., 2012). Gut bacteria also directly confer colonization resistance against pathogens by competing for food and production of acid and antibacterial peptides (Buffie and Pamer, 2013).

The SCFAs that gut bacteria produce are also important for intestinal homeostasis (Rakoff-Nahoum et al., 2004). The majority of the SCFAs produced by gut bacteria is taken up by epithelial cells of the colon and is preferentially used by the colonocytes for energy. SCFA are anti-diarrheal as they promote sodium and water absorption (Scheppach, 1994). SCFAs also regulate the expression of tight cell junction proteins (Wang et al., 2012).

Gut bacteria are seen as a therapeutic target because they can be altered by the administration of antibiotics, probiotics, and prebiotics or by fecal transplantation. Probiotics are defined as “live microorganisms, which when administered in adequate amounts confer health effect on the host” (Shen et al., 2013). Prebiotics are “non-digestible polysaccharides that promote SCFA production and the growth of beneficial gut bacteria, especially *Bifidobacterium* and *Lactobacillus*” (Shen et al., 2013).

**Benefits of modulation of gut bacteria.** Modulation of gut bacteria by antibiotics, probiotics, and prebiotics are being investigated as potential therapies for obesity and other diseases. For example, administration of the antibiotics norfloxacin and ampicillin alters gut bacteria and results in an improvement of glucose tolerance in various rodent models of obesity (Membrez et al., 2008). Treatment of high-fat diet fed mice with the probiotic VSL#3 (commercially available probiotic mixture of *Streptococcus thermophilus*, three strains of *Bifidobacterium* and four strains of *Lactobacillus*) improved glucose tolerance (Ma et al., 2008). Treatment of mice on a high-fat diet with the prebiotic oligofructose, resulted in an increase in *Bifidobacterium* species in the gut and also improved glucose tolerance (Cani et al., 2007).

Very few studies have been done in humans to test whether gut bacteria modulation can treat obesity. However, a recent human trial in the Netherlands demonstrated that transplanting fecal contents from lean donors to obese people resulted in improved glucose tolerance (Vrieze et al., 2012). This observation takes the field one step forward, but a lot needs to be done with respect to identifying the bacterial group or the bacterial byproducts responsible for the therapeutic effect.

### **1.1.5 Germ-free animals as tools to study gut bacteria**

In 1885, Louis Pasteur first proposed that to completely understand nutrition, experiments need to be performed on sterile GF animals. In 1896, Nuttal and Thierfelder generated the first GF animal, a GF guinea pig at the University of Berlin (Glimstedt, 1959; Wostmann, 1996). The following century witnessed a number of GF animal models being generated to study the function of gut bacteria. Almost half a century later, the first GF rats were generated by Gustafsson at the University of Lund

and by Reyniers at the Lobund laboratory at the University of Notre Dame (Gustafsson, 1946; Reyniers et al., 1946; Gustafsson, 1948). Soon thereafter, Pleasants at the Lobund laboratory generated the first GF mice (C3H and Swiss Webster) by maintaining mouse pups born through cesarean section in a sterile chamber, and feeding the newborns a sterile milk formula (Pleasants, 1959). At the present time, GF rats and mice are the preferred choice for research because of the relative ease in housing and handling.

GF mice are ideally suited to study the function of gut bacteria and serve as a valuable research tool to unravel the role that gut bacteria play in normal physiology and pathology. More importantly, GF mice offer a model system that can be infected with the bacteria of choice to study the function of individual bacteria.

However, as a consequence of having no gut bacteria, the anatomy and physiology of GF mice are altered to adapt to life with no microbial exposure. For example, in the gastrointestinal tract; there are many differences in the anatomy and physiology of GF and CV mice. Compared to CV mice, the GF mice have thinner small intestines with a reduced lamina propria and a reduced mucosal surface area, they have decreased number of lymphocytes and macrophages in the intestine, a reduced turnover rate of the intestinal epithelial cells, enlarged cecum, watery intestinal contents with a higher pH, slower gastric emptying, reduced intestinal motility, increased intestinal transit time, increased intestinal absorption of glucose (Abrams and Bishop, 1967; Thompson and Trexler, 1971), and decreased vasculature (Stappenbeck et al., 2002). All these changes need to be considered when interpreting the results obtained from GF mice.

### 1.1.6 Gut bacteria and BAs

BAs made by the host modify gut bacterial composition and gut bacteria modify circulating BA composition in the host. BAs are one of the host factors that shape the gut bacterial community. Therefore, BA feeding has the potential to alter gut microbial composition (Islam et al., 2011). BAs act as hormones, binding to and activating the nuclear receptor FXR. FXR in turn regulates the immune system thereby indirectly regulating gut microbial composition (Devkota et al., 2012).

Diets alter BA composition in bile and BAs alter gut bacterial composition. One view is that, the more animal fat in diet, the more BAs are secreted in bile to digest the fat, and these BAs result in the increase of BA-tolerant bacteria at the expense of other beneficial bacteria (Devkota et al., 2012). For example, consuming a diet rich in milk fat increases taurine-conjugated CA and results in dysbiosis with an overgrowth of BA-resistant bacteria such as *Bilophila wardsworthia* and development of colitis (Devkota et al., 2012). Further, these diet-induced changes in gut bacteria happen rapidly, in as little as four days (David et al., 2014).

Gut bacteria have enzymes to metabolize BAs and change the BA composition in the intestinal content and also in the circulation of the host (Narushima et al., 2000; Narushima et al., 2006). The capacity to metabolize BAs not only provides gut bacteria cellular carbon, nitrogen and sulfur, but also enables them to be bile-resistant and grow in the presence of BAs. Gut bacteria perform the following modifications of BAs in the gut lumen: deconjugation of taurine or glycine-conjugated BAs (by bile salt hydrolase), oxidation and epimerization of the 3-, 7- and 12-hydroxy groups of BAs (by

hydroxysteroid dehydrogenase) and 7-dehydroxylation (by 7-dehydratase). The enzymes bile salt hydrolase and hydroxysteroid dehydrogenase are present in a broad spectrum of gut bacteria, whereas enzymes for 7-dehydratase required to produce DCA and LCA are restricted to a small number of intestinal anaerobes such as *Clostridium* (Ridlon et al., 2006).

The relationship between gut bacteria and BAs is important to understand because modifying gut bacteria can alter BAs and BA signaling, and similarly, altering BAs can modify gut bacteria and their secondary metabolites. Thus, they may be an effective therapeutic strategy to treat obesity (Li et al., 2013), inflammatory bowel disorder, (Trauner et al., 2013), and cancer (Yoshimoto et al., 2013).

### **1.1.7 Gut bacteria and host drug metabolism**

Gut bacteria influence drug metabolism in the following ways (Morgan et al., 2008; Saad et al., 2012):

- 1) They express enzymes that directly metabolize oral drugs and make drugs less or more active.
- 2) They communicate with the immune system and regulate the expression of drug processing genes.
- 3) Their metabolites regulate xenosensing transcription factors in the host and thus regulate expression of drug processing genes.
- 4) Their metabolites compete with drugs for the same host drug metabolizing enzymes for clearance.

There are numerous examples of orally administered drugs being metabolized by gut bacteria. In fact, gut bacteria-mediated reduction of the azo bond is the basis of prodrugs such as sulfasalazine, which upon reduction in the colon gives rise to 5-aminosalicylic acid (anti-inflammatory drug) and sulfapyridine (antibiotic) (Sousa et al., 2008; Stojancevic et al., 2013). Further, consumption of probiotics such as *Bifidobacterium*, *Lactobacillus* and *Streptococcus* can increase the gut bacteria-mediated reduction of sulfasalazine (Lee et al., 2012).

In addition to directly metabolizing orally administered drugs and changing their half-lives and the efficacy of these drugs, gut bacteria also influence the drug metabolizing capacity of the host. Endotoxin from the outer wall of gram-negative bacteria has the capacity to decrease the protein level and activity of major hepatic drug metabolizing enzymes (Ueyama et al., 2005). The antibiotic ciprofloxacin reduces LCA producing gut bacteria (*Clostridium scindens*, *Clostridium sordelli*, and *Bacteroides fragilis*) and decreases the expression of major drug metabolizing enzymes in the liver (Toda et al., 2009b; Toda et al., 2009a).

Therefore, it is important to study the relationship between gut bacteria and host drug metabolism. Gut bacteria are unique for each individual and thus form another variable that determines how an individual will respond to a drug (Wilson, 2009). Metabolic byproducts of gut bacteria can be used to derive correlations to whether a person will metabolize the drug fast or slow in the gut and predict a person's response to a drug (Kaddurah-Daouk et al., 2011). Thus, gut bacterial regulation of the drug metabolizing capacity of the host is certain to become an integral part of personalized medicine.

## **1.2. Bile acids (BAs)**

BAs are amphipathic detergent-like molecules that are synthesized in the liver, secreted in bile, stored in the gallbladder, and released into the duodenum after a meal. About 95% of BAs are absorbed across the intestine, by either passive diffusion or via transporters and are transported back to the liver via the portal circulation. This process is called the enterohepatic circulation. BAs are metabolized not only by the host enzymes but also by enzymes produced by bacteria in the intestine. Historically, BAs were thought to simply act as detergents, forming micelles and trapping lipids, and lipid soluble vitamins from the ingested meal, thereby aiding their absorption. However, BAs have proved to be more interesting with the discovery that BAs activate surface and nuclear receptors that regulate several cellular functions. In the last decade, it was discovered that BAs can regulate whole-body lipids and glucose metabolism through pathways regulated by the BA nuclear receptor, Farnesoid X receptor (FXR) (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999), and the BA cell membrane receptor, Transmembrane G protein-coupled receptor 5 (TGR5) (Maruyama et al., 2002; Kawamata et al., 2003). More recently, there is a growing understanding that individual BAs with different physico-chemical properties also have different binding affinities for the BA receptors and thus have different biological functions (Chiang, 2009).

### **1.2.1 Primary BA synthesis**

BAs are synthesized from cholesterol by enzymes in the liver that convert cholesterol to the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) in humans, and also to muricholic acid (MCA) in mice. The reactions involved include:

- 1) Saturation of the double bond (between 5<sup>th</sup> and 6<sup>th</sup> position) (Fig 1A)
- 2) Epimerization of the 3 $\beta$ -OH group
- 3) Introduction of OH groups at the 6, 7 and 12 positions
- 4) Oxidation and shortening of the side chain (C<sub>27</sub> to C<sub>24</sub>)
- 5) Conjugating the carboxylic group in the side chain with a taurine or glycine moiety.

The conversion of cholesterol to BAs reflects the high capacity of the liver to convert a lipid-soluble substance to a more water-soluble product that can be secreted into bile. Cholesterol has a solubility of approximately 10 nM in water (Renshaw et al., 1983), whereas primary BAs have a solubility of approximately 1.3 M (Small et al., 1966; Shea et al., 2007). Although the type of reactions involved in the formation of BAs are similar to those involved in drug metabolism, namely hydroxylation, oxidation, and conjugation, some reactions in BA synthesis are highly specific and regulated (Staple and Gurin, 1954). The steps involved in the synthesis of primary BAs are shown in Figure 1B. The exact order of steps remains unclear because many of the intermediates serve as substrates for more than one biosynthetic enzyme (Russell, 2003). The synthesis of primary BAs is initiated by either the classic or the alternative pathway till the branch point intermediate is synthesized, which then undergoes a series of enzymatic reactions to be converted to the various primary BAs (Fig 1B).

### **Classic Pathway**

In the classic pathway of BA synthesis, the first step is the conversion of cholesterol to 7 $\alpha$ -hydroxycholesterol by the microsomal cytochrome P450 enzyme (Cyp)

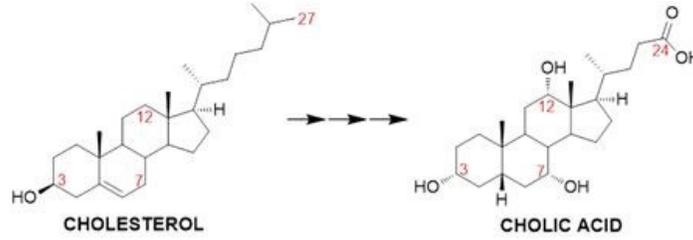
cholesterol 7 $\alpha$ -hydroxylase (Cyp7a1) that is expressed only in liver. Cyp7a1 is the rate-determining enzyme in BA synthesis and expression of Cyp7a1 is highly regulated.

### **Alternative Pathway**

The first step in the alternative pathway is the conversion of cholesterol to either 27-hydroxycholesterol by the enzyme sterol 27-hydroxylase, a mitochondrial cytochrome P450 enzyme (this enzyme can also make 24- and 25-hydroxycholesterol) or 24-hydroxycholesterol by the enzyme oxysterol 24-hydroxylase, a microsomal cytochrome P450 enzyme that is expressed largely in the brain and at much lower levels in the liver (Russell, 2003).

The second step is the 7 $\alpha$ -hydroxylation of the oxysterols. Microsomal enzyme Cyp7b1 performs 7 $\alpha$ -hydroxylation of either 27-hydroxycholesterol or 25-hydroxycholesterol and another microsomal enzyme, Cyp39a1 performs 7 $\alpha$ -hydroxylation of 24-hydroxycholesterol. In mice, about 25-30% of BAs are thought to originate through the alternative pathway whereas in humans only about 5-10% of BAs are thought to be from the alternative pathway (Russell, 2003).

A.



B.

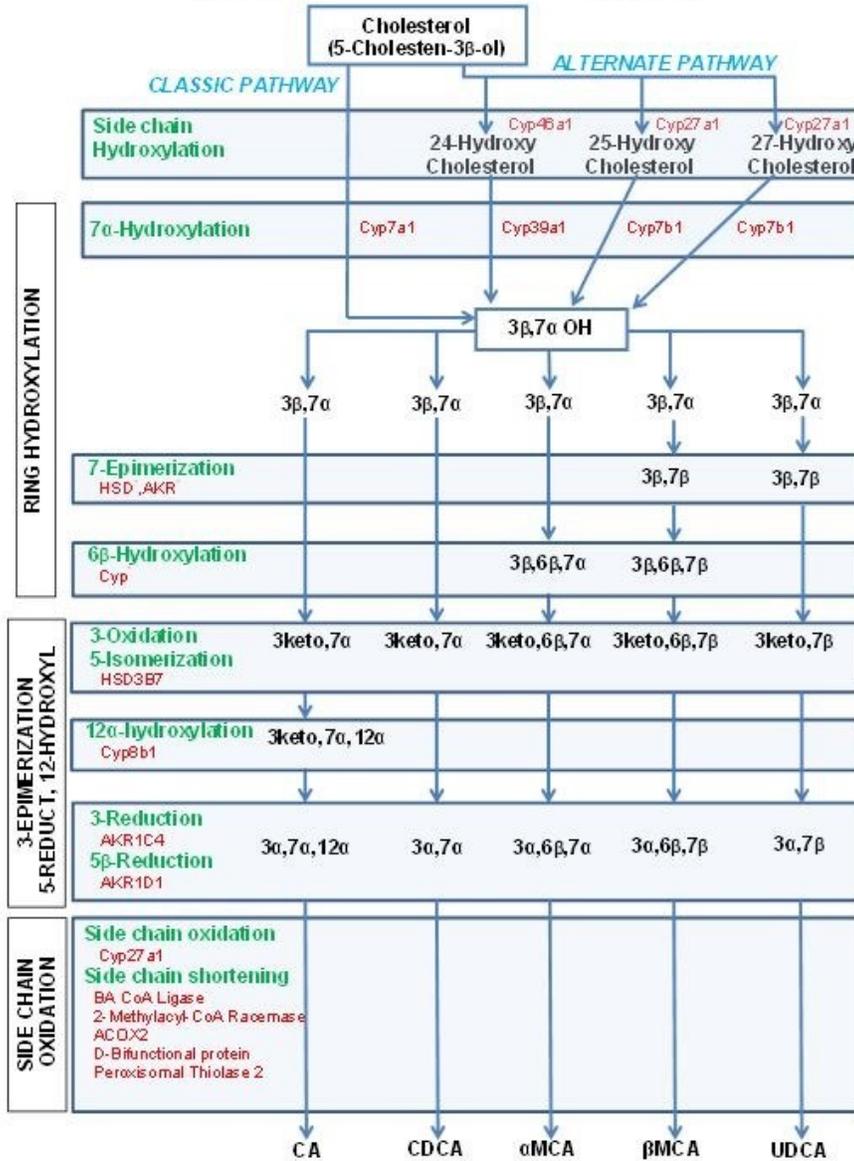


Fig.1.1. A) Conversion of cholesterol to Cholic acid B) General scheme of primary bile acid synthesis from cholesterol in the liver , \* denotes responsible enzyme is not yet identified.

## Other steps in primary BA synthesis

The other ring hydroxylations that occur during the synthesis of primary BAs include 7-epimerization (to convert 7 $\alpha$ -OH to 7 $\beta$ -OH) and 6 $\beta$ -hydroxylation. However, the enzymes that are responsible for these reactions are still unknown. The 7-hydroxylated intermediates derived from cholesterol are substrates for the enzyme 3 $\beta$  hydroxysteroid dehydrogenase 7 (HSD3 $\beta$ 7), which isomerizes the double bond from the 5<sup>th</sup> to the 4<sup>th</sup> carbon and oxidizes the 3 $\beta$ -hydroxyl group to a 3-keto group (Russell, 2003).

The products of the HSD3 $\beta$ 7 enzyme can have two fates. If acted upon by the microsomal enzyme Cyp8b1 the resulting product will have a 12-OH group and will ultimately be converted to CA. In the absence of 12-hydroxylation, the intermediate will ultimately be converted to CDCA (rat, mouse, human),  $\alpha$ - and  $\beta$ - MCA acid (mouse), and ursodeoxycholic acid (UDCA) (bear). The subsequent enzymes in the cascade are cytosolic aldo-keto reductase 1c4 (AKR1c4) and AKR1d1 that reduce the 3-keto group to an alcohol and reduce the double bond between the 4<sup>th</sup> and the 5<sup>th</sup> carbon.

The last step in the primary BA synthesis involves side chain oxidation performed by the enzyme Cyp27a1 and side chain shortening or removal of the terminal three carbon atoms in the peroxisomes by a series of reactions that are similar to  $\beta$ -oxidation of fatty acids. The enzymes involved in the side chain shortening are BA CoA ligase, 2-methylacyl-CoA racemase, branched-chain acyl-coenzyme A oxidase 2 (ACOX2), D-bifunctional protein, and peroxisomal thiolase 2 (Russell, 2003).

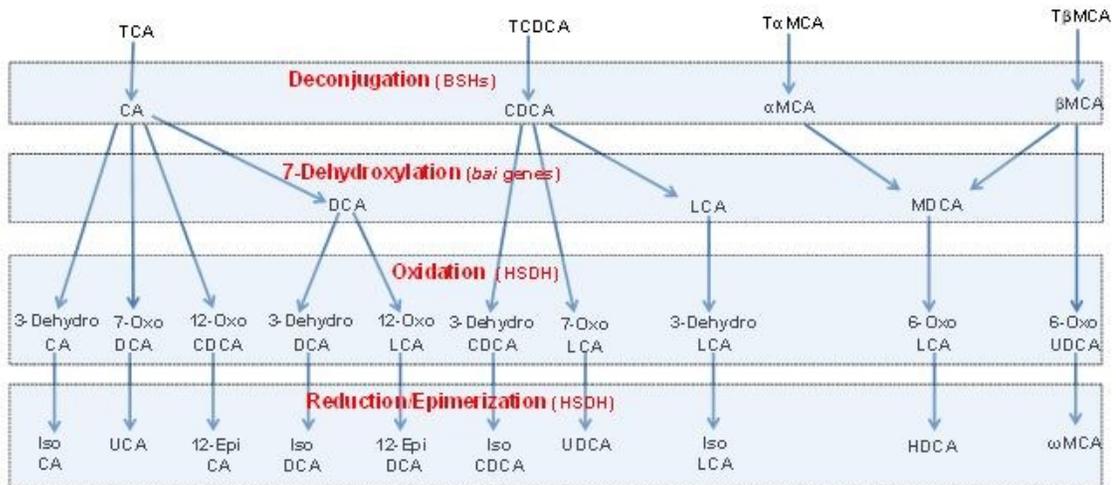


Fig.1.2 Secondary Bile acid metabolism by gut bacteria

Table 1.1 Bile acid nomenclatures and structures (adapted from Zhang et al, 2011)

Bile acids	Abbrev.	3 $\alpha$ 3 $\beta$	6 $\alpha$ 6 $\beta$	7 $\alpha$ 7 $\beta$	12 $\alpha$ 12 $\beta$
<b>Primary bile acids</b>					
cholic acid	CA	OH H	H H	OH H	OH H
chenodeoxycholic acid	CDCA	OH H	H H	OH H	H H
$\alpha$ -muricholic acid	$\alpha$ MCA	OH H	H OH	OH H	H H
$\beta$ -muricholic acid	$\beta$ MCA	OH H	H OH	H OH	H H
<b>7-Dehydroxylation products</b>					
deoxycholic acid	DCA	OH H	H H	H H	OH H
lithocholic acid	LCA	OH H	H H	H H	H H
murideoxycholic acid	MDCA	OH H	H OH	H H	H H
<b>Oxidation products</b>					
3-dehydro-cholic acid	3-DehydroCA	=O	H H	OH H	OH H
7-oxo-deoxycholic acid	7-OxoDCA	OH H	H H	=O	OH H
12-oxo-chenodeoxycholic acid	12-OxoCDCA	OH H	H H	OH H	=O
3-dehydro-deoxycholic acid	3-DehydroDCA	=O	H H	H H	OH H
12-oxo-lithocholic acid	12-OxoLCA	OH H	H H	H H	=O
3-dehydro-chenodeoxycholic acid	3-DehydroCDCA	=O	H H	OH H	H H
7-oxo-lithocholic acid	7-OxoLCA	OH H	H H	=O	H H
dehydro-lithocholic acid	DehydroLCA	=O	H H	H H	H H
6-oxo-lithocholic acid	6-OxoLCA	OH H	=O	H H	H H
6-oxo-ursodeoxycholic acid	6-OxoUDCA	OH H	=O	H OH	H H
<b>Reduction/Epimerization products</b>					
iso-cholic acid	IsoCA	H OH	H H	OH H	OH H
ursocholic acid	UCA	OH H	H H	H OH	OH H
12-epi-cholic acid	12-EpiCA	OH H	H H	OH H	H OH
iso-deoxycholic acid	IsoDCA	H OH	H H	H H	OH H
12-epi-deoxycholic acid	12-EpiDCA	OH H	H H	H H	H OH
iso-chenodeoxycholic acid	IsoCDCA	H OH	H H	OH H	H H
ursodeoxycholic acid	UDCA	OH H	H H	H OH	H H
iso-lithocholic acid	IsoLCA	H OH	H H	H H	H H
hyodeoxycholic acid	HDCA	OH H	OH H	H H	H H
$\omega$ -muricholic acid	$\omega$ MCA	OH H	OH H	H OH	H H

## **BA Conjugation**

In mice, BAs are mainly conjugated with taurine, and in humans with glycine and taurine by an amide linkage to C-24. The liver has a high capacity for conjugation and therefore more than 98% of BAs excreted from the liver are conjugated (Russell, 2003). Conjugation lowers the  $pK_a$  of BAs, enhancing aqueous solubility at the pH of the proximal intestine, and promotes a high intraluminal BA concentration leading to efficient solubilization of lipids and fat-soluble vitamins. Two enzymes catalyze the reactions leading to bile acid amidation. A CoA thioester is first formed by the rate-limiting BA-CoA ligase (BAL) enzyme and then glycine or taurine is coupled to the carboxyl group of the BA in a reaction catalyzed by cytosolic BA-CoA:amino acid *N*-acyltransferase (BAAT) (Setchell et al., 2013).

When the conjugated primary BAs reach the intestine via bile, the gut bacteria deconjugate the conjugated primary BAs and then metabolize them to secondary BAs (Ridlon et al., 2006) (Fig. 1.2). Most of these BAs enter the enterohepatic circulation where they are transported across the intestine and then reenter the liver. The BAL enzyme in the liver efficiently re-conjugates both the primary and secondary BAs (Hubbard et al., 2006).

## **Enzymes important for BA synthesis**

**Cyp7a1:** It is the first enzyme, and the rate determining enzyme, in the classic pathway of BA synthesis. Mutations in the CYP7A1 gene in humans result in a metabolic disorder with increased whole-body cholesterol, increased plasma

triglycerides, and cholesterol gallstones. Fecal BA excretion is 6% of normal, with higher CDCA compared to CA levels, increased CYP27A1 activity, and an induction in the alternate pathway of BA synthesis (Pullinger et al., 2002).

Cyp7a1-null mice have an increased rate of postnatal death. Most pups die within the first 18 days, unless the mothers are supplemented with CA and vitamins (Ishibashi et al., 1996). The enzyme Cyp7b1 (oxysterol 7 $\alpha$ -hydroxylase) of the alternate pathway of BA synthesis is induced between the third and fourth week of life and the alternate pathway takes over BA synthesis in mice that are deficient in the enzyme. Serum cholesterol and triglycerides are similar, whereas vitamin D and E levels are lower in Cyp7a1-null mice (Schwarz et al., 1996). The BA pool size and BA synthesis rate measured as excretion of BAs in feces are both reduced by more than 50% in mice, when Cyp7a1 is absent (Schwarz et al., 2001).

In Cyp7a1-null mice cholesterol feeding and cholestyramine feeding do not affect the rate of BA synthesis, which is mostly through the alternate pathway in these null mice (Schwarz et al., 2001). This indicates that the alternate pathway of BA synthesis is not inducible by changes in enterohepatic circulation of BAs in contrast to the classic pathway. The BA composition in bile and feces are very different in Cyp7a1-null mice compared to controls, with the concentration of CA being decreased more than 50% (Schwarz et al., 1996). The reduced proportion of CA in bile and subsequently in the intestine results in reduced absorption of vitamins D and E and cholesterol across the intestine in Cyp7a1-null mice, and feeding CA restores their uptake (Schwarz et al., 1998). This indicates that the proportion of CA is important for the uptake of cholesterol and fat soluble vitamins across the intestine.

**Cyp27a1:** The sterol 27-hydroxylase enzyme or Cyp27a1 is a mitochondrial enzyme that plays two important roles in BA synthesis:

- 1) it is responsible for the first step in the steroid side chain cleavage to convert  $C_{27}$  cholesterol or its metabolites to  $C_{24}$  BAs.
- 2) it is responsible for the first step in the alternate pathway of BA synthesis by generating oxysterols (25- and 27-hydroxycholesterol) from cholesterol.

Deficiency of the enzyme CYP27A1 in humans leads to an autosomal recessive disease called cerebrotendinous xanthomatosis, a rare sterol storage disease, characterized by progressive neurological dysfunction and premature atherosclerosis. (Cali et al., 1991). CDCA treatment lowers plasma cholesterol levels and decreases accumulation of abnormal BAs by repressing the activity of CYP7A1 via FXR (Honda et al., 2001).

Cyp27-null mice have markedly lower BA synthesis even though Cyp7a1 activity is increased 5-fold. BA synthesis was measured by the conversion of injected radiolabeled  $7\alpha$ -hydroxycholesterol into BAs in bile (Rosen et al., 1998). Cyp27-null mice have a decreased BA pool size, increased proportion of CA, decreased fecal BA excretion, decreased feedback regulation by FXR and decreased intestinal cholesterol absorption. There is however, a compensatory increase in hepatic cholesterol and fatty acid synthesis. Feeding CA to Cyp27-null mice restores normal intestinal absorption of cholesterol and normal hepatic cholesterol, and fatty acid synthesis (Repa et al., 2000). Cyp27-null mice do not develop the disease cerebrotendinous xanthomatosis, because unlike humans, Cyp27-null mice have a microsomal 25-hydroxylation side-chain

cleavage pathway and Cyp7a1 activity is not upregulated as much as in the cerebrotendinous xanthomatosis patients (Honda et al., 2001).

**Cyp7b1:** Oxysterol 7 $\alpha$ -hydroxylase or Cyp7b1 was identified as the 7 $\alpha$ -hydroxylase that is upregulated after 21 days of age in mice. This enzyme is part of the alternate pathway of BA synthesis and its expression is sexually dimorphic being 3-fold higher in male than in female mice (Schwarz et al., 2001).

Defects in the CYP7B1 gene in humans result in severe neonatal cholestasis. The defect in the gene results in the accumulation of high concentrations of hepatotoxic monohydroxy BAs with the 3 $\beta$ -hydroxy- $\Delta^5$  structure and a drastic reduction in normal primary BAs that provide the driving force for bile flow. This is a rare autosomal recessive condition and was fatal in the two infants diagnosed with this defect (Setchell et al., 1998; Ueki et al., 2008). CYP7A1 activity is not detected in livers of infants below one year of age, demonstrating the importance of the enzyme CYP7B1 and the importance of the alternate pathway of BA synthesis in neonates and infants (Gustafsson, 1986; Setchell et al., 1998). The ontogeny of Cyp7a1 and Cyp7b1 in mice and humans seem to be opposite; in mice Cyp7a1 is expressed at birth and Cyp7b1 develops after the third week of age, whereas in humans CYP7B1 is expressed at birth and CYP7A1 develops after 1-2 years of age.

Deficiency of Cyp7b1 in mice results in a much less significant phenotype compared to humans, as loss of Cyp7b1 in mice is compensated for by an increase in the synthesis of BAs by other pathways. There is a 30% increase in Cyp7a1 protein in livers of Cyp7b1-null mice which may result in a 30% increase in the output of BAs from the classic pathway of BA synthesis. The BA pool size, BA composition, intestinal

absorption of cholesterol, serum cholesterol, and serum triglyceride levels were similar in Cyp7b1-null mice and wild-type controls (Li-Hawkins et al., 2000).

**Cyp8b1:** Sterol 12 $\alpha$ -hydroxylase or Cyp8b1 is a microsomal enzyme that hydroxylates C-12 of cholesterol metabolites to form CA. Deletion of the Cyp8b1 gene in mice prevents the synthesis of CA and DCA. The BA synthesis and pool size increase in Cyp8b1-null mice, and the BA composition is altered with a decrease in CA and a compensatory increase in the proportion of  $\alpha$  and  $\beta$  MCA. There is a decrease in small heterodimer partner (SHP) mRNA and protein and a corresponding increase in Cyp7a1 protein and activity (Li-Hawkins et al., 2002). In Cyp8b1-null mice which have a lower proportion of CA, cholesterol absorption across the intestine is less and feeding CA to these mice reverses this situation, indicating that CA is an important determinant of cholesterol uptake across the intestine (Li-Hawkins et al., 2002; Murphy et al., 2005).

**AKRs:** Two AKR (Aldo-keto reductase) enzymes,  $\Delta^4$ -3-oxosteroid,5 $\beta$ -reductase (Akr1d1) and 3 $\alpha$ -hydroxysteroid dehydrogenase (Akr1c4) catalyze the reduction of the double bond at C-4 and the reduction of the 3-keto group to an alcohol in the stereochemical alpha configuration, respectively.

Akr1d1 deficiency in humans leads to neonatal hepatitis and cholestasis. The patients with Akr1d1 deficiency are characterized by decreased primary BA synthesis and accumulation of hepatotoxic  $\Delta^4$ -3-oxo and 5 $\alpha$ -reduced BAs. This deficiency is treated with primary BAs, which normalize liver morphology and function (Drury et al., 2010).

**Bile acid-CoA Ligase (BAL):** BAL is the enzyme that catalyzes the addition of CoA to BAs that will be conjugated with the amino acids taurine or glycine by the enzyme BA-CoA: amino acid *N*-acyltransferase (BAAT) before secretion into bile. Liver has a high capacity for conjugation, and less than 2% of BAs are unconjugated in bile under normal conditions (Matoba et al., 1986; Setchell et al., 2013).

BAL-null mice have normal BA pool size and normal BA concentrations in liver, serum, gallbladder bile, and urine compared to controls. The majority of the BAs are unconjugated in mice with BAL deficiency unlike normal mice in which the majority of the BAs are conjugated with taurine. BAL-null mice also have a small proportion of primary (less than 5% of BAs) but no secondary BAs that are conjugated and these BAs are most likely synthesized *de novo*. This suggests that there is a specific requirement for BAL enzyme in reconjugation of BAs during enterohepatic circulation (Hubbard et al., 2006).

**Bile acid-CoA:amino acid N-acyltransferase (BAAT):** Conjugation of BAs with taurine or glycine lowers the  $pK_a$  of the BA, increasing aqueous solubility, and promotes a high intraluminal BA concentration, and thus, efficient solubilization of lipids with low aqueous solubility, such as saturated fats and fat-soluble vitamins. A genetic defect in BAAT in humans disrupts BA conjugation and causes fat-soluble vitamin deficiency and growth failure, demonstrating the importance of BA conjugation in lipid absorption. In addition, unconjugated BAs are not well transported by canalicular BA transporters and may accumulate in hepatocytes causing injury in some patients (Setchell et al., 2013).

### 1.2.2 Secondary BA synthesis

When BAs pass through the intestine they encounter a low number and low diversity of facultative and anaerobic bacteria in the small intestine, where the major BA metabolism is deconjugation and hydroxyl group oxidation. 7 $\alpha$ -Dehydroxylation of the primary BAs, CA and CDCA produce the secondary BAs, DCA and LCA, respectively, are largely the action of bacteria that are present in the large intestine (Ridlon et al., 2006).

**A) Deconjugation of BAs:** Deconjugation involves the enzymatic hydrolysis of the *N*-acyl amide bond linking BAs (24-C) to taurine or glycine. The enzyme responsible is called bile salt hydrolase (BSH) and has been isolated from several species of gut bacteria. Glycine and taurine can serve as substrates for microbial metabolism. Thus, deconjugation may be a means of obtaining cellular carbon, nitrogen, and sulfur for some gut bacteria. Taurine contains a sulfonic moiety that is reduced and dissimilated to hydrogen sulfide after deconjugation. Hydrogen sulfide is highly toxic and has been implicated in the pathogenesis of colon cancer. Moreover, hydrogen sulfide inhibits butyrate metabolism in colonocytes, which is a key nutrient and regulator of cell turnover in the gut (Ridlon et al., 2006).

**B) Oxidation and epimerization of BAs:** Epimerization of the hydroxyl groups in BAs is performed by specific gut bacterial hydroxysteroid dehydrogenase (HSDH) enzymes. The gut bacteria use the reducing equivalents generated during the oxidation of hydroxyl groups in BAs (Ridlon et al., 2006). 3 $\alpha$ - and 3 $\beta$ -HSDHs catalyze the reversible, stereospecific oxidation/reduction reaction

between 3-keto-BAs and 3 $\alpha$ - or 3 $\beta$ -hydroxy BAs. This enzyme is found in members of the species *Clostridium* and *Ruminococcus* (Ridlon et al., 2006).

The 7 $\alpha$ - and 7 $\beta$ -HSDHs catalyze the reversible stereospecific oxidation/reduction reaction of the 7 $\alpha$  and 7 $\beta$ -hydroxyl groups of BAs. This enzyme is seen in bacteroides and Clostridia, as well as in *E. Coli* and *Ruminococcus* species (Ridlon et al., 2006).

The 12 $\alpha$ - and 12 $\beta$ -HSDHs are responsible for the reversible oxidation/reduction reaction of the 12 $\alpha$ -hydroxyl group of BAs. This enzyme is detected mainly in the members of the genus *Clostridium* (Ridlon et al., 2006).

**C) 7-Dehydroxylation of BAs:** Conjugated primary BAs are the most abundant BAs seen in bile and unconjugated secondary BAs are the most abundant BAs seen in feces. Therefore, the 7 $\alpha$ -dehydroxylation reaction, which forms secondary BAs from primary BAs, is quantitatively the most important bacterial BA biotransformation. It can occur only after deconjugation of the taurine or glycine moiety from the BA and is a multi-step pathway that requires multiple bile acid inducible (*bai*) genes that are often organized as an operon in bacteria. Human gut bacteria capable of BA 7 $\alpha$ -dehydroxylation have been isolated from the colon and classified to the genus *Clostridium* (Wells et al., 2003). The ability to generate secondary BAs by these bacteria must confer a growth advantage to these bacteria compared to bacteria that cannot metabolize BAs (Ridlon et al., 2006).

### 1.2.3 Enterohepatic circulation of BAs

Bile originates in the liver and serves as an excretory fluid and aids digestion. Bile contains BAs, bilirubin, glutathione conjugates, cholesterol, phospholipids-mainly phosphatidylcholine (PC), proteins, electrolytes, such as sodium, potassium, chloride, and bicarbonate, and metabolites of lipophilic xenobiotics or heavy metal cations that the body is trying to eliminate (Klaassen and Watkins, 1984).

The concept of a biliary cycle dates back to the 17<sup>th</sup> century. Mauritius Van Reverhorst, stated that a large volume of bile flow can occur daily, only if bile re-circulates, similar to William Harvey's description of blood flow. Van Reverhorst calculated that the amount of bile entering the duodenum is greater than the amount that is excreted (Reuben, 2005). Therefore, he suggested a "*motus bilis circularis* or an enterohepatic circulation of bile, meaning bile is continuously excreted and reabsorbed" (Van Reverhorst, 1696; Reuben, 2005).

Giovanni Alfonso Borelli explained enterohepatic circulation as we understand it today: bile is made in the liver, flows into the duodenum after a meal and is mixed with the food in the intestine, then most of it passes through the intestine wall to enter the portal vein to be carried back to the liver, and a small amount of bile is eliminated in feces (Reuben, 2005).

Moritz Schiff demonstrated that the daily bile salt secretion rate in bile is increased if bile is returned to the intestine instead of being excreted. (Verkade et al., 1995; Reuben, 2005). BAs are the component of bile that is responsible for the stimulation of bile flow (Foster et al., 1919; Whipple and Smith, 1928). This choleric

responses to BAs is different in various species, and this is due to differences in basal bile production (Klaassen, 1972). The bile flow that originates from the osmotic action of BAs is termed *BA-dependent bile flow*.

Treatment of rats with phenobarbital for 4 days increased bile formation by 50%. Biliary clearance of <sup>14</sup>C-labeled erythritol illustrated that the bile was of hepatocyte origin in both the control and treated rats. However, the concentration of BAs in the treated rats were lower than control and they decreased with time (Klaassen, 1969; Berthelot et al., 1970; Klaassen, 1971). So this portion of the bile flow is not due to the osmotic action of BA and is called *BA-independent bile flow*. The primary osmotic driving force responsible for the BA-independent bile flow is glutathione (Ballatori and Truong, 1992), which is pumped across the apical membrane of the hepatocytes by the transporter Mrp2 (Johnson et al., 2002).

**Bile flow begins in the liver.** The movement of BAs in the enterohepatic circulation begins in the centrolobular hepatocytes where BAs are formed *de novo* and conjugated with taurine or glycine (Hofmann, 1999). Transporters present on the apical membrane of hepatocytes, such as the bile salt export pump (Bsep), with the help of ATP, transport BAs against the concentration gradient into bile canaliculi (Green et al., 2000). The heterodimeric transporter Abcg5/Abcg8 transports cholesterol and plant sterols into the bile canaliculus (Berge et al., 2000; Repa et al., 2002). The canaliculus is surrounded by a spiral ribbon of actin filaments. The osmotic gradient generated by the actively pumped BAs, pulls water and other solutes into the canaliculus, which is then squeezed by the surrounding actin filaments.

BAs then adsorb to the phosphatidyl choline (PC) molecules on the luminal side of the canalicular membrane, and detach the PC molecules on the outer leaflet of the canalicular membrane which are then replaced by the PC floppase Mdr2 (MDR3 in humans). Initially, BAs and PC molecules form vesicles, but when the concentrations of BAs increase, the vesicles fuse to form mixed micelles (Verkade et al., 1995; Hofmann, 1999). The canaliculi empty via the canals of Hering and flow into the biliary tract and reach the gallbladder. After a meal, release of cholecystokinin from the endocrine cells of the intestine causes gallbladder contraction and relaxation of the sphincter of Oddi, causing bile to flow into the intestine (Hofmann, 1999).

Actions of the hepatic BA transporters ensure normal bile flow. Inhibition of PC biosynthesis by feeding a choline-deficient diet or anti-microtubule drugs such as colchicine, lead to inhibition of the biliary phospholipid and cholesterol secretion without affecting that of BAs (Verkade et al., 1995).

**BAs flow through the intestine where they are reabsorbed.** BAs in mixed micelles flow through the intestinal lumen. In the small intestine, BAs being detergent-like promote the solubilization of fatty acids from the diet in mixed micelles, and help them diffuse through the unstirred layer along the intestine wall, increasing lipid absorption (Hofmann, 1999).

Conjugated BAs have a lower pKa and thus are ionized at the pH of the small intestine. So the intraluminal BA concentration is high, being toxic to gut bacteria (Ridlon et al., 2006). The gut bacteria in defense deconjugate the conjugated BAs and then 7-dehydroxylate them to make secondary BAs, which are more lipid-soluble. The

terminal ileum is the site where most BAs are actively transported from the gut lumen into the enterocyte by the apical sodium dependent transporter (Asbt) (Wong et al., 1994). The unconjugated BAs are absorbed by passive absorption across the distal small intestine and large intestine. BAs are transported from inside the enterocyte to the portal vein by the basolateral organic solute transporter  $\alpha$  and  $\beta$  (Ost  $\alpha$  and Ost  $\beta$ ) (Dawson et al., 2005). Less than 5% of BAs are excreted in feces. The fecal loss is replaced by an equal daily *de novo* synthesis of BAs by the liver to maintain the BA pool size.

**BAs return to the liver.** BAs are carried from the intestine to the liver by the portal vein, mostly bound to albumin. Periportal hepatocytes efficiently extract the BAs from blood. Most conjugated BAs are thought to be transported into the hepatocytes by the sodium taurocholate cotransporting polypeptide (NTCP) (Hagenbuch et al., 1991) and unconjugated BAs are transported by the organic anion-transporting polypeptide 1b2 (Oatp1b2) (Csanaky et al., 2011).

Secondary BAs that reach the liver from the intestine are conjugated with taurine or glycine, and at times, even sulfated (especially LCA). If the secondary BAs are conjugated with taurine or glycine and sulfated, they are not efficiently reabsorbed by the intestine and are eliminated in feces. Unconjugated primary BAs returning from the intestine are also re-conjugated in the liver with taurine or glycine, and these BAs reenter the bile completing the enterohepatic circulation.

#### 1.2.4 BA transporters

Enterohepatic circulation of BAs is mediated by the action of specific transporters. Transporters are specialized membrane proteins with extracellular, transmembrane and cytoplasmic domains, which transport hydrophilic solutes across the lipid bilayer of the cell membrane. Transport of BAs is carried out by members of two superfamilies of transporters: 1) ATP-binding cassette transporters (ABC transporters) and 2) Solute carrier transporters (Slc transporters).

*Note: Homologous transporters in humans and mice having the same name are differentiated by referring to the human transporters in all capitalized letters and the mouse transporters have only the first letter capitalized.*

#### **BA Transporters in hepatocytes**

The basolateral membranes of hepatocytes have both uptake and efflux BA transporters. The BA uptake transporters on the basolateral surface of the hepatocytes extract BAs from the hepatic sinusoids. The efflux transporters on the basolateral membrane pump excess BAs out of hepatocytes into blood, which would be subsequently taken in by downstream hepatocytes to facilitate a process called “hepatocyte hopping” to prevent the periportal hepatocytes from being saturated with BAs (van de Steeg et al., 2010). Basolateral BA uptake transporters include Ntcp and Oatp1b2 and basolateral BA efflux transporters include Mrp3, Mrp4, and Ost $\alpha$ -Ost $\beta$ . The BA concentrations within the hepatocytes are presumed to be in the micromolar range and the canalicular BA concentrations are about 1000-fold higher, therefore necessitating active transport across the apical membrane (Dawson et al., 2009). The

apical BA efflux transporters include Bsep and Mrp2, which transport BAs into bile canaliculi. No apical BA uptake transporter has been identified on hepatocytes.

**A. Sodium taurocholate cotransporting polypeptide (Ntcp):** The transporter Ntcp (*Slc10a1*) is present on the basolateral membrane of hepatocytes (Stieger et al., 1994; Aleksunes et al., 2006) and is thought to be responsible for the sodium-dependent uptake of conjugated BAs in a sodium-dependent manner from portal blood. Ntcp was first cloned from rat liver (Hagenbuch et al., 1991).

Currently no human diseases are known that are caused by mutations in the *SIC10A1* gene (Shneider et al., 1997; Carlton et al., 2003; Stieger, 2011). This could be either because NTCP expression is so important that its absence would lead to early death, or there is another transporter that mediates hepatic uptake of BAs. However, down regulation of the *SLC10A1* gene and protein expression are seen in several pathophysiological conditions, such as progressive obstructive cholestasis due to biliary atresia (Shneider et al., 1997).

Animal models of cholestasis, such as bile-duct ligation also lead to a decrease in the expression of the *Slc10a1* gene, probably to prevent the liver from accumulating toxic BAs (Donner et al., 2007; Slitt et al., 2007). Ntcp shows gender divergent expression in mice. Female mice have higher Ntcp protein and mRNA levels in liver, and this is due to the inhibitory effect of male-pattern growth hormone secretion (Cheng et al., 2007). BA homeostasis in Ntcp-null mice has not yet been characterized.

**B. Organic anion transporting polypeptides (Oatps):** The transporters OATP1B1 and OATP1B3 in humans and its homolog in rodents Oatp1b2 (*Slco1b2*) are located on the basolateral membrane of hepatocytes and play an important role in the sodium-independent uptake of endogenous substrates, such as BAs into hepatocytes (Jacquemin et al., 1994). These Oatps were isolated exclusively in human and rodent liver and so was previously termed liver-specific organic anion transporter (LST-1/Lst-1) (Abe et al., 1999). Humans with low-activity OATP1B1 polymorphisms have higher serum BAs, indicating that OATP1B1 plays an important role in the hepatic uptake of BAs in humans (Xiang et al., 2009). OATP1B3 may play a role in the hepatic uptake of BAs in humans, but polymorphisms in the *S/CO1B3* gene have not been reported.

Oatp1b2-null mice have 3- to 45-fold higher unconjugated BAs in serum. They also have decreased clearance of intravenously injected CA but not TCA. This demonstrates that Oatp1b2 in mice is important for the basolateral uptake of unconjugated BAs (Csanaky et al., 2011).

**C. Multidrug resistance-associated proteins (Mrps):** Several members of the Mrp family are expressed in hepatocytes. Mrp2 (*Abcc2*) is important for BA transport. Mrp2 is present on the canalicular (apical) membrane of hepatocytes (Scheffer et al., 2000) and is responsible for the efflux of sulfated and/or glucuronidated BAs. Mrp2 is a major determinant of BA-independent bile flow by transporting glutathione.

Human MRP2 can transport UDCA and TUDCA when expressed in Sf9 cell membranes (Gerk et al., 2007). Mutations in the MRP2 transporter in

humans are linked to the Dubin-Johnson syndrome, characterized by reduced efflux of conjugated bilirubin into bile (Kartenbeck et al., 1996; Paulusma et al., 1997).

Mrp2-null mice exhibit biochemical abnormalities that are similar to those described in Dubin-Johnson syndrome patients, such as increased serum and urine bilirubin glucuronide, increased hepatic glutathione concentrations, and reduced bile flow rate (Chu et al., 2006; Kruh et al., 2007).

**D. Bile salt export pump (Bsep):** The transporter Bsep (*Abcb11*), which was originally called “sister” of P-glycoprotein, is localized on the apical or canalicular membrane of hepatocytes (Childs et al., 1998; Jansen et al., 1999; Green et al., 2000) and functions to efflux conjugated BAs from hepatocytes into bile. Bsep was first identified in a pig liver cDNA screening (Childs et al., 1995) and in a rat hepatoma cell line that was resistant to the methyl ester of glycocholic acid (Brown et al., 1995). The full-length cDNA of Bsep was cloned from rat liver (Gerloff et al., 1998). Humans with mutations in BSEP that lead to the absence of BSEP function develop progressive familial intrahepatic cholestasis type 2 (PFIC2), which often progresses to hepatic failure. Total BA secretion in these patients is only 1% of normal (Jansen et al., 1999), with a decrease in synthesis of both CA and CDCA. Administration of UDCA helps to improve the liver function of some of these patients (Takahashi et al., 2007). This demonstrates that there is no backup in humans for BSEP’s function of transporting BAs from the hepatocytes into bile (Stieger, 2011).

Knockout of Bsep in mice led to some unexpected findings. Bsep-null mice have decreased secretion of BAs into bile, but that did not affect bile flow in these mice, which is surprising because BAs are assumed to be the major driving force for bile flow. Although canalicular secretion of conjugated TCA and other hydrophobic BAs decreased in Bsep-null mice, the mice displayed only mild nonprogressive cholestasis. In humans, absence of BSEP causes a more severe progressive cholestasis. This species difference could be due to the presence of alternate BA canalicular transport system to transport hydrophilic BAs in mice and not in humans (Wang et al., 2001a).

### **BA transporters in cholangiocytes**

Cholangiocytes express the BA uptake transporter Asbt on the apical side to reabsorb BAs from bile and the BA efflux transporter Ost $\alpha$ -Ost $\beta$  on the basolateral side to transport the reabsorbed BAs into blood. The descriptions of Ost $\alpha$ / $\beta$  are included in the next section.

### **BA transporters on intestinal enterocytes**

Enterocytes express the BA uptake transporter Asbt on the brush border membrane and BA efflux transporters Ost $\alpha$ -Ost $\beta$  on the basolateral side. Asbt facilitates the reabsorption of conjugated BAs from the intestinal lumen into the enterocytes. Ost $\alpha$ -Ost $\beta$  effluxes BAs absorbed from the intestinal lumen into the portal vein to be carried back to the liver.

**A. Apical sodium-dependent BA transporter (Asbt):** The first step in BA reabsorption in the intestine is the active uptake of BAs across the apical

membrane of enterocytes by the transporter Asbt (*Slc10a2*) (Wong et al., 1994). In addition, Asbt is also localized on the apical membrane of large cholangiocytes in intrahepatic bile ducts and plays a role in cholehepatic circulation of BAs (Alpini et al., 1997; Lazaridis et al., 1997). The cholehepatic shunt/circulation was proposed to explain the hypercholerisis induced by some unconjugated BAs, and it refers to the reuptake of BAs from the bile duct lumen by cholangiocytes and the return of those BAs to hepatocytes (Yoon et al., 1986; Gurantz et al., 1991).

Human ASBT has a higher affinity for dihydroxy BAs and taurine- and glycine-conjugated BAs compared to trihydroxy BAs and unconjugated BAs (Craddock et al., 1998). Mutations in human ASBT, leads to primary BA malabsorption, refractory infantile diarrhea, steatorrhea, interruption of enterohepatic circulation of BAs, reduced plasma cholesterol levels and growth defects (Heubi et al., 1982; Oelkers et al., 1997). This underscores the importance of ASBT's role in the reuptake of BAs from the intestine.

Mice deficient in *Asbt* have decreased or very minimal intestinal BA absorption, 10- to 20-fold increase in fecal BA excretion, 80% reduction in BA pool size despite an increase in BA synthesis and a BA pool enriched in CA (Dawson et al., 2003). Treatment with a BA-binding resin, cholestyramine did not further increase fecal BA excretion in these *Asbt*-null mice. This demonstrates that there is no or very minimal alternative (non-*Asbt*) mechanism for intestinal BA absorption in mice (Dawson et al., 2009).

**B. Organic solute transporters (Ost):** BAs that are reabsorbed from the intestinal lumen are translocated to the basolateral membrane by the intestinal bile acid binding protein and then effluxed across the basolateral membrane by the heterodimer Ost $\alpha$ -Ost $\beta$  transporters. Ost $\alpha$  and  $\beta$  were first cloned from a liver cDNA library of the marine skate (Wang et al., 2001b). The two subunits of the transporter Ost $\alpha$  and Ost $\beta$  are expressed together in enterocytes, hepatocytes, and cholangiocytes and localize to the basolateral membrane in cells that express Asbt on the apical membrane (Ballatori et al., 2005; Dawson et al., 2005). Interaction between the Ost $\alpha$  and Ost $\beta$  protein is required for the proper delivery to the plasma membrane, and heterodimerization is important for their transport function (Li et al., 2007). No inherited defects have been reported for the OST $\alpha$  and OST $\beta$  genes in humans (Dawson et al., 2009).

Ost $\alpha$ -null mice have undetectable Ost $\beta$  levels. Absence of the basolateral BA transporters Ost $\alpha$  and  $\beta$  results in impaired BA transport across the basolateral membrane of enterocytes, altered feedback regulation of BAs, decreased hepatic BA synthesis leading to increased plasma cholesterol levels and decreased BA pool size (Rao et al., 2008).

### **1.2.5 Regulation of BA synthesis**

Early research revealed that cholesterol and BAs in portal blood regulate the synthesis of primary BAs. Later, it was found that cholesterol feeding, BA feeding, and cholestyramine treatment could induce the activity of the enzyme cholesterol 7 $\alpha$ -hydroxylase or Cyp7a1, which is the rate-limiting enzyme of primary BA synthesis (Danielsson and Sjovall, 1975; Russell, 2009). The 3'-untranslated region in the Cyp7a1

mRNA transcripts are unusually long and the mRNA has a very short half-life of about 30 mins. BAs via the BA response element present on the 3'-untranslated region can reduce Cyp7a1 mRNA stability (Agellon and Cheema, 1997; Chiang, 2009).

FXR was discovered as an orphan receptor that forms a heterodimer complex with RXR (Retinoid X receptor) and was activated by farnesol and its metabolites (Forman et al., 1995). In 1999, three laboratories found BAs to be endogenous ligands of this orphan nuclear receptor, and by binding to FXR, BAs were able to regulate their own synthesis, transport and cholesterol metabolism (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999)

In humans, functional variants of FXR have been associated with susceptibility to developing intrahepatic cholestasis of pregnancy and cholesterol gallstone formation (Van Mil et al., 2007; Kovacs et al., 2008). Synthetic FXR agonists such as INT-747, 6-ECDCA (6 $\alpha$ -ethyl-chenodeoxycholic acid) and GW4064 protect animal models of cholestasis, liver fibrosis, and diabetes (Pellicciari et al., 2002; Liu et al., 2003; Fiorucci et al., 2004; Zhang et al., 2006; Gadaleta et al., 2010). Hence, FXR agonists are considered potential therapeutic drugs. FXR present in the liver and FXR present in the ileum can both regulate enzymes involved in BA synthesis and BA transporters.

**A) FXR-SHP-LRH-1 liver pathway:** FXR is a nuclear receptor activated by BAs that plays a critical role in the feedback regulation of BA synthesis as well as BA-mediated regulation of glucose and lipid homeostasis. When BA concentrations increase in the enterohepatic circulation, the excess BAs binds to the nuclear receptor FXR in hepatocytes, and that leads to an increase in expression of short

heterodimer partner (SHP), an atypical nuclear receptor lacking a DNA binding domain. SHP then binds to and antagonizes the binding of the transcription factors liver receptor homolog-1 (LRH-1) and hepatocyte nuclear receptor 4 $\alpha$  (HNF4 $\alpha$ ) to the promoter region of the target gene Cyp7a1, thus repressing the expression of Cyp7a1 and decreasing BA synthesis (Chiang, 2009) (see Fig.1.3, pathway I). Other target genes of FXR signaling in liver include suppression of Cyp8b1, and induction of BSEP and Ost $\alpha$ -Ost $\beta$  transporters.

Dr. Gonzalez's group has generated liver specific FXR-null mice and compared them to whole-body FXR-null mice to understand the importance of liver-specific FXR regulation in BA homeostasis. From that mouse model, it is known that Cyp8b1, Bsep and SHP are targets of FXR activation in the liver, whereas Cyp7a1 is not (Kim et al., 2007; Kong et al., 2012).

**FXR-FGF15/19-FGFR4/ $\beta$ -klotho intestinal pathway:** Excess BAs also bind to the nuclear receptor FXR in the ileum, which leads to an increase in synthesis and secretion of fibroblast growth factor 15 (Fgf15) in mice (or FGF19 in humans). Fgf15 secreted from the intestine, reaches the liver and suppresses BA synthesis by binding to the heterodimer receptor composed of FGF receptor 4 (FGFR4) and  $\beta$ -klotho expressed on hepatocytes. Activation of the FGFR4 +  $\beta$ -klotho complex stimulates the c-jun *N*-terminal kinases (JNK) and extracellular-signal-regulated kinase (ERK) pathways and SHP, eventually suppressing Cyp7a1. Using an intestinal specific FXR-null mouse model, it was demonstrated that FXR regulation in the intestine is important for hepatic Cyp7a1 regulation (see Fig.1.3, pathway II).

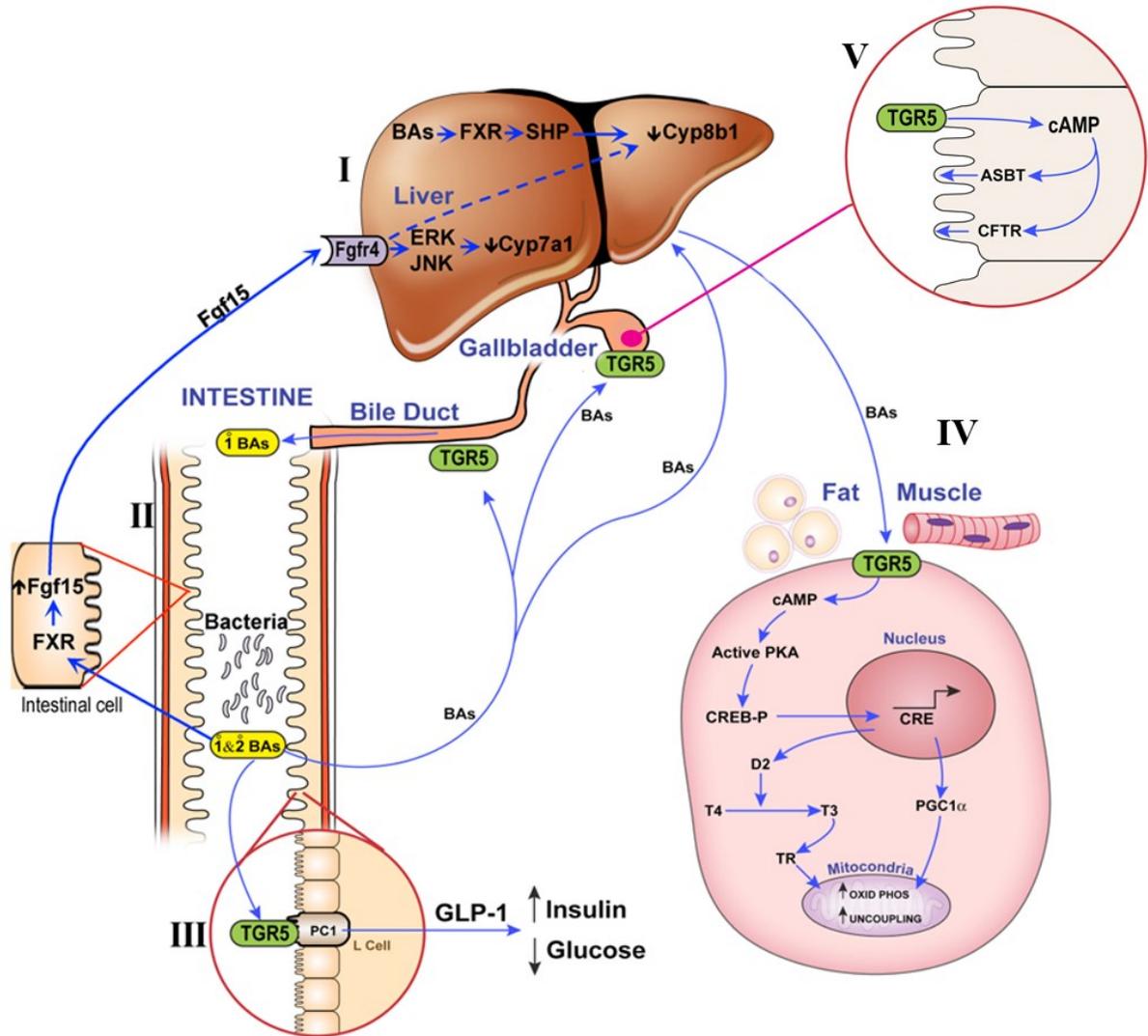


Fig.1.3. Schematic diagram depicting BA signaling through FXR (pathway I and II) and TGR5 (pathway III, IV and V).

Targets of intestinal FXR regulation include Fgf15, SHP, Ileum BA binding protein (IBABP), and organic solute transporter Ost $\alpha$ -Ost $\beta$  in the ileum and hepatic Cyp7a1 (Kim et al., 2007; Matsubara et al., 2013).

### **1.2.6 BA signaling through TGR5**

TGR5, also known as M-BAR (Membrane-bound BA receptor) or Gpbar-1 (G protein-coupled BA receptor) or BG37 is a plasma membrane-bound G protein-coupled BA receptor. Two independent groups discovered TGR5 by searching GenBank database for DNA sequence coding for novel GPCRs. BAs are the endogenous ligand for TGR5, and upon binding to the receptor, increases intracellular cAMP (Maruyama et al., 2002; Kawamata et al., 2003).

TGR5 was found to be expressed ubiquitously in human tissues including heart, skeletal muscle, spleen, kidney, liver, small intestine, colon, placenta, leukocytes, thymus, and lung (Maruyama et al., 2002). In mice, TGR5 is expressed in jejunum, stomach, kidney, spleen, lung, white and brown adipose tissue, muscle, brain, heart, enteroendocrine cells in ileum and colon, gallbladder epithelial cells, sinusoidal endothelial cells, and kupffer cells in liver (Maruyama et al., 2006; Vassileva et al., 2006; Keitel et al., 2007; Keitel et al., 2008; Reimann et al., 2008; Keitel et al., 2010).

TGR5 gene variants have been identified in patients with primary sclerosing cholangitis, which is an inflammatory disease of the bile ducts, indicating that TGR5 may contribute to disease susceptibility (Keitel and Haussinger, 2012).

TGR5 agonists such as 6 $\alpha$ -ethyl-23(S)-methyl-cholic acid (EMSA or INT-777) are promising drug candidates to treat metabolic disorders such as type 2 diabetes, obesity,

and steatohepatitis as they improve glucose tolerance and reduce inflammation and steatosis in mice (Keitel and Haussinger, 2012).

### **TGR5 activation and GLP-1 secretion**

TGR5 can promote glucagon-like peptide-1 (GLP-1) secretion from the murine enteroendocrine cell line STC-1 upon activation by BAs (Katsuma et al., 2005; Thomas et al., 2009). Patients treated with a BA-binding resin to reduce cholesterol levels also had improved glycemic control, and these BA-binding resins also improved hyperglycemia in rodent models of obesity (Kobayashi et al., 2007; Chen et al., 2010). TGR5 overexpressing mouse models demonstrate enhanced GLP-1 secretion and insulin release in response to a glucose load, and TGR5-null mice have impaired glucose tolerance (Thomas et al., 2009). Thus, BAs through their receptor TGR5 regulate GLP-1 release and subsequently regulate glucose homeostasis (see Fig.1.3, pathway III).

Oral administration of glucose results in more insulin release compared to intravenous glucose administration. Glucagon-like peptide-1 (GLP-1) is an incretin or gut derived hormone that is released in response to nutrient sensing and increases the insulin release after a meal. GLP-1 is a potent stimulator of insulin release and also reduces gastric motility, suppresses appetite, slows gastric emptying, increases hepatic fatty acid oxidation, and reduces hepatic gluconeogenesis.

**GLP-1 discovery.** GLP-1 was discovered after cloning and sequencing mammalian proglucagon genes and cDNAs (Mojsov et al., 1987). The levels of intracellular cAMP and activation of cAMP/PKA signaling are major determinants of

intestinal proglucagon gene expression. The proglucagon mRNA is translated into a single 180 amino acid precursor protein that undergoes tissue specific posttranslational processing to produce specific peptides in pancreas, intestine, and brain (Drucker and Brubaker, 1989; Drucker et al., 1994).

**GLP-1 biosynthesis.** Prohormone convertase 1 (PC1) is important for proglucagon processing in the intestine and PC1-null mice have a marked decrease in posttranslational products of the proglucagon gene (Ugleholdt et al., 2006). Posttranslational products of proglucagon in the enteroendocrine cells of the intestine are: glicentin, oxyntomodulin, GLP-1, intervening peptide-2, and GLP-2. The physiological actions of glicentin and the intervening peptide-2 are not known. Oxyntomodulin inhibits gastrointestinal secretion and motility, stimulates pancreatic secretions and glucose uptake. GLP-2 up-regulates intestinal glucose transport, and stimulates cell proliferation and inhibits apoptosis in the intestinal crypt compartment (Baggio and Drucker, 2007).

**GLP-1 forms.** Multiple forms of GLP-1 are secreted *in vivo*: GLP-1 (1-37) and GLP-1 (1-36) NH<sub>2</sub> are inactive, whereas GLP-1 (7-37) and GLP-1 (7-36) NH<sub>2</sub> are biologically active and are products of PC1/3 cleavage (Baggio and Drucker, 2007). The enzyme peptidyl-glycine  $\alpha$ -amidating monooxygenase is responsible for the addition of the amide group to GLP-1, which may enhance the survival of GLP-1 in plasma (Wettergren et al., 1998). The major GLP-1 in human circulation is GLP-1 (7-36) NH<sub>2</sub> (Orskov et al., 1994).

**GLP-1 clearance.** GLP-1 (7-36) has a penultimate alanine residue and thus is rapidly inactivated by a ubiquitous proteolytic enzyme dipeptidyl peptidase-4 [(DPP-4) DPP-4 is also known as CD26] to bioinactive GLP-1 (9-36) amide or GLP-1 (9-37). DPP-4 is found lining the blood vessels near the intestinal mucosa adjacent to the sites of GLP-1 secretion; therefore GLP-1 has a half-life of 2 minutes due to the action of DPP-4 (Baggio and Drucker, 2007). The C-terminal degradation product of GLP-1, the nonapeptide GLP-1(28-36) amide, can also repress hepatic gluconeogenesis involving the activation of the cAMP/PKA signaling pathway, and reduced body weight gain in diet-induced obese mice (Ip et al., 2013), raising concerns about the physiological action of the other degradation products of GLP-1.

**GLP-1 receptor.** GLP-1 receptor (GLP-1R) belongs to the class B family of 7-transmembrane-spanning, heterotrimeric G-protein-coupled receptor. Upon activation there are increases in PKA activity, Akt and AMPK phosphorylation, and a PKA-dependent increase in peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) activity. GLP-1R is expressed in a wide range of tissues including pancreatic islets, kidney, stomach, intestine and several regions of the hypothalamus, and brain. The presence of GLP-1R in liver was questionable until a recent study demonstrated the presence of GLP-1R in both human and rodent hepatocytes, proving GLP-1 has a direct effect on hepatocytes (Svegliati-Baroni et al., 2011).

**Therapeutic use of GLP-1.** GLP-1 has beneficial effects in the treatment of type-2 diabetes. Recent reports also suggest that GLP-1 can also be beneficial in treating fatty liver disorders. The only problem is the short half-life of GLP-1. Several strategies have been tried and tested to help sustain a therapeutic concentration of

GLP-1 in serum. The DPP4-inhibitor sitagliptin and GLP-1R agonist (or GLP-1 mimetics) exenatide and liraglutide are licensed drugs to treat type 2 diabetes. They do not cause hypoglycemia like the insulin secretagogues, but they create concerns because of their proliferative effects in the pancreas. Another recent approach is the continuous expression of GLP-1 *in vivo* by a gene therapy strategy, and it has shown promise in maintaining normoglycemia and aids in the treatment of type-2 diabetes in rodent models (Lee et al., 2007).

### **TGR5 regulation of energy expenditure**

BAs bind to TGR5 expressed in brown adipose tissue and skeletal muscle, and increase intracellular cAMP, which in turn increases type 2 iodothyronine deiodinase (D2) activity. D2 is a thyroid hormone activating enzyme, which increases uncoupling protein (UCP)1 and UCP3, and increases energy expenditure as heat (Watanabe et al., 2006). Treating diet-induced obese mice with a TGR5 agonist INT-777, reduced body weight gain and increased energy expenditure in brown adipose tissue by increasing the gene expression of the genes D2, UCP1, and UCP3 (Thomas et al., 2009). Thus, activation of the BA receptor TGR5 influences energy expenditure in skeletal muscle and brown adipose tissue (see Fig.1.3, pathway IV).

### **TGR5 and gallbladder**

TGR5 is expressed highly in the apical membrane of epithelial cells in the gallbladder (Keitel et al., 2009). Cholesterol precipitates in bile when its concentration increases relative to BAs and phospholipids and formation of cholesterol microcrystals from supersaturated bile is a critical step in gallstone formation. In supersaturated bile,

cholesterol is kept in vesicles consisting of cholesterol and phospholipids, and in TGR5-null mice, the bile had an unexplained increase in the proportion of phospholipids, and thus kept cholesterol in vesicles and prevented microcrystal formation. (Vassileva et al., 2006). Thus, TGR5-null mice that were fed a lithogenic diet were protected from cholesterol gallstone formation (Vassileva et al., 2006).

In the gallbladder, TGR5 agonists increase cAMP levels and decrease intracellular chloride concentrations by increasing chloride secretion via the cAMP-regulated chloride channel, that is the cystic fibrosis transmembrane conductance regulator (CFTR) (Keitel et al., 2009). TGR5-null mice have reduced gallbladder size. TGR5 agonist INT-777 increases gallbladder filling and gallbladder size in control mice but not in TGR5-null mice, illustrating that TGR5 activation promotes smooth muscle relaxation in gallbladder leading to gallbladder filling (Li et al., 2011) (see Fig.1.3, pathway V).

### **1.3 Drug-metabolizing enzymes in liver**

A xenobiotic is any substance that is found in an organism that is not normally produced by the organism. Xenobiotic biotransformation/detoxification occurs via a series of enzyme-catalyzed processes that alter the physiochemical properties of xenobiotics. As it 'filters' the blood coming from the intestines, the liver is the primary organ responsible for detoxification. It contains a large number of enzymes dedicated for xenobiotic detoxification with the ultimate goal of making the xenobiotic more water-soluble, and hence easier to eliminate from the body. These enzymes are called drug-

metabolizing enzymes and can be classified as phase-1 and phase-2 enzymes (Parkinson and Ogilvie, 2013).

### 1.3.1 Phase-1 enzymes

Phase-1 enzymes perform hydrolysis, reduction, and oxidation reactions and as a consequence, this either increases or decreases the toxicity of a xenobiotic. Some examples of phase-1 drug metabolizing enzymes are summarized below:

**Carboxylesterases.** Carboxylesterases (Ces) are enzymes with broad substrate specificities that cleave carboxylic esters to the corresponding alcohol and carboxylic acid. Ces help hydrolyze prodrugs with ester groups (increases hydrophilicity of drug) such as the cancer chemotherapeutic drug CPT-11 to its active form SN38 (Redinbo and Potter, 2005).

**Epoxide hydrolases.** Epoxide hydrolases (Ephx) catalyze the addition of water to epoxides to form dihydrodiols. Ephx play an important role in detoxifying electrophilic epoxides that could bind to proteins and nucleic acids causing cell damage and genetic mutations. Ephx1 is a microsomal enzyme whereas Ephx2 is a soluble enzyme (Seidegard and Ekstrom, 1997).

**Aldo-keto reductases.** Aldo-keto reductase (Akr) enzymes are cytosolic NADPH-dependent oxidoreductases that catalyzes the reduction of aldehydes and ketones to primary and secondary alcohols respectively (Chen and Zhang, 2012).

**Quinone reductases.** Quinone reductase (Nqo) is a cytosolic, NADPH-dependent enzyme. Nqos can catalyze a two-electron reduction and detoxify a quinone to a hydroquinone or catalyze a one-electron reduction and convert a quinone into a semiquinone free radical (Parkinson and Ogilvie, 2013).

**Aldehyde dehydrogenases.** Aldehyde dehydrogenase (Aldh) enzymes, as the name suggests catalyze the oxidation of aldehydes such as acetaldehyde to carboxylic acids (acetate) and water. The mitochondrial Aldh2 and cytosolic Aldh1a1 are responsible for ethanol clearance. Polymorphisms in ALDH2 gene result in loss of enzyme activity and lead to the alcohol flushing syndrome (Stagos et al., 2010).

**Aldehyde oxidases.** Aldehyde oxidase (Aox) is a cytosolic molybdozyme drug metabolizing enzyme. Aox are homodimers and require FAD for their activity. Substrates of Aox include xenobiotics with aldehyde groups, nitro/nitroso groups or heterocyclic moieties (Pryde et al., 2010).

**Flavin monooxygenases.** Flavin monooxygenase (Fmo) is a FAD-containing monooxygenase that catalyzes NADPH-dependent N- or S-oxygenation of xenobiotics that possess heteroatoms. Many reactions catalyzed by Fmos can also be catalyzed by cytochrome P450s. Fmos are found in the endoplasmic reticulum (Cashman, 2005)

**Cytochrome P450s.** Cytochrome P450 (Cyp) enzymes are responsible for 75-80% of all phase-1 drug metabolism reactions and for the clearance of 65-70% of clinically used drugs. Cyp enzymes are grouped into families based on their amino acid homology. The first three families (Cyp1, Cyp2, and Cyp3) are generally involved in the metabolism of xenobiotics, and Cyp families with higher numbers are usually involved in endobiotic metabolism (Sim and Ingelman-Sundberg, 2010). Cyp enzymes are found in the endoplasmic reticulum (most important for xenobiotic metabolism) and in mitochondria (most important for endobiotic metabolism). NADPH-cytochrome P450 reductase (POR) is the obligate redox partner of all Cyps. Deletion of the POR leads to suppression of all Cyp enzyme activity (Gu et al., 2003).

### 1.3.2 Phase- 2 enzymes

Phase-2 enzymes perform conjugation reactions that generally results in decreased toxicity of xenobiotics. Furthermore, conjugation in general markedly increases the water-solubility, and promotes the excretion of the xenobiotic (exceptions include methylation and acetylation). The major phase-2 enzymes in liver include uridinediphosphate (UDP)-glucuronosyltransferases, sulfotransferases, and glutathione S-transferases.

**UDP-glucuronosyltransferases (UGTs).** UGTs are enzymes found in the endoplasmic reticulum and cytosol, that catalyze the transfer of a glucuronic acid from the cosubstrate UDP-glucuronic acid (UDPGA) to the xenobiotic with functional groups such as aliphatic alcohols or phenols, carboxylic acid, primary or secondary amines, and free sulfhydryl groups. Glucuronide conjugates of xenobiotics are polar and are eliminated in urine or bile. If eliminated in bile, some gut bacteria with the enzyme  $\beta$ -glucuronidase can hydrolyze the glycosidic bond between the glucuronic acid and the xenobiotic, thus delaying the elimination of the xenobiotic because it might enter the enterohepatic circulation (Parkinson and Ogilvie, 2013).

**Sulfotransferases (Sults):** Sults catalyze the transfer of a sulfonic acid group from the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the xenobiotic with an alcohol functional group. Sults can be either in the cytosol or bound to the golgi membranes. However, the cytosolic Sults are responsible for the sulfation of drugs and other xenobiotics. Sulfonic acid conjugated xenobiotics are excreted mainly in urine (Parkinson and Ogilvie, 2013).

**Glutathione-S-transferases (Gsts):** Gsts are enzymes that catalyze the transfer of the sulfhydryl group from the cofactor glutathione (GSH) to xenobiotics with halide, sulfate, sulfonate, phosphate, or a nitro group attached to an allylic or benzylic carbon atom, arene or alkene oxides, or electrophilic heteroatoms (O, N, and S). Gsts are localized in the cytosol (involved in xenobiotic metabolism), endoplasmic reticulum or in the mitochondria. The cofactor GSH is a tripeptide composed of glycine, cysteine, and glutamic acid. Glutamate-cysteine ligase catalytic subunit (Gclc) is the rate limiting enzyme of glutathione synthesis (Parkinson and Ogilvie, 2013).

### 1.3.3 Regulation of hepatic drug metabolizing enzymes

**Nuclear receptors as xenosensors.** Nuclear receptors are proteins found in the cytosol or nucleus of cells that upon activation act as transcription factors and induce the expression of a battery of its target genes. Some nuclear receptors act as sensors that can monitor the presence of a xenobiotic and induce drug metabolizing enzymes and transporters to detoxify and eliminate the xenobiotic. Some xenobiotics activate the xenobiotic sensor and thus induce the expression of the gene and subsequent drug metabolizing enzyme.

The xenobiotic sensors that can regulate hepatic drug metabolizing enzymes include steroid receptors such as estrogen and androgen receptors; nonsteroid receptors, such as constitutive androstane receptor (CAR, NR1I3), pregnane X receptor (PXR, NR1I2), and peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ , NR1C1); transcription factor, aryl hydrocarbon receptor (Ahr); and oxidative stress sensor, nuclear factor erythroid 2-related factor 2 (Nrf2, Nfe2L2). CAR, PXR, and PPAR $\alpha$

function by heterodimerizing with the retinoid X receptor (RXR, NR2B1). Ahr functions by binding to the aryl hydrocarbon receptor nuclear translocator (ARNT) (Omiiecinski et al., 2011; Aleksunes and Klaassen, 2012). Table 1.2 shows a list of some target genes of xenosensors (Aleksunes and Klaassen, 2012).

**Table 1.2 Examples of xenosensor target genes.**

<b>Xenosensor</b>	<b>Examples of target genes</b>
Ahr	Cyp1a1, Nqo1, Aldh7a1, Ugt1a1, Ugt1a6, Ugt1a9, Sult5a1, and Gstm3
CAR	Cyp2b10, Aldh1a1, Aldh1a7, Ugt1a1, Ugt2b34, Sult1e1, Sult3a1, Sult5a1, Papps2, Gsta1, Gsta4, and Gstm1-4
PXR	Cyp3a11, Ugt1a1, Ugt1a5, Ugt1a9, Gsta1, and Gstm1-3
PPAR $\alpha$	Cyp4a14, Aldh1a1, mGst3, and Gstm4
Nrf2	Nqo1, Aldh1a1, Gsta1, Gsta4, Gstm1-4, and mGst3

**CHAPTER 2**  
**STATEMENT OF PURPOSE**

## Statement of purpose

BAs are hormones; they circulate in the blood and regulate host physiology by activating the FXR and TGR5 receptors. The diversity of BAs in the circulation is influenced by their metabolism by the liver as well as by gut bacteria. Similarly, the disposition of drugs and other xenobiotics are also influenced by metabolism of the drugs by the liver as well as by gut bacteria.

There is a growing appreciation of gut bacteria as being an influential player in human physiology. Dysbiosis or alterations in the gut bacterial community are associated with many diseases. Altering gut bacteria by probiotics, prebiotics, and by fecal transplantation is viewed as a valuable therapeutic strategy for diseases associated with dysbiosis. Alterations in gut bacteria will result in alterations in the host BA profile and the host drug metabolizing capacity.

Mouse models have shed light on the players and pathways involved in BA and drug metabolism. To understand how certain bacteria regulate BA and drug metabolism, it is important to know how BAs and drug metabolism are altered in GF mice, in which gut bacteria are absent. Studying GF mice will reveal target genes that are altered by gut bacteria. Therefore, the **objectives** of this dissertation are to:

1. Determine the alterations in BA metabolism and BA signaling in GF mice.
2. Determine the alterations in hepatic drug metabolizing enzymes at the transcriptome level in GF mice.

In **Specific Aim 1**, I determined the changes in BA homeostasis and BA signaling in GF mice. This was accomplished by establishing a GF animal facility to

breed and raise GF mice. The BA composition in the various BA enterohepatic tissue compartments (liver, gallbladder bile, ileum, and serum) and feces were compared between CV and GF mice. **Chapter 4** summarizes the differences in BA composition in the different tissues and mRNA expression of 1) enzymes involved in BA synthesis, 2) BA transporters, and 3) genes involved in BA feedback regulation in CV and GF mice.

Further, **Specific Aim 1** also involved determining the differences in BA mediated TGR5 signaling in CV and GF mice by quantifying the downstream targets of TGR5 receptor activation. A striking observation was that GF mice have enlarged gallbladders, a phenomenon that was demonstrated to be possibly due to TGR5 activation. Another major observation regarding BAs in GF mice was the increase in UDCA, which so far was thought to be a secondary BA made with help of gut bacteria. Because GF mice do not have gut bacteria to synthesize secondary BAs, our findings suggest that UDCA most likely must be a primary BA made in the livers of mice.

To further support this suggestion, I studied the formation of UDCA by incubating microsomal fractions from livers of both CV and GF mice with other primary BAs, to determine whether enzymes present in the liver microsomes can biotransform primary BAs to UDCA. **Chapter 4** also describes the observations that demonstrate that this is indeed the case and support the finding that UDCA is a primary BA in mice.

In **Specific Aim 2**, I determined the alterations in hepatic drug metabolizing enzymes at the transcriptome level by quantifying their mRNA levels in the livers of GF and CV mice by RNA-Seq. **Chapter 5** describes the observed differences in mRNA expression of phase-1 and phase-2 enzymes. To determine whether these mRNA

changes resulted in changed enzyme activities *in vivo*, I then performed the pentobarbital sleeping test in GF and CV mice.

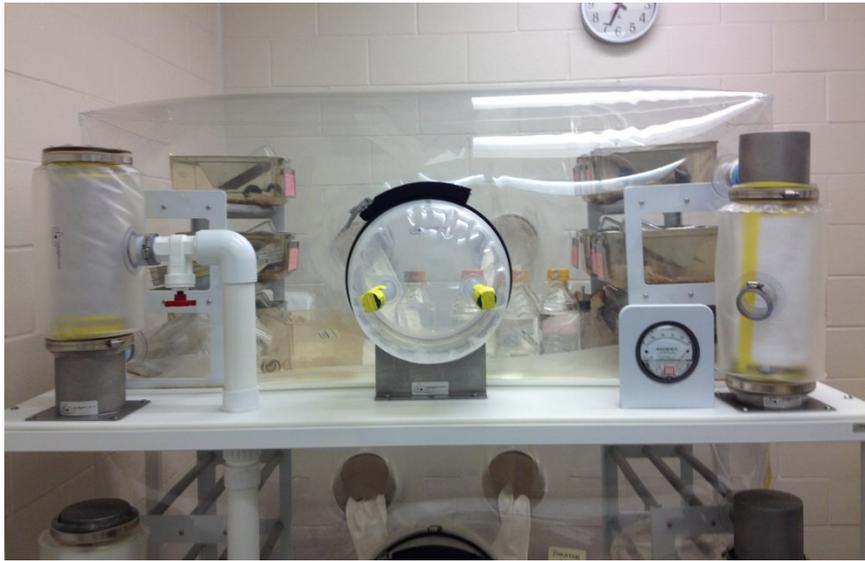
**CHAPTER 3**  
**MATERIALS AND METHODS**

### **3.1 Germ-free isolator, supplies and sterility testing**

Vinyl flexible film isolators were purchased from Class Biologically Clean Ltd. (Madison, WI) to house the GF mice (Fig 3.1.A, B). The GF mice received autoclaved, irradiated Harlan Sani chips (#7990.BG Irradiated Teklad Sani Chips, Harlan Teklad, Madison, WI) as bedding and autoclaved paper towels or autoclaved nestlets as enrichment. The GF mice received autoclaved breeder or regular chow (autoclavable Rodent diet # 5K67, or autoclavable mouse breeder diet # 5021, Labdiet, Richmond, IN) and autoclaved water. The autoclave conditions for feed were standardized in Dr. Dan Peterson's Germ-free animal facility at the University of Nebraska to minimize nutrient loss from food and prevent vitamin deficiency. The supplies were autoclaved and transferred using a supply cylinder (Fig 3.1.C, D).

The GF status of the mice were monitored by sampling the feces and water in the drinking bottles and culturing them to detect the presence of bacteria in the following ways : 1) Fluid thioglycolate medium at 37°C for a week (detects facultative anaerobes) (Fig 3.2.A), 2) Brucella agar with 5% horse blood plates and placing them in Anaerobic pouches at 37°C for a week (detects anaerobes) (Fig 3.2.B), 3) Sheep blood agar plates at 37°C for a week (detects enterobacteria) (Fig 3.2.C) and 4) LB agar plates at room temperature for a week (detects environmental bacteria) (Fig 3.2.D). Ammonia is released from the bacterial breakdown of urea in urine, therefore ammonia levels were monitored in the germ-free room using ammonia test strips (Fig 3.2.E).

**A**



**B**



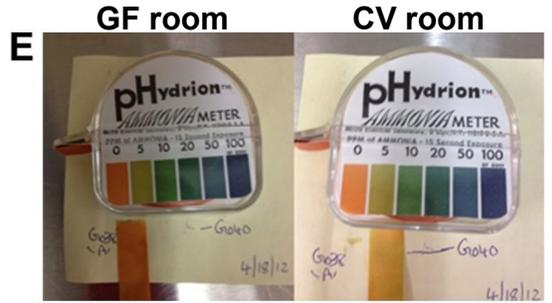
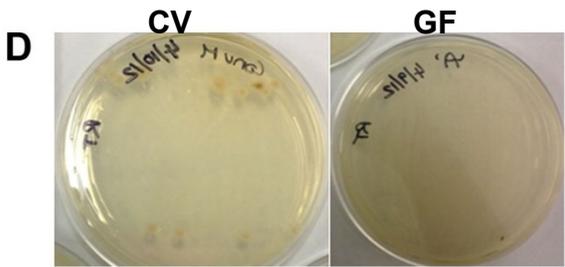
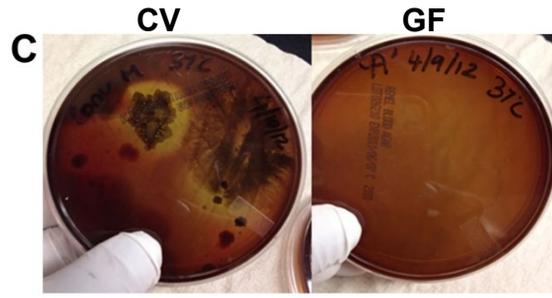
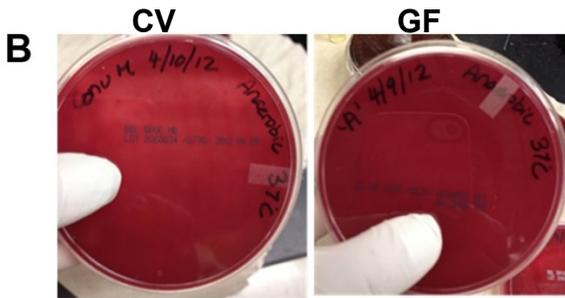
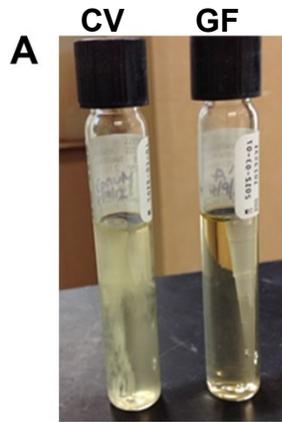
**C**



**D**



**Fig 3.1. Pictures of the GF isolator and supply cylinder.** (A) Front view of the germ-free isolator. (B) Side view of the germ-free isolator. (C) Front view of the supply cylinder with autoclaved cage units. (D) Side view of the supply cylinder.



**Fig 3.2. Representative pictures of the results of Sterility Testing.** Feces from GF mice and water from their drinking bottles were tested routinely for the presence of bacteria. The results obtained with GF and CV mice are shown. (A) Fluid thioglycolate medium incubated for at 37°C for a week. (B) Brucella agar with 5% horse blood plates incubated in anaerobic pouches at 37°C for a week. (C) Sheep blood agar plates incubated at 37°C for a week, and (D) LB agar plates incubated at room temperature for a week. (E) Ammonia test strips representing ammonia levels in the GF and CV mice room. GF represents germ-free mice and CV represents conventional mice.

### **3.2 Animals**

All mice used in the studies were about 3-months old unless otherwise mentioned. All mice were housed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)-accredited facility at the University of Kansas Medical Center, with a 14-h light/10-h dark-cycle, in a temperature- and humidity-controlled environment, and *ad libitum* access to water. The initial breeding colony of GF C57BL/6J/UNC mice was established with mice purchased from the National Gnotobiotic Rodent Resource Center (University of North Carolina, Chapel Hill). All conventional mice were purchased from Jackson Laboratories and received autoclaved rodent diet and autoclaved water for a week before and during the study. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

### **3.3 Chemicals and reagents**

The sources of individual BA standards and internal standards were described previously by Zhang and Klaassen (Zhang and Klaassen, 2010). The monoclonal mouse anti-rat Cyp2b1/2b2 antibody, which also detects mouse Cyp2b10, was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). A Cyp3a11 antibody was a generous gift from Dr. Xiaochao Ma (University of Pittsburgh). Secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO). The glucagon-like peptide-1(GLP-1) total ELISA kit was purchased from EMD Millipore (St. Charles, MO). The fluid thioglycollate medium, brucella agar plates with 5% horse blood and anaerobic pouches were purchased from BD (Sparks, MD), sheep blood agar plates were purchased from Remel (Lenexa, KS) and ammonia strips were purchased from

Microessential Laboratory (Brooklyn, NY). All other chemicals and reagents, unless indicated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

### **3.4 Bile collection**

Five-month old CV and GF mice were used for bile collection. The protocol for bile duct cannulation and bile collection was standardized in our laboratory (Csanaky et al., 2011). Mice were anesthetized intraperitoneally with a ketamine/midazolam mixture (100 and 5 mg/kg, respectively), and the common bile duct of each mouse was cannulated through a high abdominal incision with the shaft of a 30-gauge needle attached to PE-10 tubing. Bile was collected in the dark for 40 mins into preweighed microcentrifuge tubes on ice. The volume of bile was determined gravimetrically, using the value 1 for specific gravity.

### **3.5 Pentobarbital sleeping test**

The pentobarbital sleeping test was performed as described previously (Bjorkholm et al., 2009). Briefly, the anesthetic pentobarbital was injected intraperitoneally into CV and GF mice (50 mg/Kg), and the time between losing and then regaining the righting reflex was quantified.

### **3.6 Animal sacrifice and tissue collection**

All animal sacrifices and tissue collections were performed between 9:00 am and noon to minimize the variations in drug metabolizing enzyme gene expression and BA metabolism due to the circadian rhythm (Zhang et al., 2009; Zhang et al., 2011b).

### **3.7 Bile acid extraction**

Internal standards (40 µg/mL d4-G-CDCA and 20 µg/mL d4-CDCA in MeOH) were added to the samples, and BAs were extracted from serum, gallbladder bile, livers,

biliary bile, ileum, and feces using methods reported previously by our laboratory (Alnouti et al., 2008; Zhang and Klaassen, 2010; Zhang et al., 2011a; Zhang et al., 2012).

### **3.8 BA Quantification by Ultra-performance Liquid Chromatography-Tandem**

#### **Mass Spectrometry (UPLC-MS/MS)**

BAs were quantified by UPLC-MS/MS using methods reported previously by our laboratory (Zhang and Klaassen, 2010). BAs quantified include: TCA, TDCA, TCDCA, TLCA, TUDCA, T $\alpha$ MCA, TMDCA, T $\beta$ MCA, T $\omega$ MCA, CA, DCA, CDCA, LCA, UDCA,  $\alpha$ MCA, MDCA,  $\beta$ MCA, and  $\omega$ MCA. The concentrations of individual BAs were summed to derive the concentrations of conjugated, unconjugated, and total BAs in each compartment.

### **3.9 Preparation of hepatic microsomal fractions**

Hepatic microsomal fractions were isolated following a previously published protocol (MacGeoch et al., 1984). Briefly, livers were homogenized with a glass dounce homogenizer on ice with the homogenization buffer (sucrose, 250 mM; Tris-HCl, 50 mM, pH 7.4; EDTA (ethylenediaminetetraacetic acid), 1 mM; PMSF (phenylmethylsulfonyl fluoride), 0.2 mM) and centrifuged at 10,000xg for 25 min at 4°C. The supernatant was decanted and centrifuged at 100,000 g for 60 min at 4°C. The pellet was washed with potassium chloride Buffer (KCl buffer) (KCl, 150 mM; Tris-HCl, 10 mM, pH 7.4; EDTA, 1 mM) and again centrifuged at 100,000xg for 60 min at 4°C. The resulting microsomes were resuspended in resuspension buffer (Tris-HCl, 10 mM, pH 7.4; EDTA, 1 mM; PMSF, 0.2 mM; glycerol, 20% w/v). Protein concentrations were

determined using the bicinchoninic acid assay (BCA assay) according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL).

### **3.10 Microsomal biotransformation assay**

Final reaction mixtures contained CDCA, 200  $\mu$ M; 0.5 mg of liver microsomal protein; 100 mM potassium phosphate buffer, pH 7.4 and 2 mM NADPH in a final volume of 250  $\mu$ L. After preincubation of microsomal protein and CDCA for 10 min at room temperature, reactions were initiated with NADPH and incubated for 60 min at 37°C. Reactions were terminated by adding 1 mL of ice-cold acetonitrile, dried under vacuum and resuspended in 80  $\mu$ L of 50% methanol. Tubes were vortex-mixed and then centrifuged at 20,000xg for 10 min. The BAs were quantified using the method reported previously by our laboratory (Zhang and Klaassen, 2010). UDCA formation was reported as the ratio of the area under the curve (AUC) of UDCA formed to the AUC of CDCA used in the incubation mixture.

### **3.11 GLP-1 quantification**

Total serum GLP-1 was quantified using an ELISA kit from EMD Millipore (St. Charles, MO) following the manufacturer's protocol. The antibody pair used in the assay is specific for GLP-1(7-36) and (9-36), and has no cross-reactivity with GLP-2, GIP, glucagon or oxyntomodulin.

### **3.12 RNA isolation**

Total RNA was isolated from tissues using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. Quality of RNA was assessed by running the sample on a denaturing agarose gel and visualizing two

discrete 18S and 28S ribosomal RNA bands, with the 28S band double the intensity of the 18S band.

### **3.13 Multiplex Suspension Assay**

The mRNAs of the BA biosynthetic enzymes and BA transporters in liver and ileum described in Chapter 4 were quantified using Panomics 2.0 QuantiGene Plex technology (Panomics/Affymetrix Inc., Fremont, CA), following the manufacturer's protocol. Individual gene information can be found on Panomics web site (<http://www.panomics.com>) with Panel numbers 21330 and 21383. Fluorescence was analyzed using a Bio-Plex 200 system array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex data manager software 5.0 (Bio-Rad, Hercules, CA). The mRNAs of target genes were normalized to the housekeeping gene 60S ribosomal protein L13a (Rpl13a).

### **3.14 cDNA library preparation and RNA-sequencing**

The cDNA library preparation and sequencing of the transcriptome were performed with the help of the KUMC-Genome Sequencing Facility. The cDNA libraries from total RNA samples were prepared using an Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA). The average size of the cDNAs were approximately 160bp (excluding the adapters). The cDNA libraries were validated for RNA-integrity and quantity using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) before sequencing. The cDNA libraries were clustered onto a TruSeq paired-end flow cell and sequenced (2×50bp) using a TruSeq SBS kit (Illumina, San Diego, CA) on the Illumina HiSeq2000 sequencer (KUMC – Genome Sequencing Facility) with a multi-plex strategy of 4 samples per lane.

### **3.15 RNA-Seq Data Analysis**

After the sequencing platform generated the sequencing images, the pixel-level raw data collection, image analysis, and base calling were performed by Illumina's Real Time Analysis (RTA) software on a Dell PC attached to the HiSeq2000 sequencer. The base call files (\*.BCL) were converted to qseq files by the Illumina's BCL Converter, and the qseq files were subsequently converted to FASTQ files for downstream analysis. The RNA-Seq reads from the FASTQ files were mapped to the mouse mm10 reference genome and the splice junctions were identified by TopHat. The output files in BAM (binary sequence alignment) format were analyzed by Cufflinks to estimate the transcript abundance and the differential expression (Cuffdiff, FDR-BH<0.05). The mRNA abundance was expressed in FPKM (fragments per kilobase of exon per million reads mapped).

### **3.16 Reverse Transcription and Real-time PCR Analysis**

Total RNA was transcribed to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit 1001073 (Applied Biosystems, Foster City, CA). The resulting cDNA products were amplified by PCR, using Power SYBR Green PCR Master Mix in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers for all real-time PCR reactions were synthesized by Integrated DNA Technologies (Coralville, IA).

### **3.17 Western blotting**

Western blots of Cyp2b10 and Cyp3a11 were performed as previously described with minor modifications (Renaud et al., 2011). Liver homogenates were prepared in radioimmunoprecipitation assay buffer (RIPA buffer) (Sigma-Aldrich, St. Louis,

MO). Protein concentrations were determined using the BCA assay reagents according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The samples were subjected to polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were stripped and reprobed with  $\beta$ -actin antibody as the loading control. Proteins were detected using chemiluminescence (Pierce Biotechnology, Rockford, IL). Intensities of protein bands were quantified using Image J software (National Institutes of Health, Bethesda, MD).

### **3.18 Statistical Analysis.**

Data are presented as mean  $\pm$  SEM. Asterisks (\*) represent significant differences between CV and GF mice, determined by Student's *t*-test ( $p < 0.05$ ).

**CHAPTER 4**  
**ALTERATIONS IN BILE ACID HOMEOSTASIS AND BILE ACID SIGNALING IN**  
**GERM-FREE MICE.**

## I. BACKGROUND

Bile acids are amphipathic cholesterol metabolites that are synthesized in the liver and secreted into bile. BAs are metabolized by gut bacteria to produce secondary BAs. Most of the BAs are reabsorbed from the intestine and enter the portal vein. Therefore, the BA profile in a person is the net result of the host hepatic enzyme activity and the gut bacterial enzyme activity. The BA profile is important because, in addition to helping in the absorption of fat and fat soluble vitamins, BAs also regulate host physiology. Therefore, alterations in gut bacteria can result in alterations in BA profiles leading to alterations in host physiology. The best animal model to study the function of gut bacteria is GF mice, and this dissertation provides the first information on BA homeostasis in GF C57BL/6 mice, which is the most widely used mouse strain for derivation of transgenic mouse models.

Therefore, the **purpose of this study** is to determine the alterations in BA homeostasis and BA signaling in GF C57BL/6 mice. The **hypothesis** I tested is that absence of gut bacteria would alter BA homeostasis. Therefore, a gnotobiotic facility was established to breed and raise GF mice used for the study.

## II. RESULTS

### A. General characterization of GF C57BL/6 mice

The GF mice are a unique animal model as they are raised in an artificially clean enclosure, preventing exposure to microorganisms from the environment, food, and water. To better understand the differences between GF and CV mice it is important to characterize the general features of our GF mice.

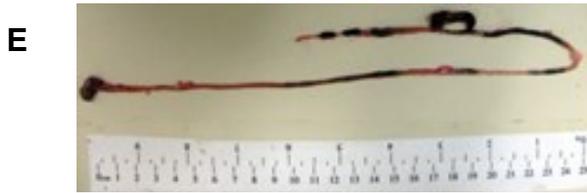
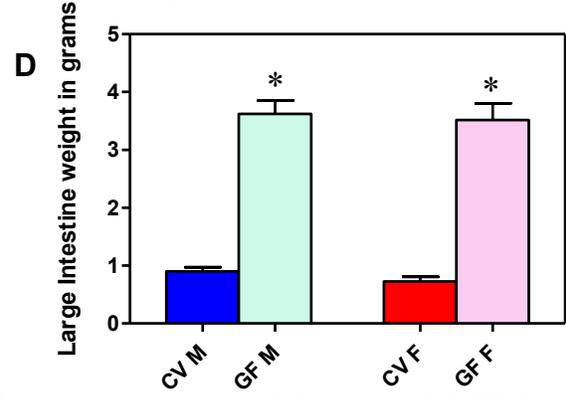
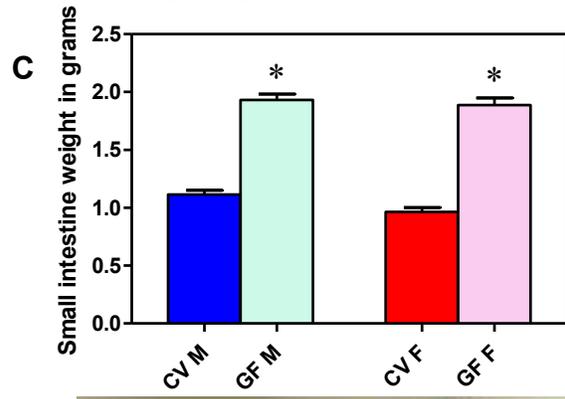
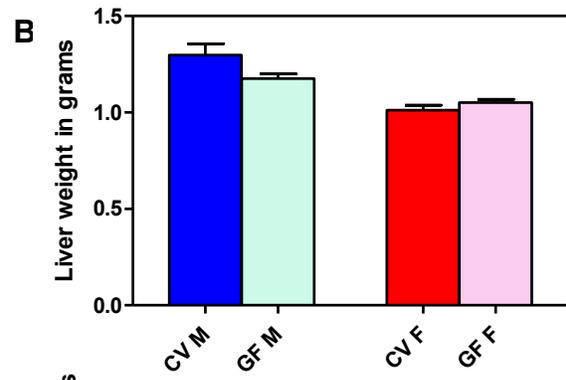
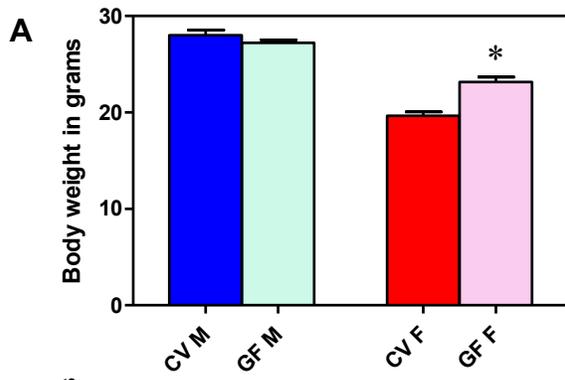
The body weights of adult (3-months old) GF male mice are comparable to the body weights of adult CV male mice. However, adult GF female mice tend to be heavier than the CV mice at the same age (Fig. 4.1.A). Both male and female GF mice have similar liver weights as CV mice (Fig. 4.1.B). The liver weight to body weight percentage is similar in male CV and GF mice but is lower in female GF mice compared to CV ( 5.1% in CV and 4.6% in GF female mice) (data not shown).

The GF mice display enlarged cecum and watery intestinal contents as previously described in the literature (Asano, 1969). The small and large intestines of GF mice weigh more than the intestines of CV mice (Fig.4.1.C and D) and the intestinal tract of GF mice is longer than in CV mice (representative pictures in Fig.4.1.E and F).

The other distinguishing feature between GF and CV mice is the gallbladder. GF mice have a gallbladder that is about 4-5 fold larger than in CV mice (Fig. 4.1.G). Bile was collected over a 40-min period from five-month-old male and female CV and GF mice. Bile flow was increased in GF male mice compared to CV mice. In female GF mice, bile flow was increased compared to CV mice but due to increased variation within the group this difference was not statistically significant (Fig. 4.1.H). The increased bile flow could explain the increased gallbladder size seen in GF mice.

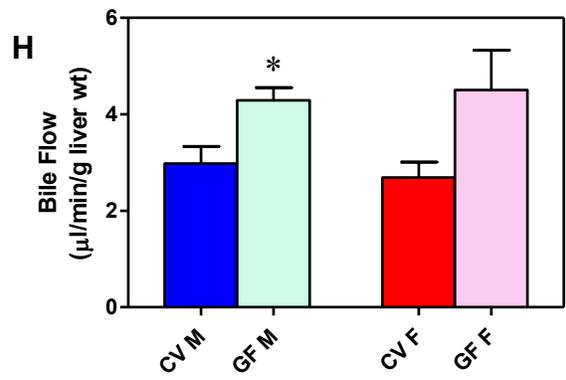
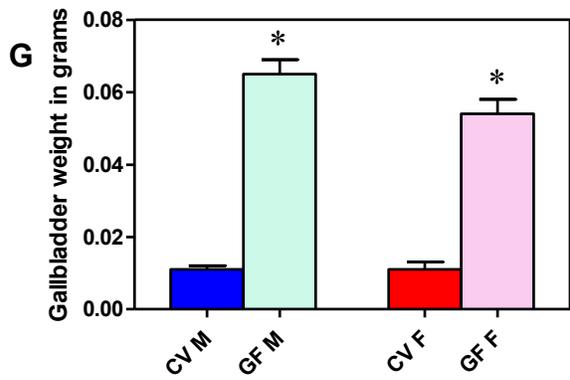
#### **B. BA concentrations in the various BA enterohepatic compartments of CV and GF mice.**

Individual BAs have physicochemical properties based on their structure. Increase in BAs that have a -OH group in the 12 position (12-OH BAs such as CA and DCA), increase the cholesterol absorption across the intestine (Li-Hawkins et al., 2002).



CV mouse intestine

GF mouse intestine

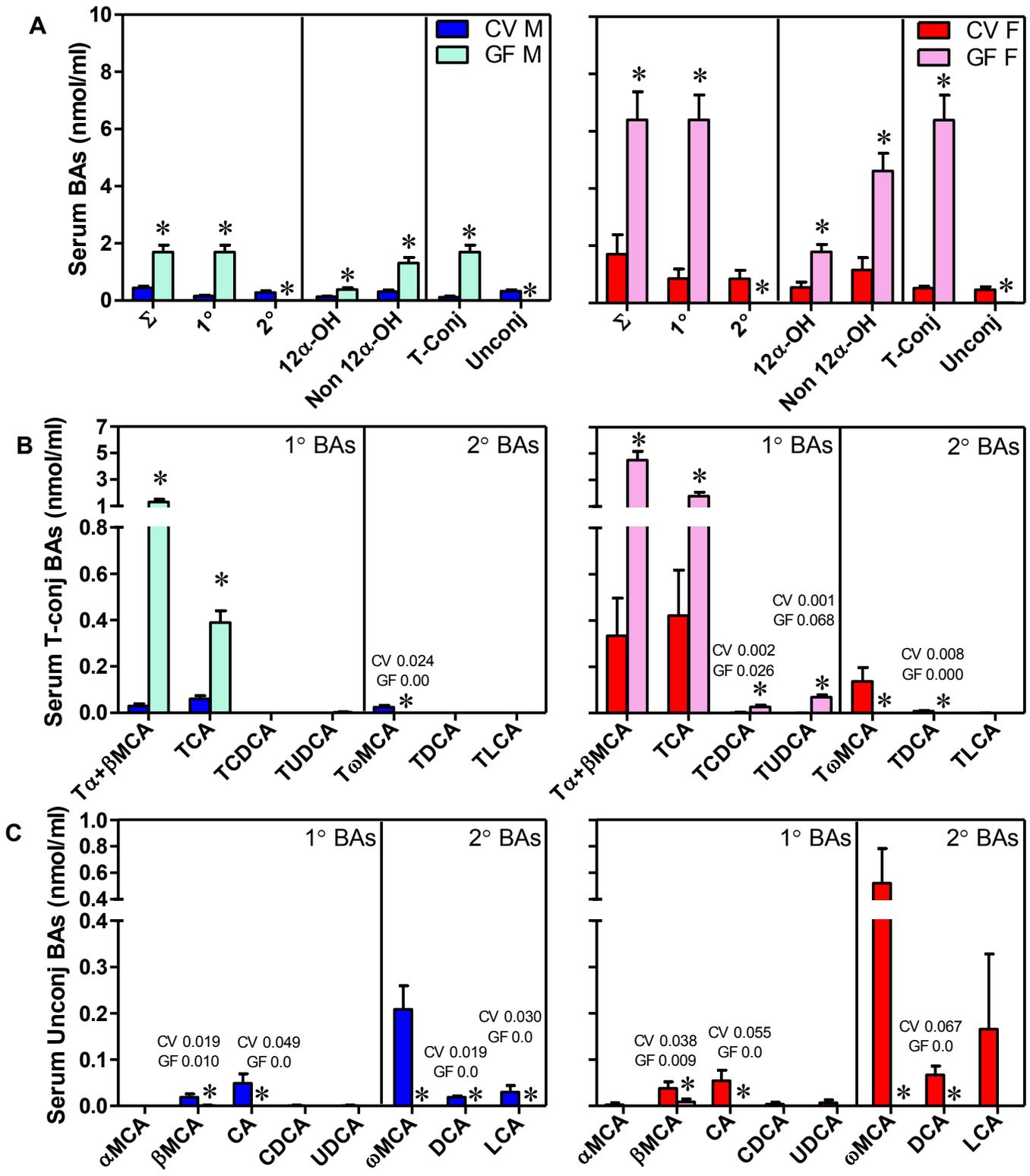


**Fig. 4.1. General characterization of GF mice.** (A) Body weight of CV and GF mice. (B) Liver weight of CV and GF mice. (C) Small intestine weight of CV and GF mice. (D) Large intestine weight of CV and GF mice. (E) and (F) Representative pictures of the intestines of CV and GF mice. (G) Gallbladder weight of CV and GF mice, n = 10/group. (H) Bile flow in CV and GF mice, n = 3-6/group. Data are presented as means  $\pm$  SEM. Asterisks (\*) represent statistically significant difference between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. CV - conventional mice, GF - germ-free mice, M - males, F- females.

Taurine conjugation lowers the  $pK_a$  of BAs, increases the aqueous solubility at the pH of the proximal intestine, and promotes a high intraluminal BA concentration leading to efficient solubilization of lipids and lipid-soluble vitamins (Setchell et al., 2013). Therefore, in addition to quantifying total BAs (summation of individual BA concentrations), the total concentrations of 12-OH BAs and non 12-OH BAs as well as total concentrations of taurine-conjugated and unconjugated BAs were quantified. In general, GF mice had higher concentrations of BAs in all the tissue compartments tested but have lower concentration of BAs excreted in feces compared to CV mice. Female GF and CV mice had higher BA concentrations than the respective male mice.

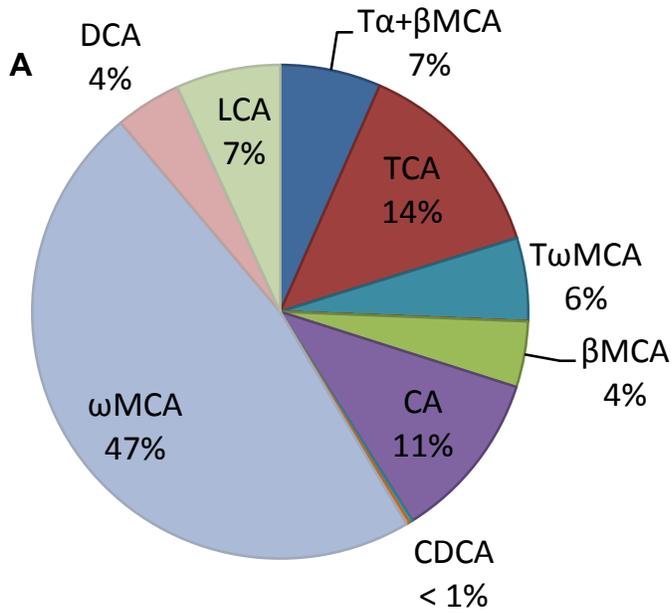
**BAs in Serum:** GF male and female mice have a 4-fold increase in total BAs in serum compared to CV mice (Fig. 4.2.A). This increase in total BAs in GF mice is due to an increase in primary BAs. Secondary BAs were not detected in GF mice, confirming that gut bacteria are absent in these mice. GF mice have increased total 12 $\alpha$ -OH BAs (CA, DCA, and their taurine conjugates) and non 12 $\alpha$ -OH BAs (BAs other than CA, DCA, and their taurine-conjugates) compared to CV mice. Gut bacteria are known to deconjugate taurine-conjugated BAs, therefore in GF mice there is an increase in taurine-conjugated BAs and a decrease in unconjugated BAs (Fig. 4.2.A).

In serum of male and female GF mice, the concentration of taurine-conjugated primary BAs, T $\alpha$ + $\beta$ MCA, and TCA are higher, whereas the taurine-conjugated secondary BA, T $\omega$ MCA are lower than in the CV mice (Fig. 4.2.B). In addition, female GF mice also have increased primary BAs, TUDCA, and TCDCA, and decreased secondary BA, TDCA, compared to CV mice. Most unconjugated BAs decrease in the serum of male and female GF mice compared to CV mice (Fig. 4.2.C).

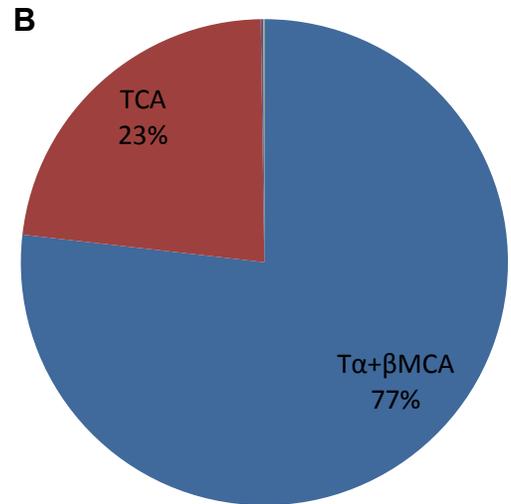


**Fig. 4.2. Concentrations of BAs in the serum of CV and GF mice.** (A) Concentrations of total BAs ( $\Sigma$ ), total 1° (primary) BAs and 2° (secondary) BAs, total 12 $\alpha$ -OH BAs (CA, TCA, DCA, and TDCA) and non 12 $\alpha$ -OH BAs (all other BAs), total T-Conj (taurine- conjugated) and Unconj (unconjugated) BAs. (B) Concentrations of individual T-conj (taurine-conjugated) BAs. (C) Concentrations of individual Unconj (unconjugated) BAs. Data are presented as means  $\pm$  SEM, n = 7-10 mice/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice; red and pink bars represent CV and GF female mice, respectively. CV- conventional mice. GF- germ-free mice. M- males. F- females.

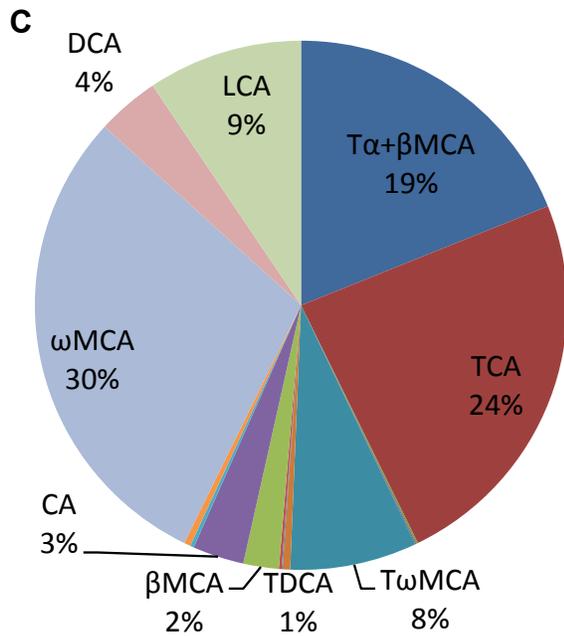
### Conventional Males



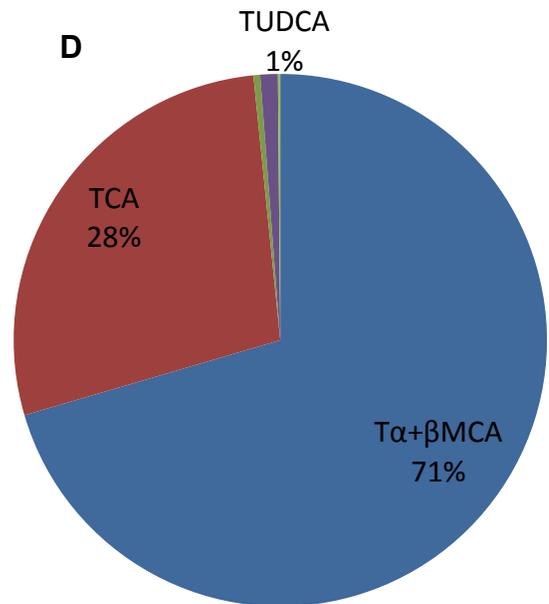
### Germ-Free Males



### Conventional Females



### Germ-Free Females



**Fig. 4.3. Proportion of individual BAs in the serum of CV and GF mice.** Proportions of individual BAs in serum of (A) conventional male mice, (B) germ-free male mice, (C) conventional female mice, and (D) germ-free female mice.

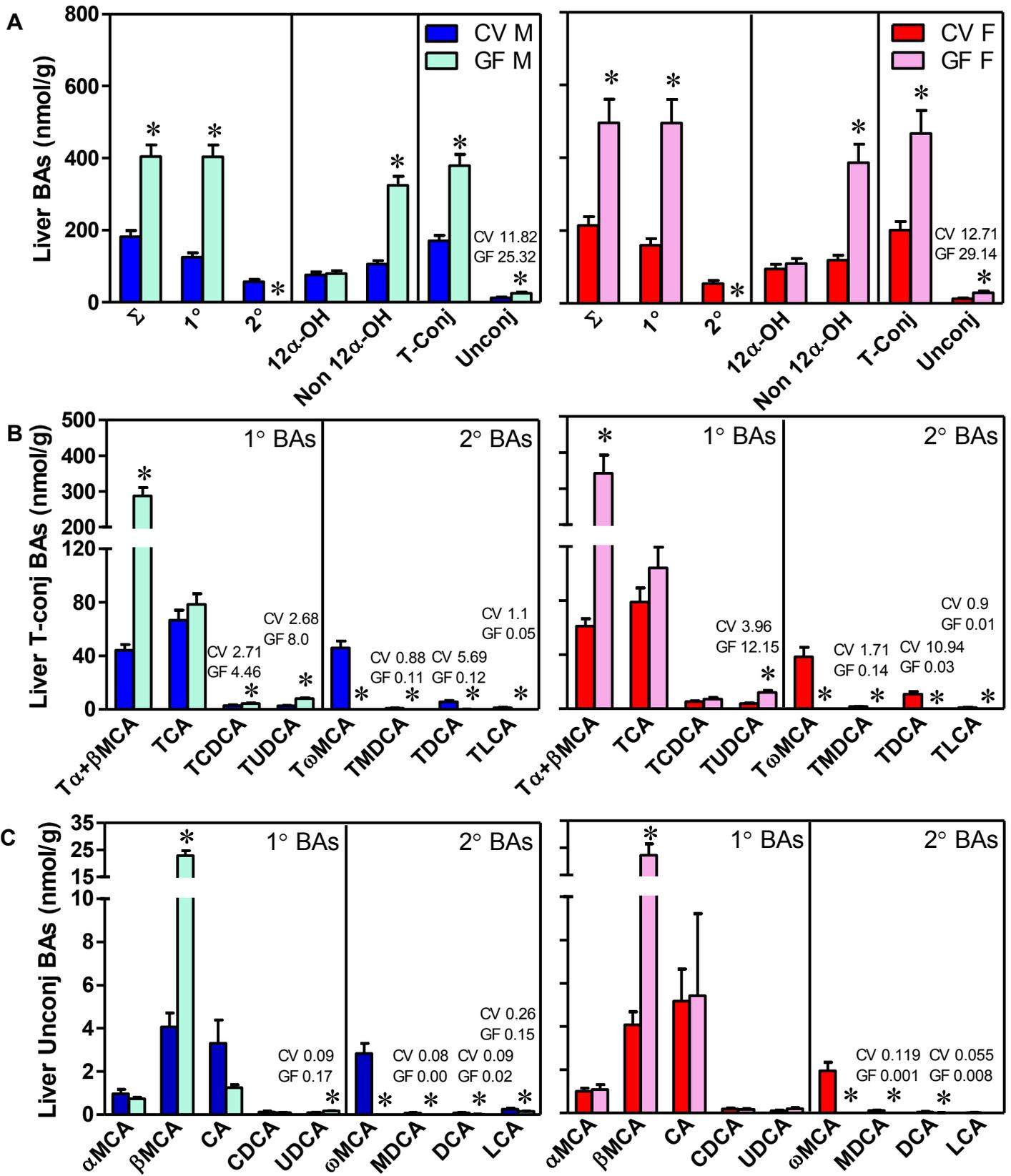
The proportions of individual BAs in the serum of GF mice change markedly. T- $\alpha$ + $\beta$  MCA and TCA are the major BAs in the serum of GF male and female mice (Fig. 4.3.B and D). T- $\alpha$ + $\beta$  MCAs make up 7% and 19% of BAs in the serum of CV male and female mice respectively, but are 77% and 71% of the total BAs in the serum of GF male and female mice (Fig. 4.3.A, B, C, and D).

**BAs in Liver:** Liver is responsible for the majority of BA synthesis. Interestingly, GF mice livers had a 2-fold higher concentration of primary BAs and total BAs, compared to CV mice. As expected, bacteria-derived secondary BAs are essentially absent in GF mice. GF mice have an increased non 12 $\alpha$ -OH BAs, but not the 12 $\alpha$ -OH BAs. Both taurine-conjugated and unconjugated BAs are approximately twice as high in the livers of GF mice compared to CV mice (Fig. 4.4.A).

GF male and female mice have increased taurine-conjugated primary BAs, such as T $\alpha$ + $\beta$ MCA and TUDCA, and decreased taurine-conjugated secondary BAs in the livers compared to CV mice. In addition, livers of GF male mice have increased TCDCA than CV mice (Fig. 4.4.B).

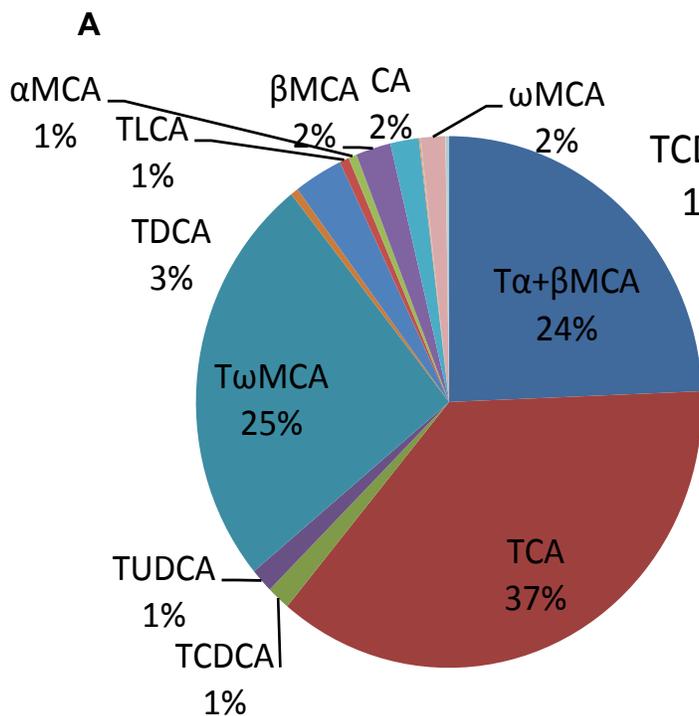
Concentrations of  $\beta$ MCA in livers of GF male and female mice are higher than in CV mice. Unconjugated secondary BAs are essentially absent in the livers of GF mice in comparison to CV mice (Fig. 4.4.C).

Proportions of the various BAs in livers of GF mice are altered. T $\alpha$ + $\beta$ MCA increase and form 71% and 69% of the total liver BAs in GF male and female mice, respectively compared to 24% and 29% of total BAs in CV male and female mice (Fig. 4.5.A, B, C and D).

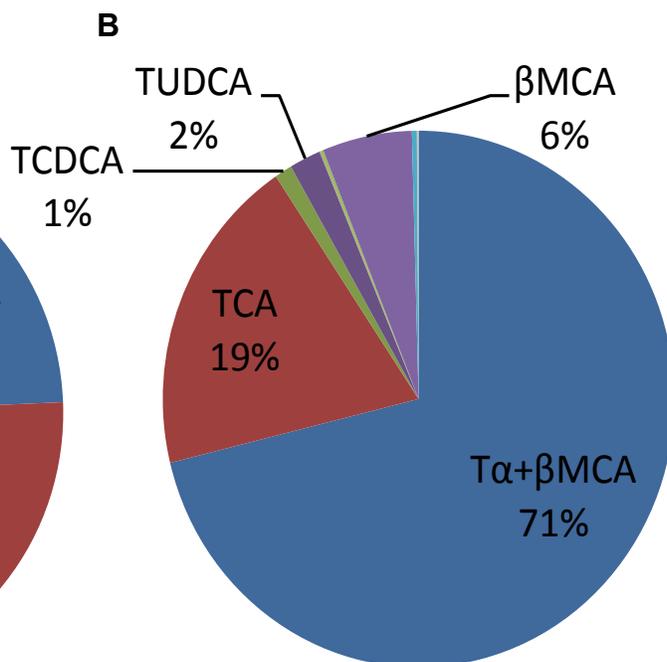


**Fig. 4.4. Concentrations of BAs in the livers of CV and GF mice.** (A) Concentrations of total BAs ( $\Sigma$ ), total 1° (primary) BAs and 2° (secondary) BAs, total 12 $\alpha$ -OH BAs (CA, TCA, DCA, and TDCA) and non 12 $\alpha$ -OH BAs (all other BAs), total T-Conj (taurine-conjugated) and Unconj (unconjugated) BAs. (B) Concentrations of individual T-conj (taurine-conjugated) BAs. (C) Concentrations of individual Unconj (unconjugated) BAs. Data are presented as means  $\pm$  SEM, n = 7-10 mice/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice; red and pink bars represent CV and GF female mice, respectively. CV- conventional mice. GF- germ-free mice. M- males. F- females.

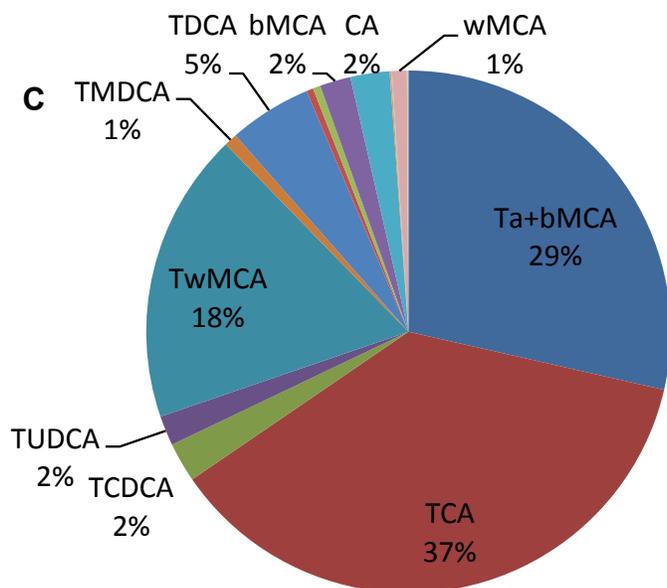
### Conventional Males



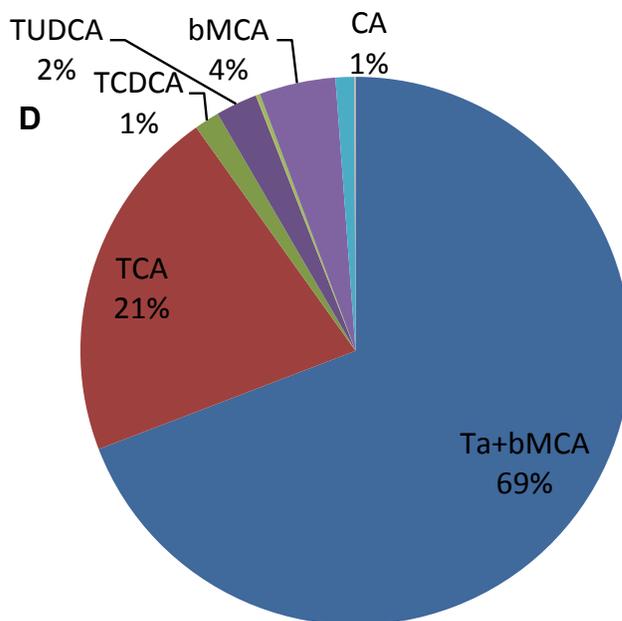
### Germ-Free Males



### Conventional Females



### Germ-Free Females



**Fig. 4.5. Proportion of individual BAs in the livers of CV and GF mice.** Proportions of individual BAs in livers of (A) conventional male mice, (B) germ-free male mice, (C) conventional female mice, and (D) germ-free female mice.

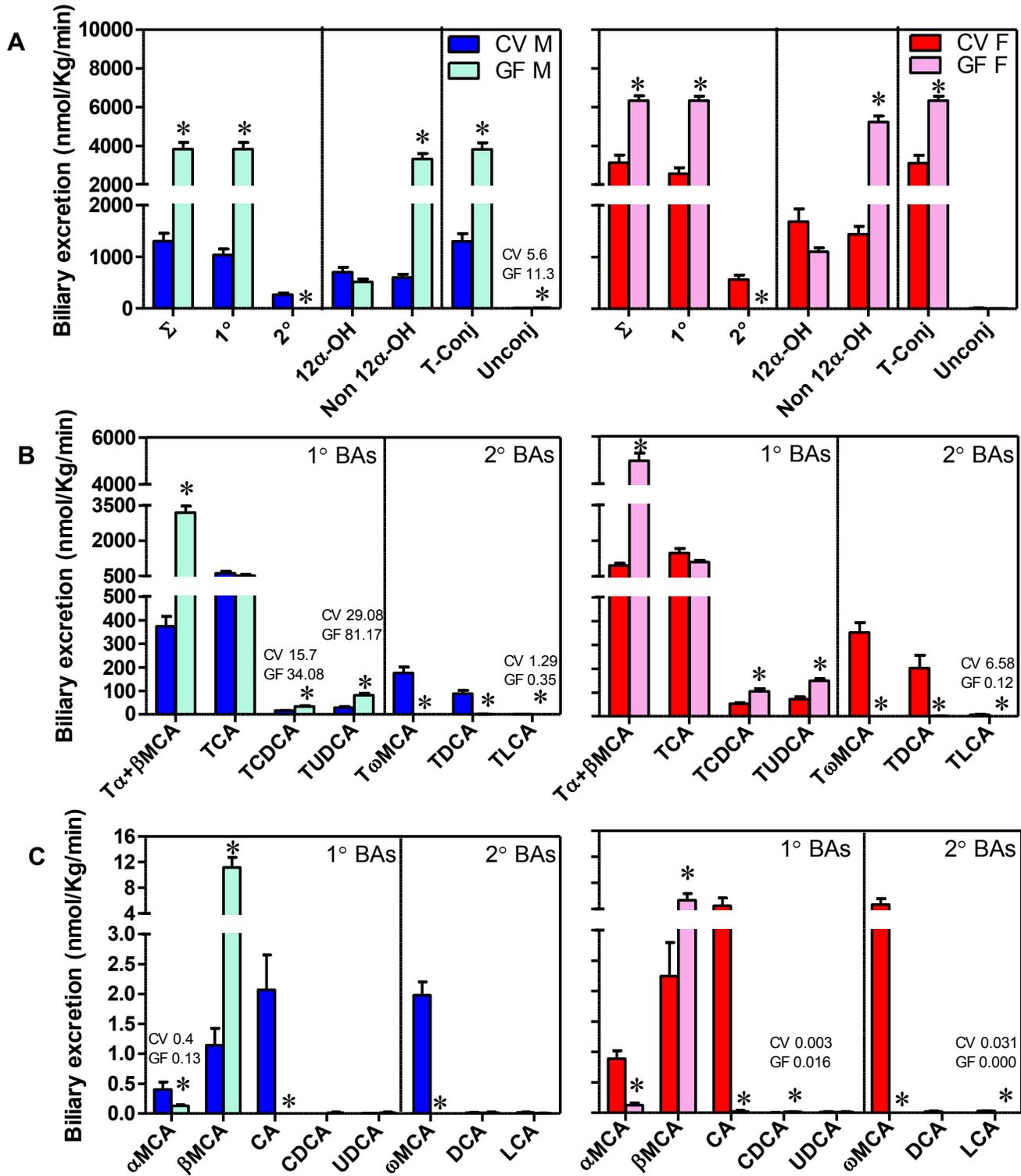
Interestingly, concentration of TUDCA, a known therapeutic secondary BA is increased in the livers of GF mice compared to CV mice (Fig. 4.4.B and Fig. 4.5.A, B, C and D). This suggests that in mice, TUDCA can also be synthesized in the liver and therefore is a primary BA.

**BA<sub>s</sub> in Bile:** BA<sub>s</sub> formed in the liver are effluxed into bile. Therefore, it is not surprising that the BA composition of GF livers and GF bile is similar. Biliary excretion of total BA<sub>s</sub> in GF mice was 3-fold higher in male GF mice and 2-fold higher in female GF mice compared to CV mice (Fig. 4.6.A). This increase is due to an increase in primary BA<sub>s</sub>, because secondary BA<sub>s</sub> are again essentially absent. There is an increase in the concentration of total non 12 $\alpha$ -OH BA<sub>s</sub> in the bile of male and female GF mice. Both taurine-conjugated and unconjugated BA<sub>s</sub> are increased in the bile of GF mice compared to CV mice (Fig. 4.6.A).

Concentrations of primary conjugated BA<sub>s</sub>, such as T $\alpha$ + $\beta$ MCA, TCDCA, and TUDCA are higher in bile of both GF male and female mice compared to CV mice. As expected, the concentrations of secondary conjugated BA<sub>s</sub> such as, T $\omega$ MCA, TDCA and TLCA are essentially absent in bile of both GF male and female mice compared to CV mice (Fig. 4.6.B).

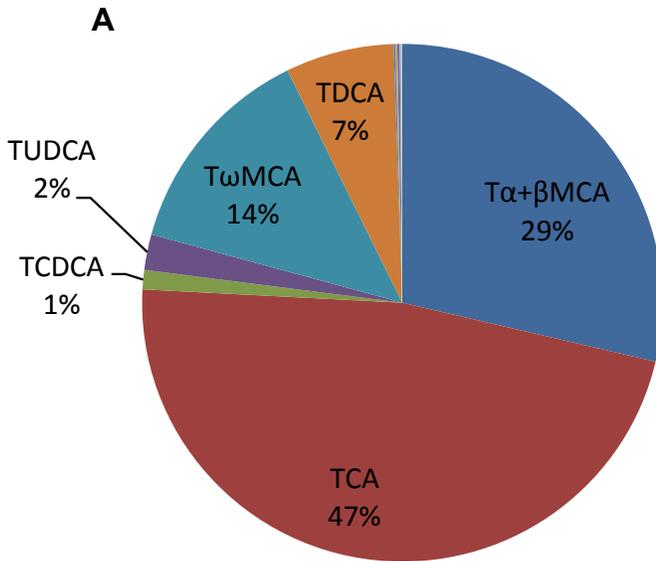
Concentrations of the primary unconjugated BA<sub>s</sub> ( $\alpha$ MCA and CA) are decreased, whereas  $\beta$ MCA is increased in both male and female GF mice compared to CV mice. Again, the secondary unconjugated BA<sub>s</sub> ( $\omega$ MCA, DCA and LCA) are essentially absent in bile of GF male and female mice (Fig. 4.6.C).

The proportions of the various BA<sub>s</sub> in the bile of GF and CV mice are very different. Apart from the decrease in secondary BA<sub>s</sub>, the proportion of the primary BA,

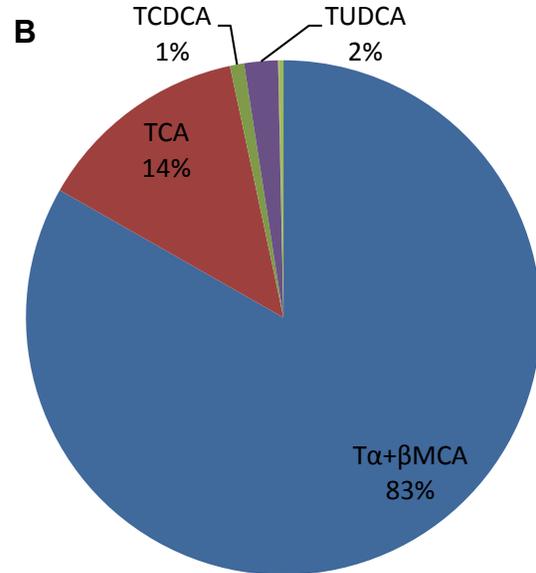


**Fig. 4.6. Concentrations of BAs in bile of CV and GF mice.** BAs in bile are expressed as nMol/Kg body weight/min. (A) Concentrations of total BAs ( $\Sigma$ ), total 1° (primary) BAs and 2° (secondary) BAs, total 12 $\alpha$ -OH BAs (CA, TCA, DCA, and TDCA) and non 12 $\alpha$ -OH BAs (all other BAs), total T-Conj (taurine-conjugated) and Unconj (unconjugated) BAs. (B) Concentrations of individual T-conj (taurine-conjugated) BAs. (C) Concentrations of individual Unconj (unconjugated) BAs. Bile was collected for 40 mins from each mouse. Data are presented as means  $\pm$  SEM, n =3-6 mice/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice; red and pink bars represent CV and GF female mice, respectively. CV- conventional mice. GF- germ-free mice. M- males. F- females.

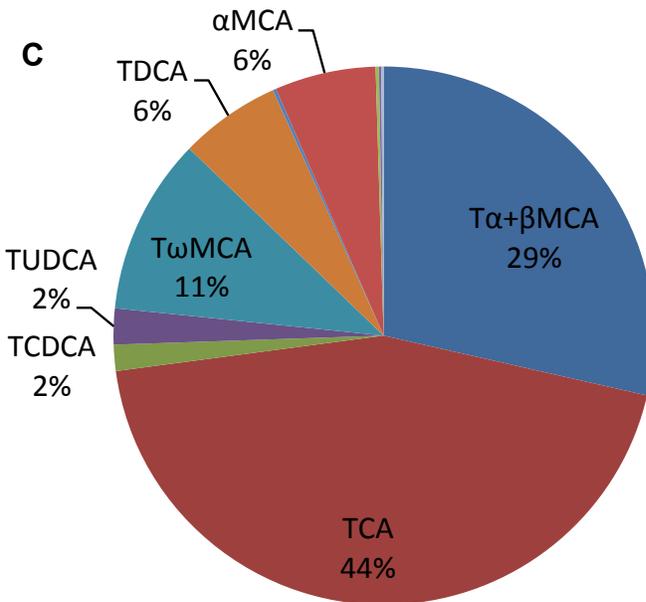
### Conventional Males



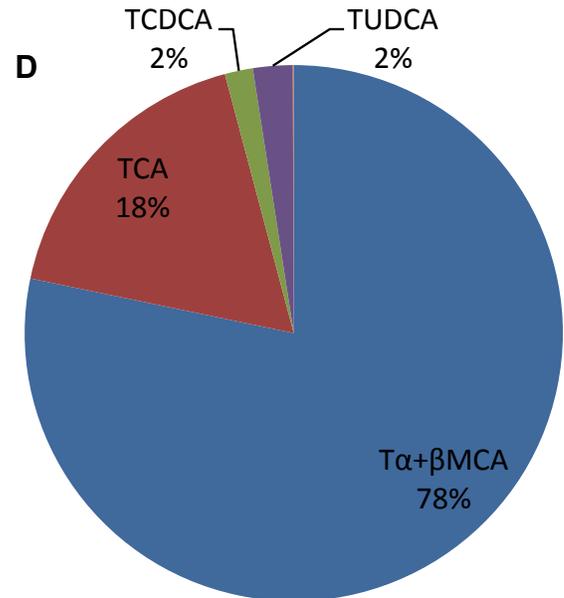
### Germ-Free Males



### Conventional Females



### Germ-Free Females



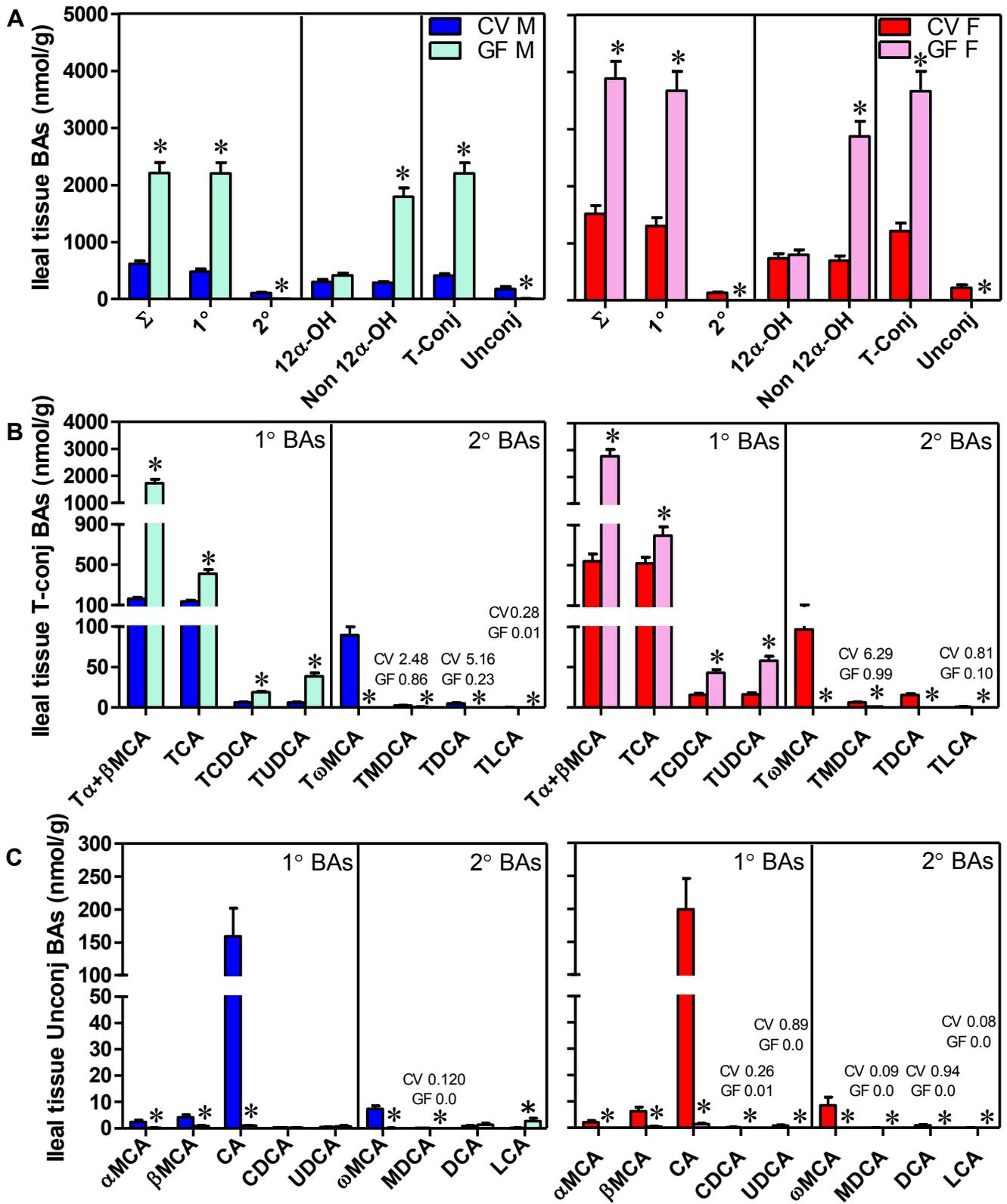
**Fig. 4.7. Proportion of individual BAs in the bile of CV and GF mice.** Proportions of individual BAs in bile of (A) conventional male mice, (B) germ-free male mice, (C) conventional female mice, and (D) germ-free female mice.

TCA also decreases from 47% and 44% in CV male and female mice to 14 and 18% in GF male and female mice, respectively. In contrast,  $T\alpha+\beta$ MCA which make up 29% of BAs in bile of CV male and female mice, are increased and form 83% and 78% of total BAs in the bile of GF male and female mice, respectively (Fig 4.6. A, B, C, and D).

**BAs in ileal tissue:** Normally, BAs in the intestine are metabolized by gut bacteria, to their deconjugated form and/or to secondary BAs. The majority of BAs return to the liver via the portal circulation. Some of the unconjugated BAs can diffuse across the enterocytes, while the rest and in particular the conjugated BAs are taken up into enterocytes by Asbt across the apical membrane. Transport across the basolateral membrane is mediated by  $Ost\alpha$ - $Ost\beta$ . Similar to the other tissue compartments, there are increased levels of primary and taurine-conjugated BAs, and decreased levels of secondary and unconjugated BAs in GF mice compared to CV mice (Fig. 4.8.A)

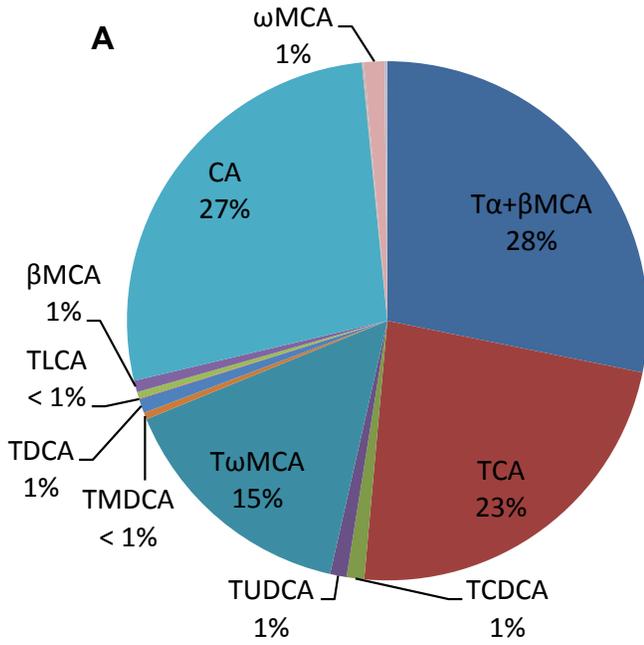
Total BAs concentrations were 4-fold higher in male GF mice and 3-fold higher in female GF mice compared to their CV controls (Fig. 4.8.A). This suggests increased uptake of BAs from the intestinal lumen or decreased transport across the basolateral membrane. Similar to the other tissue compartments, there are increased levels of primary and taurine-conjugated BAs and decreased levels of secondary and unconjugated BAs in GF mice compared to CV mice (Fig. 4.8.A).

Concentrations of the primary taurine-conjugated BAs, ( $T\alpha+\beta$ MCA, TCA, TCDCA, and TUDCA) are increased, whereas the concentrations of the secondary taurine-conjugated BAS ( $T\omega$ MCA, TMDCA, TDCA, and TLCA) are decreased in both male and female GF mice compared to CV mice (Fig. 4.8.B). Concentrations of both primary and secondary unconjugated BAs are lower in the ileal tissue of GF mice

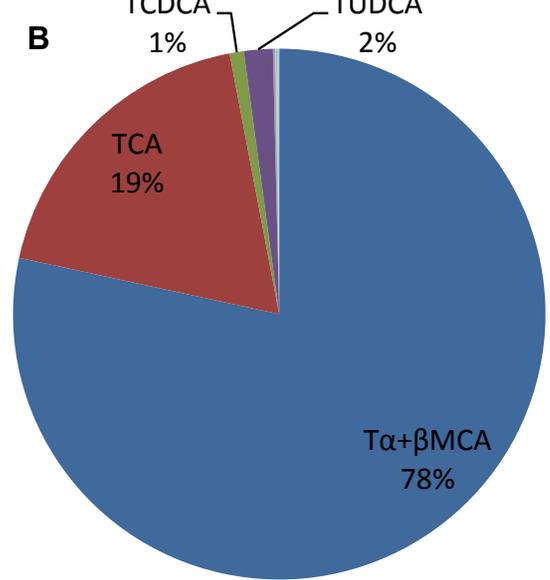


**Fig. 4.8. Concentrations of BAs in the ileal tissue of CV and GF mice.** (A) Concentrations of total BAs ( $\Sigma$ ), total 1° (primary) BAs and 2° (secondary) BAs, total 12 $\alpha$ -OH BAs (CA, TCA, DCA, and TDCA) and non 12 $\alpha$ -OH BAs (all other BAs), total T-Conj (taurine- conjugated) and Unconj (unconjugated) BAs, (B) Concentrations of individual T-conj (taurine-conjugated) BAs, and (C) Concentrations of individual Unconj (unconjugated) BAs. Ileal tissue refers to the last 1/3rd of the small intestine. Data are presented as means  $\pm$  SEM, n = 7-10 mice/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice; red and pink bars represent CV and GF female mice, respectively. CV- conventional mice. GF- germ-free mice. M- males. F- females.

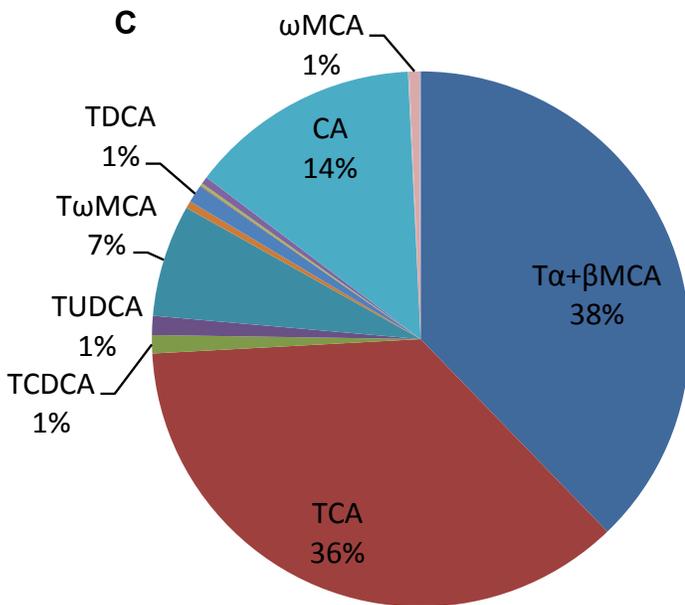
### Conventional Males



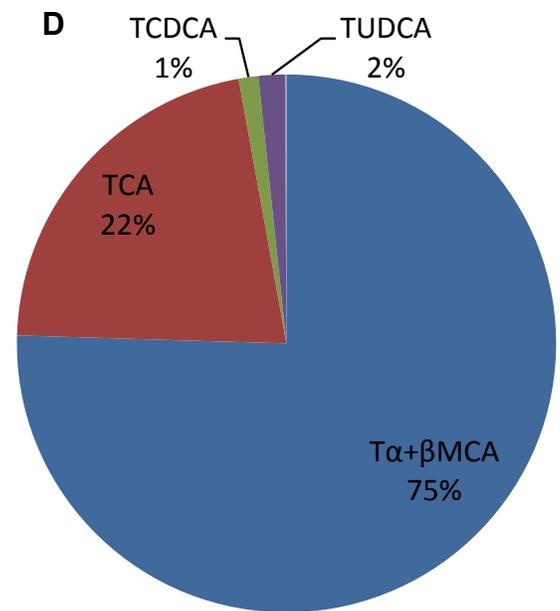
### Germ-Free Males



### Conventional Females



### Germ-Free Females



**Fig. 4.9. Proportion of individual BAs in the ileal tissue of CV and GF mice.**

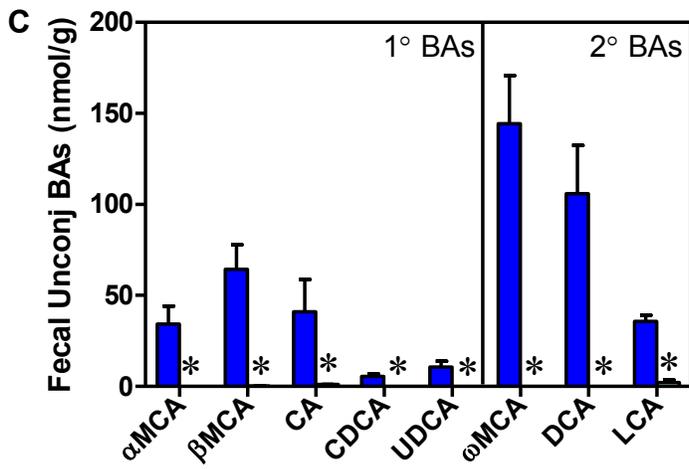
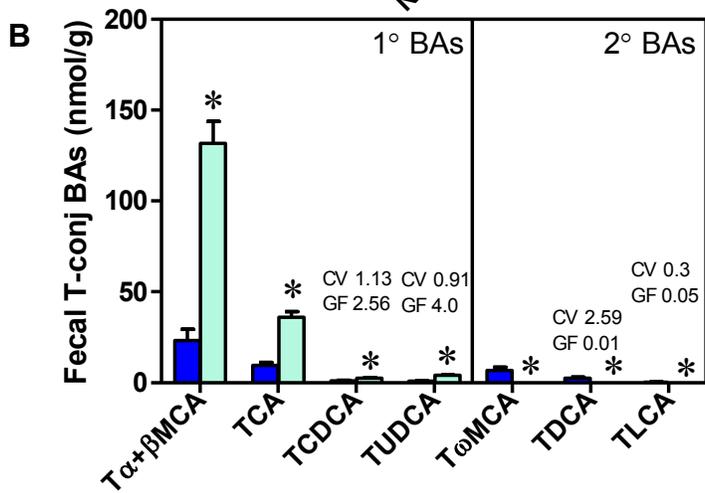
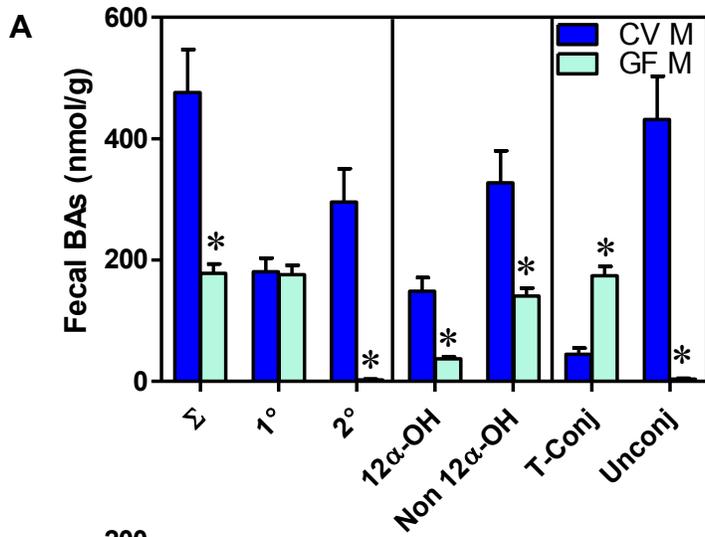
Proportions of individual BAs in ileal tissue of (A) conventional male mice, (B) germ-free male mice, (C) conventional female mice, and (D) germ-free female mice.

compared to CV mice (Fig. 4.8.C). The proportions of BAs in the ileal tissue are altered in GF mice compared to CV mice.  $T\alpha+\beta$ MCA are increased to 78 and 75% of the total BAs in ileal tissue of GF male mice, compared to 28 and 38% in CV mice (Fig. 4.9.A, B, C, D).

**BAs in Feces:** Mice were housed in individual metabolic cages and feces were collected over a 24-hr period. Generally, more than 90% of BAs in the intestinal lumen are reabsorbed from the intestine and the remaining BAs are excreted in feces. There was a 63% decrease in the amount of total BAs in feces of GF mice compared to CV mice, which is mainly due to the absence of secondary BAs (Fig. 4.10.A). This can in part be explained by an increased ileal uptake of BAs in the absence of gut bacteria in GF mice. Furthermore, the absence of gut bacteria for deconjugation also leads to increased levels of conjugated BAs in feces of GF mice (Fig. 4.10.A).

The amount of primary taurine-conjugated BAs (TCA,  $T\alpha+\beta$ MCA, TCDCA, and TUDCA) are increased whereas the amount of the secondary conjugated BAs ( $T\omega$ MCA, TDCA, and TLCA) are decreased in the feces of GF mice (Fig. 4.10.B). The amount of all unconjugated BAs quantified in feces of GF mice, were much lower than CV mice (Fig. 4.10.C).

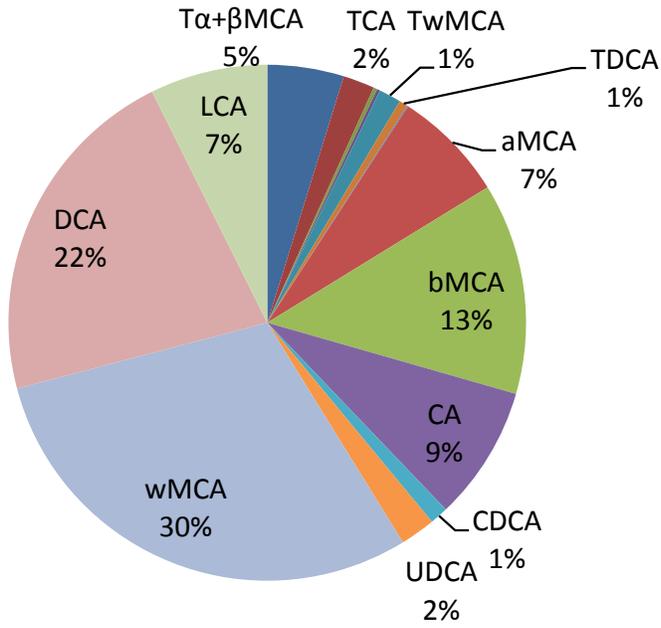
Finally, the proportions of BAs in feces of GF mice are very different compared to CV mice.  $T\alpha+\beta$ MCA are increased and form 74% of the total BAs, compared to 5% in CV mice (Fig. 4.11.A and B).



**Fig. 4.10. Concentrations of BAs in feces of CV and GF mice.** BAs are expressed as nMols/gram of feces. (A) Concentrations of total BAs ( $\Sigma$ ), total 1 (primary) BAs and 2 (secondary) BAs, total 12 $\alpha$ -OH BAs (CA, TCA, DCA, and TDCA) and non 12 $\alpha$ -OH BAs (all other BAs), total T-Conj (taurine-conjugated) and Unconj (unconjugated) BAs. (B) Concentrations of individual T-conj (taurine-conjugated) BAs. (C) Concentrations of individual Unconj (unconjugated) BAs. Mice were housed individually in metabolic chambers and feces were collected over 24 hrs. Data are presented as means  $\pm$  SEM, n = 4 mice/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice. CV- conventional mice. GF- germ-free mice. M- males.

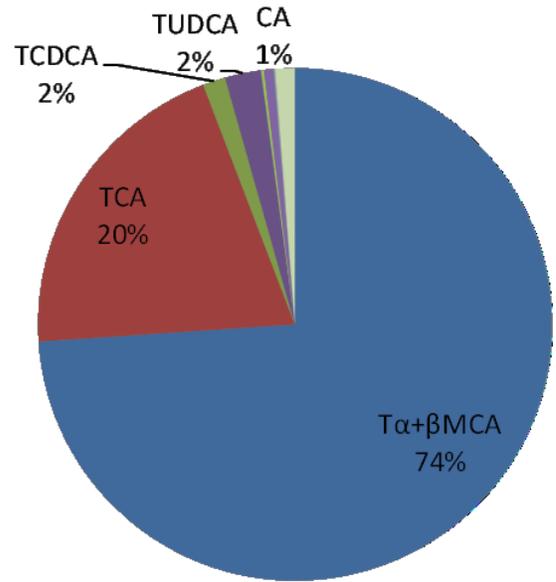
### Conventional Males

A



### Germ-Free Males

B



**Fig. 4.11. Proportion of individual BAs in feces of CV and GF mice.** Proportions of individual BAs in feces of (A) conventional male mice, and (B) germ-free male mice.

### **C. BA-related gene expression in livers and ileum of CV and GF mice**

The gene expression of BA biosynthetic enzymes, BA transporters, and genes involved in BA feedback regulation were quantified in liver and ileal tissue of GF mice and compared to CV mice.

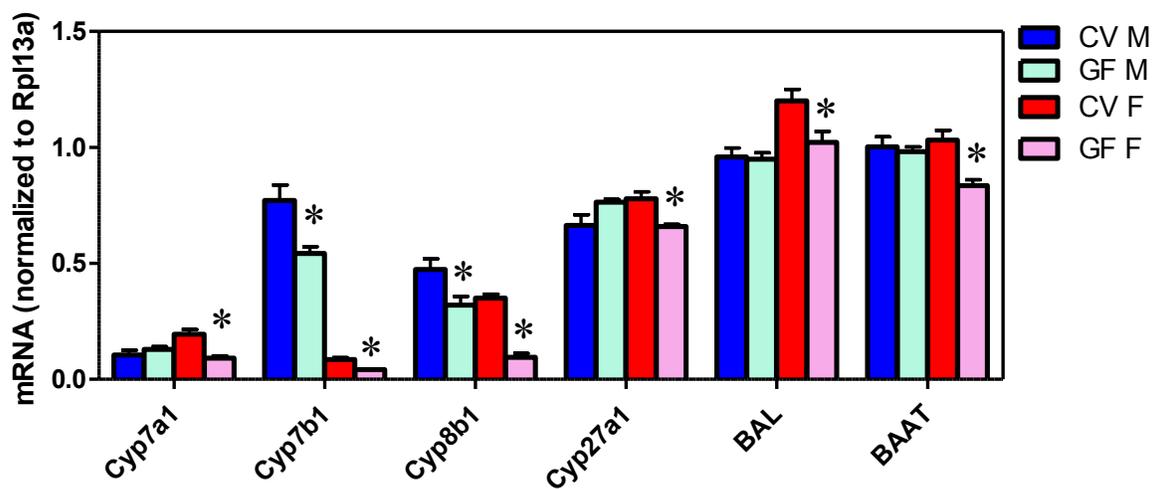
#### **Enzymes involved in BA biosynthesis.**

The main enzymes involved in BA biosynthesis include Cyp7a1, Cyp7b1, Cyp8b1, Cyp27a1 and the BA conjugation enzymes BAL and BAAT. Cyp7a1 is the rate-limiting enzyme in BA synthesis and is responsible for the 7 $\alpha$ -hydroxylation of cholesterol metabolites in the classic pathway of BA synthesis. The mRNA levels of Cyp7a1 are similar in livers of CV and GF male mice. However, in female mice Cyp7a1 mRNA levels are about 50% of the levels detected in GF mice (Fig. 4.12).

Cyp7b1 is the enzyme responsible for 7 $\alpha$ -hydroxylation of cholesterol metabolites in the alternate pathway of BA synthesis. Cyp7b1 mRNA is decreased by 30% and 52% in livers of male and female GF mice, respectively, compared to CV mice (Fig. 4.12). This may be an attempt to decrease the alternate pathway of BA synthesis to ultimately decrease concentrations of BAs in the livers of GF mice.

Cyp8b1, which is also known as sterol 12 $\alpha$ -hydroxylase, is an important enzyme that is responsible for the 12 $\alpha$ -hydroxylation of cholesterol to form CA. The mRNA levels of Cyp8b1 are decreased 32% in the livers of male GF mice and decreased 73% in livers of female GF mice compared to their CV controls (Fig. 4.12). This correlates well with the decrease in concentration of 12 $\alpha$ -OH BAs (TCA and CA) in liver and the decrease in proportion of TCA to T $\alpha$  +  $\beta$ MCA in livers of GF mice (Fig. 4.5.A, B, C, D).

### Enzymes in BA synthesis



**Fig. 4.12. Gene expression of enzymes involved in BA synthesis in CV and GF mice.** mRNA expression of genes involved in BA homeostasis in livers of male and female CV and GF mice. mRNA was quantified by beadplex assay, n = 6/group. Data are presented as means  $\pm$  SEM. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. CV - conventional mice, GF - germ-free mice, M - males, F - females. Dark blue and light blue bars represent CV and GF male mice, respectively. Red and pink bars represent CV and GF females, respectively.

Cyp27a1 is a mitochondrial enzyme that plays two important roles in BA synthesis: 1) it is responsible in the classical pathway for steroid side chain cleavage, that is to convert C<sub>27</sub> cholesterol or its metabolites to C<sub>24</sub> BAs, and 2) is also responsible for the first step in the alternate pathway of BA synthesis by generating oxysterols (25- and 27-hydroxycholesterol) from cholesterol. The mRNA of Cyp27a1 decreased by 15% in the livers of female GF mice compared to CV mice, but is similar in livers of male CV and GF mice.

The enzyme BAL catalyzes the addition of CoA to BAs, and subsequently the enzyme BAAT conjugates BA-CoA to the amino acids taurine or glycine before BA secretion into bile. In GF mice, there is an increase in taurine-conjugated BAs compared to CV mice as GF mice lack gut bacteria that deconjugate BAs. The mRNA levels of BAL were decreased 15% and the mRNA levels of BAAT were decreased 19% in the livers of female GF mice compared to the CV controls. However, BAL and BAAT mRNA levels do not change in male GF mice compared to CV mice (Fig. 4.12).

### **BA Transporters**

The main BA uptake transporters in livers are Ntcp and Oatp1b2, whereas Asbt is considered to be the main uptake system in ileum. BAs efflux into bile is mediated by Bsep, BAs efflux back into blood is mediated by Mrp3 and Mrp4 and BAs efflux into portal circulation is by Ost $\alpha$ -Ost $\beta$  in ileum.

The hepatic BA transporters Ntcp and Oatp1b2 are responsible for the uptake of conjugated and unconjugated BAs, respectively, across the basolateral membrane of hepatocytes. In male GF mice, the mRNA of Ntcp increased 42% and the mRNA levels of Oatp1b2 increased 48%, compared to CV mice (Fig. 4.13.A). This increase in mRNA

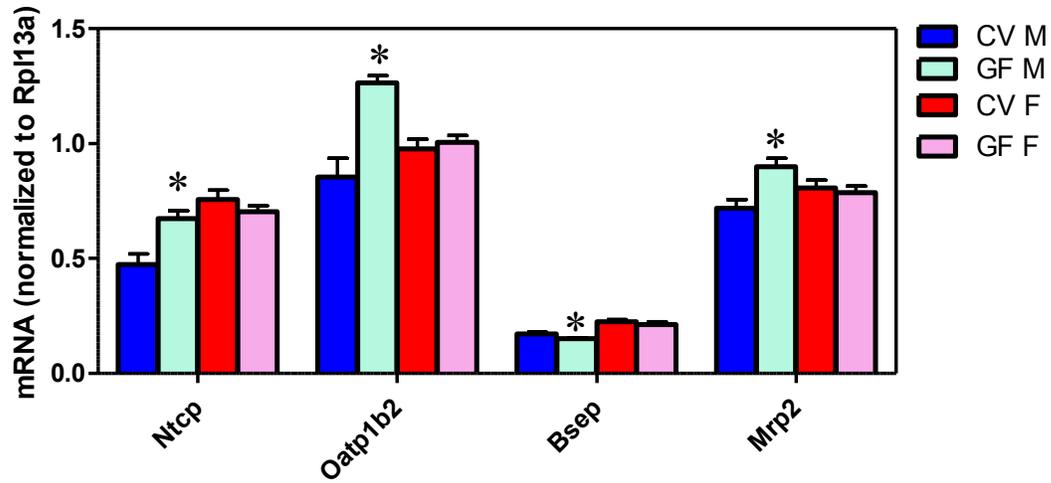
of hepatic BA uptake transporters enable the liver to remove more BAs from the circulation, given that total BAs are higher in serum of both male and female GF mice. However, in female GF mice, the mRNA of Ntcp, and Oatp1b2 are similar to that in CV mice (Fig. 4.13.A).

The mRNA of the major hepatic BA efflux transporter Bsep was decreased by 11% in male GF mice but does not change in livers of female GF mice compared to their respective CV controls (Fig. 4.13.A). The gene expression of Mrp2 increased by 25% in livers of male GF mice but does not change in livers of female GF mice compared to their respective controls (Fig. 4.13.A).

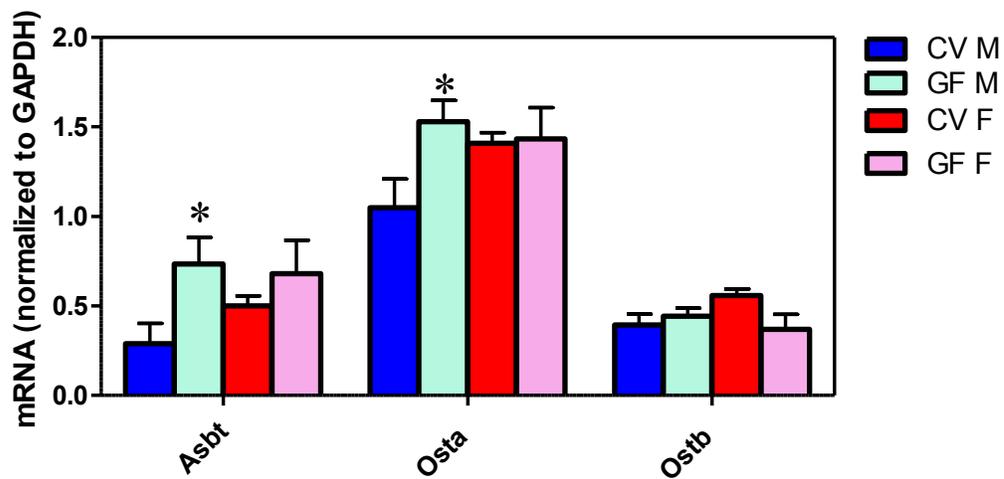
Asbt is the only known apical BA uptake transporter and the mRNA levels of Asbt increased by 154% in the ileum of male GF mice compared to CV mice (Fig. 4.13.B). This correlates well with the increased total BA concentrations in the ileal tissue and decreased total BA content in feces observed in the GF mice compared to CV mice (Fig. 4.8.A and Fig. 4.10.A). However, in females, there is no difference in expression of Asbt in both groups of mice.

The ileal BA transporters Ost $\alpha$  and Ost $\beta$  mediate efflux of BAs into the portal circulation. There are no differences in the mRNA levels of Ost $\alpha$  and Ost $\beta$  in the ileal tissue of GF mice compared to CV mice. In male GF mice, although mRNA of Ost $\alpha$  increased by 46% compared to CV mice, the mRNA of Ost $\beta$  did not change (Fig. 4.13.B).

**A** Hepatic BA transporters



**B** Ileal BA transporters



**Fig. 4.13. Gene expression of BA transporters in CV and GF mice.** (A) mRNA expression of BA transporters in the livers of male and female CV and GF mice. (B) mRNA expression of BA transporters in the ileum of male and female CV and GF mice. mRNA was quantified by beadplex assay,  $n = 6/\text{group}$ . Data are presented as means  $\pm$  SEM. Asterisks (\*) represents statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's  $t$ -test. CV - conventional mice, GF - germ-free mice, M - males, F - females. Dark blue and light blue bars represent CV and GF male mice respectively. Red and pink bars represent CV and GF females, respectively.

## **Genes involved in BA feedback regulation**

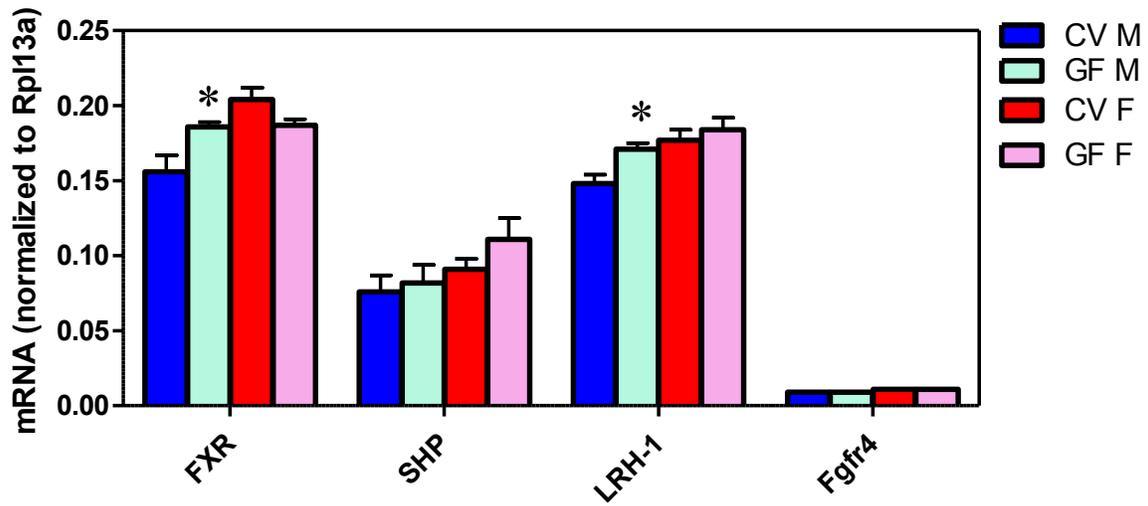
BAs are known to regulate their own synthesis by binding to the intracellular BA receptor FXR that suppresses Cyp7a1, the rate limiting enzyme in BA synthesis. FXR is expressed both in liver and ileum. In liver, binding of BAs to FXR, results in an increase in SHP, which displaces Lrh-1 from the promoter region of Cyp7a1 and suppresses Cyp7a1 transcription. In the ileum, BA binding to FXR increases the secretion of Fgf15, which travels via the portal circulation to the liver and activates the hepatic receptor Fgfr4. It is known that activation of the Fgfr4 receptor, leads to an increase in SHP and subsequently also suppresses Cyp7a1.

The only changes in the mRNA levels of genes involved in BA feedback regulation were increased mRNA levels of FXR and LRH-1 in the livers of male GF mice compared to their CV controls. However, this increase may not be biologically significant as there was no subsequent decrease in the mRNA of Cyp7a1 in the livers of male GF mice compared to CV mice (Fig. 4.14.A). There are no changes in the mRNA of the other important genes involved in BA feedback regulation through FXR activation (Fig. 4.14).

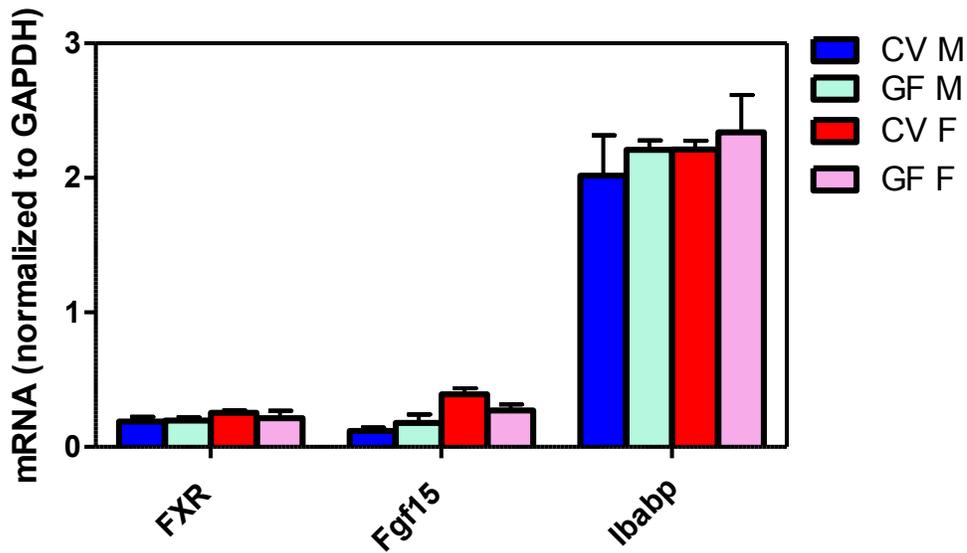
## **D. Targets of TGR5 signaling in CV and GF mice**

The plasma membrane-bound G protein-coupled receptor TGR5 is activated by BAs which results in an increase in intracellular cAMP levels, which in turn activate the enzyme protein kinase A (PKA). PKA can increase or decrease the activity of target enzymes by direct phosphorylation.

### A Genes involved in BA feedback regulation in liver



### B Genes involved in BA feedback regulation in ileum



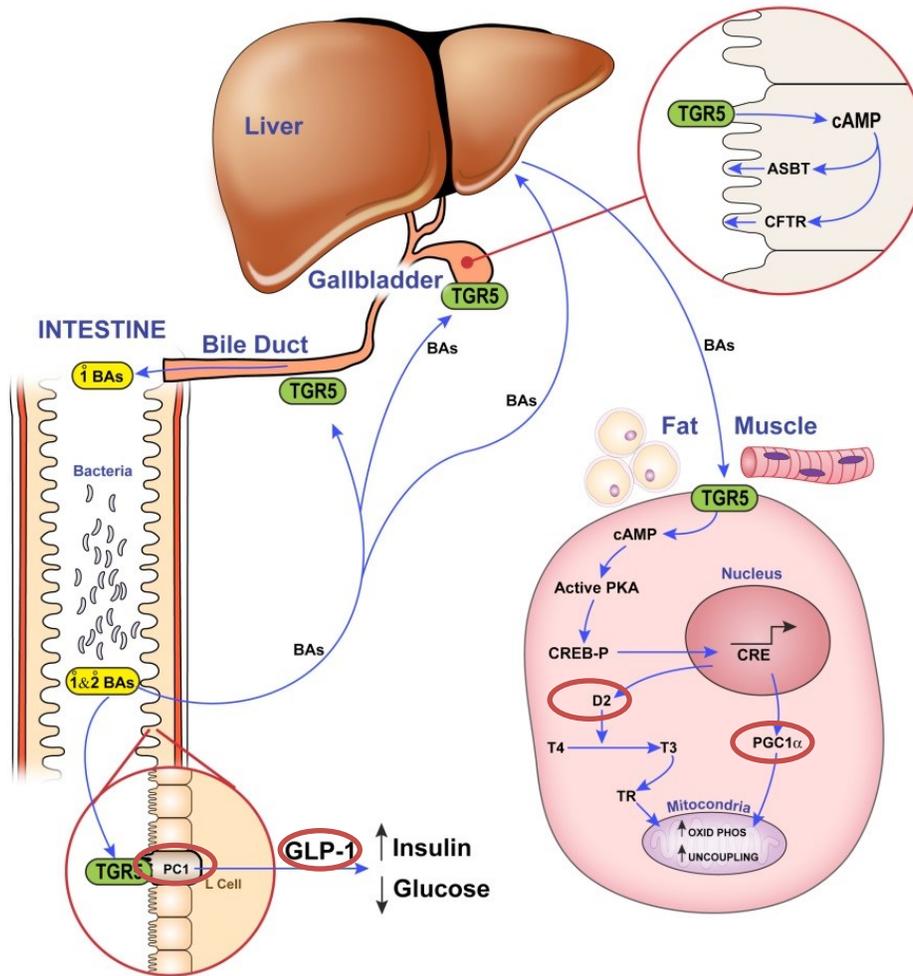
**Fig. 4.14. Expression of genes involved in BA feedback regulation in CV and GF mice.** (A) mRNA expression of genes involved in BA feedback regulation in livers of male and female CV and GF mice. (B) mRNA expression of genes involved in BA feedback regulation in ileum of male and female CV and GF mice. mRNA was quantified by beadplex assay,  $n = 6/\text{group}$ . Data are presented as means  $\pm$  SEM. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice, respectively. Red and pink bars represent CV and GF females, respectively. CV- conventional mice, GF- germ-free mice, M- males, F- females.

An increase in intracellular cAMP also results in activation of the transcription factor cAMP response element-binding protein (CREB), which increases the transcription of its target proteins. The major effects of TGR5 activation include 1) increased bile flow and gallbladder size (Li et al., 2011); 2) increased GLP-1 secretion from ileum (Katsuma et al., 2005) and, 3) increased energy expenditure as heat (Thomas et al., 2009) (Fig. 4.15 and Table 4.1).

**Bile flow and gallbladder size.** GF mice have a 5-fold increase in gallbladder weight compared to CV mice (Fig. 4.16.A and B). In addition, bile flow was higher by 44% in GF male mice compared to their CV controls (Fig. 4.16.C).

**GLP-1.** Serum GLP-1 concentrations were quantified by ELISA (Millipore, MO). GLP-1 was minimally detectable in the serum of CV mice but the concentration in the serum of GF mice was clearly higher (Fig. 4.17.A). GLP-1 is a cleavage product of the proglucagon peptide in the ileum, and the enzyme responsible for this cleavage is prohormone convertase (PC1). The mRNA of PC1 was also significantly higher in GF mice compared to CV mice (Fig. 4.17.B).

**Genes involved in thermogenesis.** Activation of TGR5 in brown adipose tissue and muscle increases the expression of type 2 iodothyronine deiodinase (D2), which subsequently increases energy expenditure as heat in the mitochondria. The mRNA of D2 was increased 3-fold in the brown adipose tissue of GF mice (Fig. 4.17.C), which suggests that the TGR5 receptor is activated in the brown adipose tissue of GF mice. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1 $\alpha$ ) is a transcription factor that regulates mitochondrial biogenesis. The mRNA of PGC1 $\alpha$  also



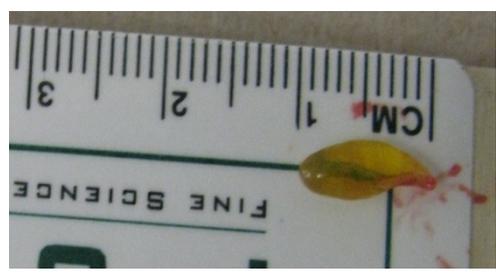
**Fig 4.15. Schematic diagram displaying the TGR5 signaling pathways.**

**Table 4.1. Targets of TGR5 signaling**

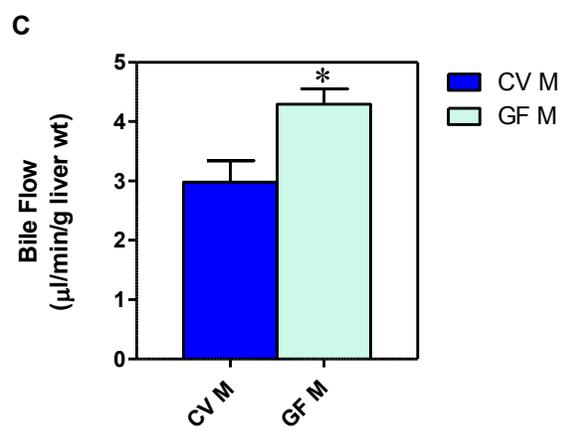
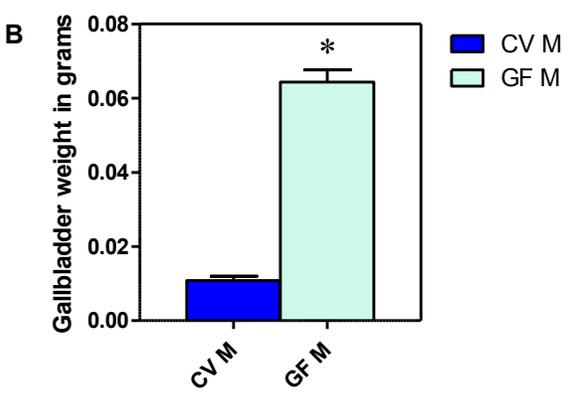
	Target organs	Effect
1	Gallbladder	Increase gallbladder volume/weight
2	Ileum	Increase GLP-1 release
3	Muscle	Thermogenesis
4	Brown fat	Thermogenesis



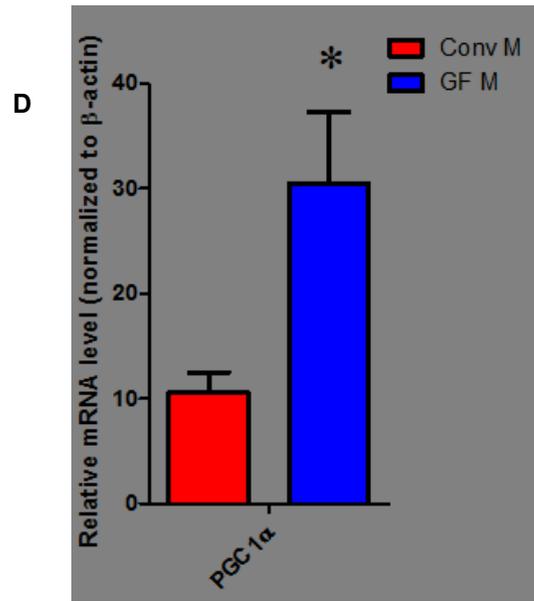
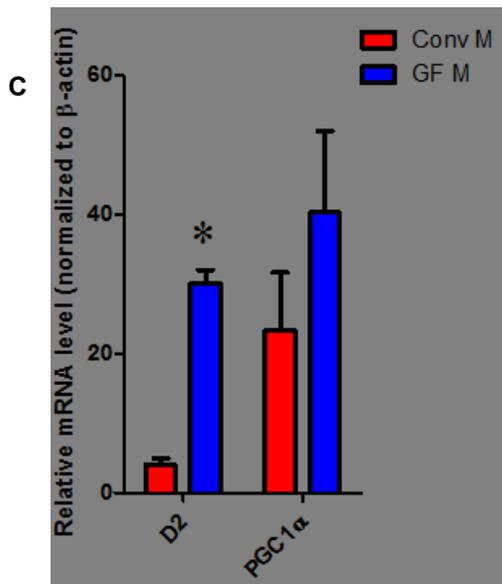
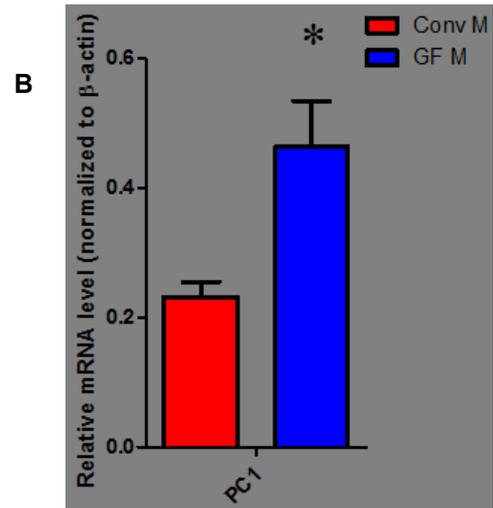
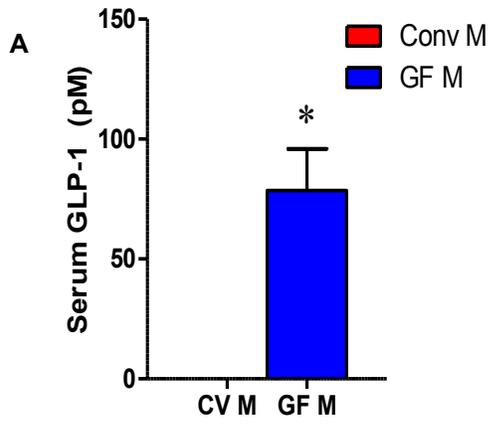
CV mice



GF mice



**Fig.4.16. Gallbladder weights in GF mice.** (A) Representative pictures of the gallbladders of CV and GF mice. (B) Weights of the gallbladder in CV and GF mice, n = 7-10 mice/group. (C) Bile flow in CV and GF mice, n = 3-6 mice/group. Data are presented as means  $\pm$  SEM. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. CV - conventional mice, GF - germ-free mice, M – males. Dark blue and light blue bars represent CV and GF male mice, respectively.



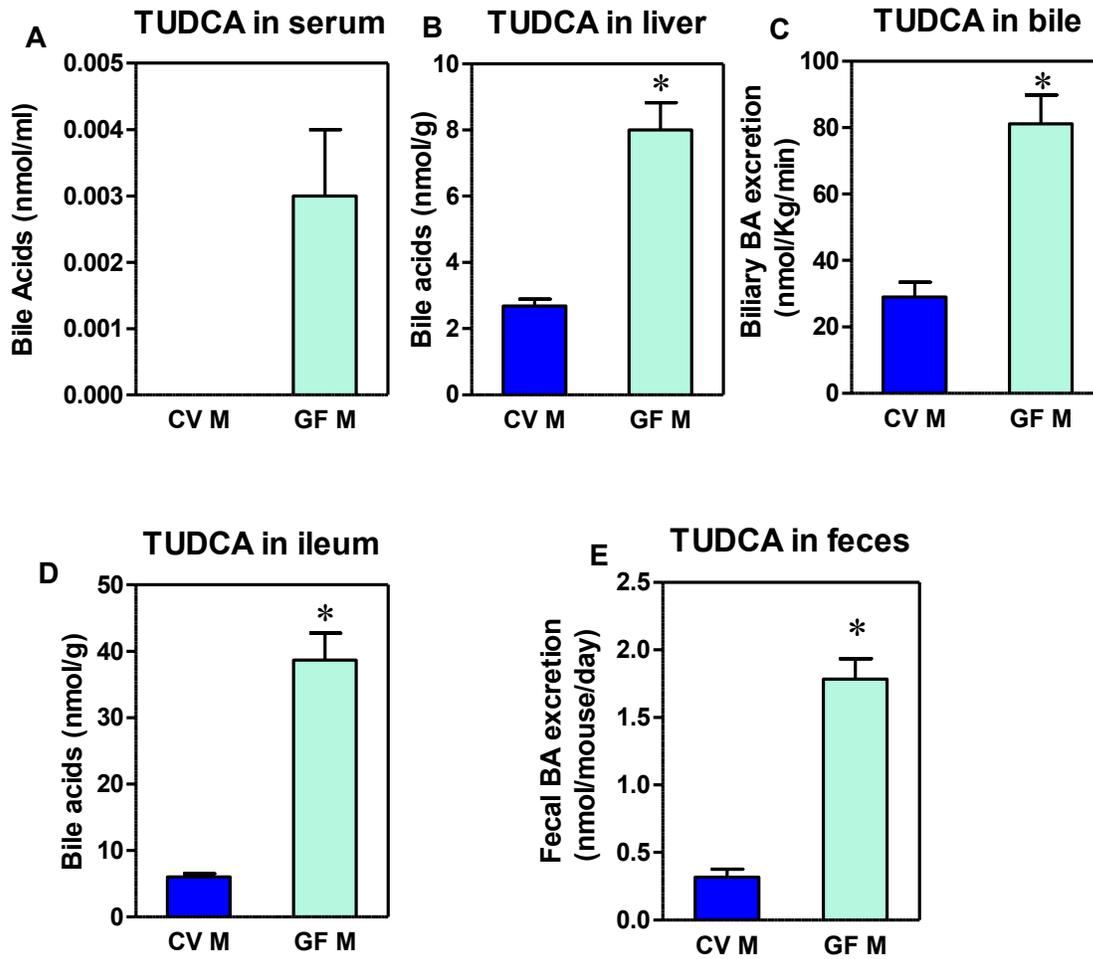
**Fig.4.17. Targets of TGR5 signaling.** (A) Serum GLP-1 quantification by ELISA. mRNA quantification by RT-PCR in (B) ileum, (C) brown adipose tissue and (D) muscle. Data are presented as means  $\pm$  SEM, n = 5/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Conv - conventional mice, GF - germ-free mice, M – males. Red bars represent CV mice and blue bars represent GF mice, respectively.

increased in muscle and brown adipose tissue of GF mice (Fig. 4.17.C and D) which suggests increased thermogenesis occurring in the brown adipose tissue and muscle of GF mice compared to CV mice.

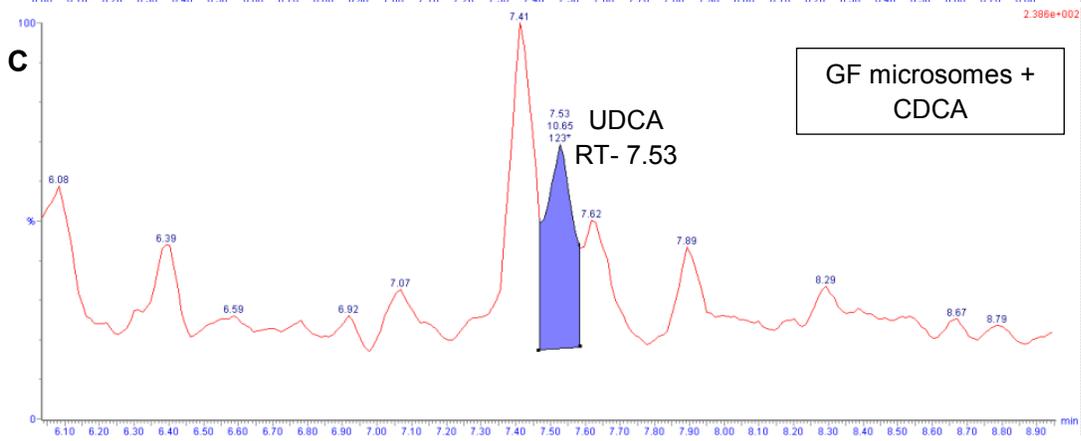
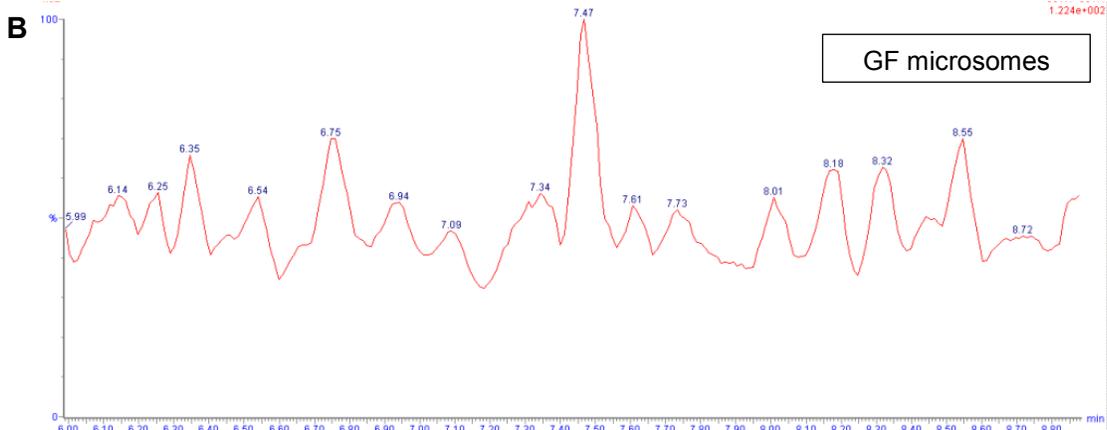
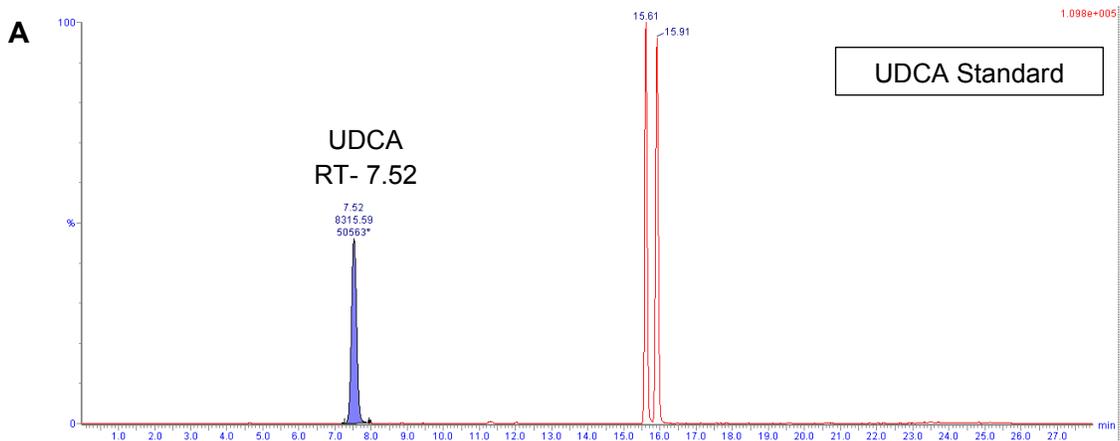
#### **E. UDCA synthesis by enzymes in mouse hepatic microsomes**

One of the striking findings of this study was that GF mice, without any gut bacteria, still synthesized UDCA, and had high TUDCA concentrations. TUDCA levels in serum were not detectable in CV male mice, but could easily be quantified in GF mice (Fig. 4.18.A). Compared to CV mice, GF mice had a 3-fold increase in TUDCA in the liver and bile (Fig. 4.18.B and C), and a 6-fold increase in TUDCA in the ileal tissue and in feces (Fig. 4.18.D and E). Therefore, I wanted to test the hypothesis that microsomal enzymes prepared from GF mice livers can synthesize UDCA from a primary BA. Using a traditional biotransformation assay (Description in Chapter 3), I found that incubating CDCA with hepatic microsomal protein from GF mice resulted in the appearance of a detectable peak with the same retention time and mass as the UDCA standard (Fig. 4.19). No UDCA peak was detected if the assay mixture did not contain CDCA or if CA was used instead of CDCA (data not shown), clearly suggesting that UDCA is a primary BA.

Because the concentration of TUDCA was higher in livers of GF mice compared to CV mice, I next wanted to determine whether hepatic microsomal enzymes from GF mice would synthesize more UDCA than enzymes isolated from CV mice. Therefore, I incubated the same concentration of hepatic microsomal protein from both CV and GF mice with CDCA in the biotransformation assay and analyzed the products.



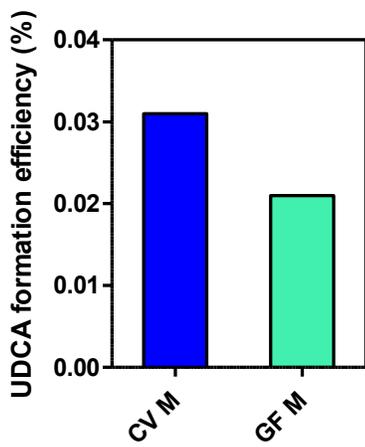
**Fig. 4.18. UDCA concentrations in CV and GF mice.** Bile acid concentrations in (A) serum, (B) liver, (C) bile, (D) ileal tissue, and (E) feces of CV and GF mice. Data are presented as means  $\pm$  SEM. Serum and liver n = 10/group; ileal tissue n = 6-10/group; bile n = 5/group; feces n = 4/group. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. CV - conventional mice, GF - germ-free mice, M - males. Dark blue and light blue bars represent CV and GF male mice, respectively.



**Fig. 4.19. Representative chromatograms.** UDCA was quantified using the LC-MS method developed in our laboratory to quantify bile acids. (A) Retention time of UDCA standard. (B) Control reaction mixture that does not have the BA substrate-CDCA added to the GF hepatic microsomal protein. (C) Reaction mixture containing GF hepatic microsomal protein + BA substrate CDCA. GF = germ-free.

Microsome sample	AUC of CDCA	AUC of UDCA	UDCA formation efficiency (%) (AUC UDCA / AUC CDCA) x100
CV M1	83214.5	29	0.035
CV M2	74308.2	20.6	0.028
		CV Mean	<b>0.031</b>
GF M1	138598.9	26.7	0.019
GF M2	127108.7	28.8	0.023
		GF Mean	<b>0.021</b>

**Table 4.2. Comparison of the efficiency of UDCA formation by conventional (CV) and germ-free (GF) hepatic microsomal enzymes**



**Fig 4.20. Comparison of the efficiency of UDCA formation by conventional (CV) and germ-free (GF) hepatic microsomal enzymes.** Reaction mixtures contained CDCA (200  $\mu$ M); 0.5 mg of liver microsomal protein; 100 mM potassium phosphate buffer, pH 7.4; 2 mM NADPH in a final volume of 250  $\mu$ L. After preincubation for 10 min at room temperature, reactions were initiated with NADPH and incubated for 60 min at 37°C. Reactions were terminated by adding 1 mL of ice-cold acetonitrile, dried under vacuum and resuspended in 80  $\mu$ L of 50% methanol. UDCA and CDCA were quantified using LC-MS. The efficiency of UDCA formation was reported as the ratio of the area under the curve (AUC) of the product BA (UDCA) formed to the AUC of the substrate BA (CDCA) used in the incubation mixture.

UDCA formation efficiency, was calculated as the ratio of the area under the curve (AUC) of UDCA (product) formed to the AUC of CDCA (substrate) (Table 4.2). The results demonstrate that hepatic microsomes from CV mice had a UDCA formation efficiency of 0.03% and hepatic microsomes from GF mice had a UDCA formation efficiency of 0.02% (Fig. 4.20). This suggests that both GF and CV mice have a similar capacity to synthesize UDCA.

### **III. Discussion**

The first report of quantification of BAs in GF mice was published in 1976, using crude analytical techniques (Eyssen et al., 1976). Last year, the second report of quantification of BAs in GF mice was published as we were finishing our studies. This recent publication described the BA composition in GF Swiss Webster mice (Sayin et al., 2013), whereas our studies were done with a widely used animal strain in BA research, the C57BL/6 mice in which the BA profiles in GF mice have not yet been characterized.

In our study, I observed that GF mice have a longer intestinal tract and an enlarged cecum. The enlarged cecum phenotype can be considered to be a morphological marker for the absence of gut bacteria, as the cecum decreases to its normal size upon colonization of GF mice with gut bacteria (Schaedler et al., 1965).

I also observed that gallbladders of GF mice are visually much larger and weigh about 4-5 fold more than gallbladders in CV mice. Bile flow is higher in GF mice compared to CV mice and this increase in bile flow might lead to the increase in gallbladder size. Enlarged gallbladders were also recently reported in GF Swiss

Webster mice (Sayin et al., 2013). BA activation of the TGR5 receptor in the gallbladder leads to an increase in gallbladder filling and gallbladder size (Li et al., 2011). So, I decided to determine whether BA-induced receptor TGR5 signaling is increased in GF mice. TGR5 activation will increase GLP-1 secretion from the enteroendocrine cells in the intestine, and increase expression of thyroid hormone activating enzyme (D2) and uncoupling protein (UCP3) in muscle and brown adipose tissue (Katsuma et al., 2005; Watanabe et al., 2006; Thomas et al., 2009). I observed an increase in the mRNA of PC1, which is the enzyme that cleaves the proglucagon peptide to make GLP-1, as well as a corresponding increase in serum GLP-1 and increase in the mRNA of the enzyme D2 in brown adipose tissue of GF mice. These observations suggest that increased concentrations and altered BA profiles in GF mice results in activation of TGR5 signaling. This increased TGR5 signaling seen in GF mice is probably a major reason for the better glycemic control and resistance to obesity reported in GF mice (Backhed et al., 2007; Rabot et al., 2010).

The BA profile of GF mice is strikingly different than that of CV mice. Compared to CV mice, GF male and female mice have increased total BAs in serum (4-fold), liver (2-fold), bile (2 to 3-fold), and ileal tissue (3 to 4-fold), and decreased total BAs in feces (3-fold), indicating that the BA pool size is larger in GF mice compared to CV mice. The decrease in total BAs in the feces of GF mice suggests that there is increased reabsorption and recirculation of BAs, which is supported by the finding that the mRNA of Asbt, the ileal BA uptake transporter is also increased about 150% in GF mice. An earlier study performed in CV and GF rats used radioactive CA to determine the biological half-life of CA. The results showed that the half-life was about 2 days in CV

rats but 11 days in GF rats (Gustafsson et al., 1957). Although the half-life of CA is known to increase in germ-free rodents, the concentration of TCA or CA were not markedly increased in livers of GF mice, suggesting lower basal levels of CA synthesis in GF mice, which is in agreement with the decreased mRNA levels of Cyp8b1 observed.

As expected, secondary BAs were essentially absent in all tissue compartments in GF mice. However, UDCA, which was thought to be a secondary BA in mice, was readily detected in tissues of GF mice suggesting that it could be a primary BA in mice. Biotransformation studies with liver microsomes isolated from both CV and GF mice verified that UDCA can be synthesized by metabolizing CDCA but not CA. Both GF and CV mouse livers appear to have a similar efficiency to convert CDCA to UDCA. But the conversion is small, and the enzymatic reaction has not been fully characterized. Nonetheless, this is the first report that enzymes in mouse liver microsome preparations can synthesize UDCA from the primary BA CDCA

Taurine-conjugated BAs increase and T $\alpha$ + $\beta$  MCA becomes the dominant BAs in all compartments analyzed in GF mice compared to CV mice. The increase in taurine-conjugated BAs could be because bile salt hydrolase enzymes that deconjugate BAs present in gut bacteria are absent in GF mice. In addition, taurine-conjugation of BAs also decreases its hydrophobicity. As hydrophobic BAs cause tissue injury, this increase in taurine-conjugated BAs could be an adaptive response to elevated total BAs. Recently, T $\beta$ MCA was identified to be an antagonist of the BA receptor FXR (Sayin et al., 2013). Increased levels of T $\beta$ MCA in the intestine suppress intestinal FXR feedback regulation of BAs, and results in an increase in hepatic Cyp7a1 expression and activity

(Li et al., 2013; Sayin et al., 2013). However, my observations are not in line with this finding. In my study, although T $\beta$ MCA levels increase in ileal tissue, there was not a decrease in the intestinal FXR pathway. Intestinal FXR inhibition should result in a decrease in ileal Fgf15 mRNA and hepatic SHP mRNA, as well as an increase in hepatic Cyp7a1 mRNA. But I observed no change in the mRNA of Fgf15, SHP and Cyp7a1 in GF and CV mice. Further, the mRNA of Cyp7a1 decreases in livers of female GF mice compared to CV mice.

The mRNA of important enzymes involved in BA synthesis was quantified. The mRNA of the rate-limiting enzyme (Cyp7a1) are similar in livers of male GF and CV mice, even though there was an increase in total BAs in all tissues of the GF mice. The mRNA of Cyp8b1, the enzyme responsible for 12 $\alpha$ -hydroxylation of BAs to make CA and the levels of CA are lower in GF mice compared to CV mice. The enzyme Cyp7b1 is responsible for 7 $\alpha$ -hydroxylation of cholesterol metabolites in the alternate pathway of BA synthesis, and the mRNA levels of Cyp7b1 are 30-50% lower in GF mice. This might result in a decreased BA output via the alternate pathway in GF mice

As a result of increased total BAs in GF mice, one might predict that mRNA levels of genes involved in the FXR-mediated BA-feedback mechanisms would be activated. However, targets of FXR activation, such as SHP (in liver) and Fgf15 (in ileum) did not change. This suggests that both liver and ileal FXR receptor signaling are not different in GF compared to CV mice. As mentioned above, T $\beta$ MCA can antagonize ileal FXR and the markedly increased concentrations of T $\alpha$ + $\beta$  MCA found in ileum could indeed lead to inactivation of FXR, explaining why its target gene mRNAs were not altered.

The absence of gut bacteria leads to slower intestinal propulsion and increased intestinal transit time (Abrams and Bishop, 1967). In the ileum, mRNA of the BA uptake transporter *Asbt* increased in GF male mice compared to CV mice. Therefore, the increased total BAs in tissues of GF mice could be due to increased reabsorption of BAs from the intestine, facilitated by the increased intestinal transit time and increased BA uptake by *Asbt*. Increased reabsorption of BAs in the intestine will result in increased enterohepatic circulation and increased half-life of BAs in GF rodents, as reported by others (Gustafsson et al., 1957). Decreasing the number of gut bacteria by administration of antibiotics, results in increased ileal *Asbt* expression, decreased fecal BA excretion and increased hepatic BA concentration, similar to what we observe in GF mice (Hu et al., 2013). Further, *Asbt*-null mice that have limited enterohepatic circulation of BAs have increased fecal BA excretion and a decreased BA pool size, just the opposite of what is observed in GF mice (Dawson et al., 2003).

Taken together, the BA profiles of both male and female GF mice are markedly altered compared to CV mice. GF mice have slower intestinal propulsion rates and increased BA reabsorption, which probably result in increased total BAs in all tissues analyzed. GF mice have increased proportions of MCAs, UDCA and taurine-conjugated BAs compared to CV mice. These BA changes render the BA pool more hydrophilic and the altered BA profile in GF mice results in the activation of TGR5 signaling which results in enlarged gallbladders, increased serum GLP-1 concentration and increased mRNA of thermogenesis related genes in brown adipose tissue and muscle.

**CHAPTER 5**  
**ALTERATIONS IN THE EXPRESSION OF HEPATIC**  
**DRUG METABOLIZING ENZYMES IN GERM-FREE MICE**

## I. Background

There are about  $10^{14}$  bacteria that call the intestinal tract of each human their home. These bacteria grow and divide inside the gut lumen alongside ingested food, drugs, bile and GI secretions. To survive, gut bacteria have to metabolize food, bile, etc to extract energy from them. In general the host liver provides an ideal environment for oxidation and conjugation reactions, making polar and high-molecular-weight metabolites, whereas the gut bacteria provides an environment suited for reduction and hydrolysis reactions, making nonpolar and lower-molecular-weight metabolites (Sousa et al., 2008). These gut bacterial enzymes, metabolize drugs as well as some endobiotic substances, such as conjugated hormones, conjugated bilirubin and BAs.

Gut bacterial metabolism of orally administered drugs can alter their efficacy and clearance. For example, a prodrug such as sulfasalazine on reduction by the bacteria in the colon, gives rise to 5-aminosalicylic acid (anti-inflammatory drug) and sulfapyridine (antibiotic) (Sousa et al., 2008). Liver is the major organ for drug metabolism. Phase-1 drug metabolizing enzymes perform oxidation, hydrolysis, and reduction reactions on drugs and phase-2 drug metabolizing enzymes perform conjugation reactions. While the action of hepatic phase-1 and phase-2 enzymes generally make the drugs more hydrophilic, the enzymes of gut bacteria often make the drug more hydrophobic by deconjugating the conjugated drug metabolites, favoring intestinal uptake and increasing the half-life of drugs (Stojancevic et al., 2013).

In addition, gut bacteria can also alter the expression of hepatic drug metabolizing enzymes. Bacterial infections are known to down-regulate the expression

and activities of drug metabolizing enzymes such as the Cyps (Morgan, 1997). Endotoxin of common gram-negative bacteria, when injected into rats, can decrease hepatic drug metabolism and Cyp expression (Ueyama et al., 2005). Oral antibiotics, such as ciprofloxacin, can alter the metabolism of other drugs co-administered to the host (Xie et al., 2003). It was later noted that ciprofloxacin decreases the gut bacteria that make the secondary BA, LCA which decreases the activation of the nuclear receptor FXR and lowers Cyp expression in the liver (Toda et al., 2009b).

The drug metabolizing capacity of an individual varies not only because of polymorphisms in genes encoding host drug metabolizing enzymes, but probably also because of individual differences in gut bacterial species. Further, therapeutic modulation of gut bacteria by probiotics, prebiotics, and by fecal transplantation has the potential to alter the drug metabolizing capacity of the host and thus affect the pharmacokinetics and pharmacodynamics of orally administered drugs taken simultaneously by the host. Thus, there is a need to identify drug metabolizing enzymes that are altered by gut bacteria. The function of gut bacteria is best studied in a model system that lacks gut bacteria, such as GF mice. Very little is known about the hepatic drug metabolizing enzymes that are regulated by gut bacteria. The studies published so far, have only analyzed a small subset of hepatic drug metabolizing enzymes in GF mice and their results are contradictory (Bjorkholm et al., 2009; Toda et al., 2009c).

Therefore, the **purpose of this study** was to determine the alterations in hepatic drug metabolizing enzymes at the transcriptome level in GF mice as compared to CV mice. The **hypothesis** I tested is that absence of gut bacteria would alter drug metabolizing capacity of the host. This was accomplished by comparing the mRNA of

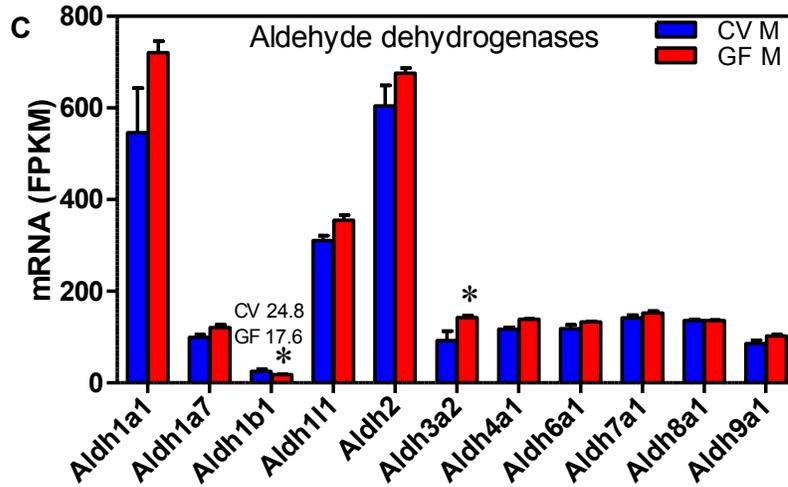
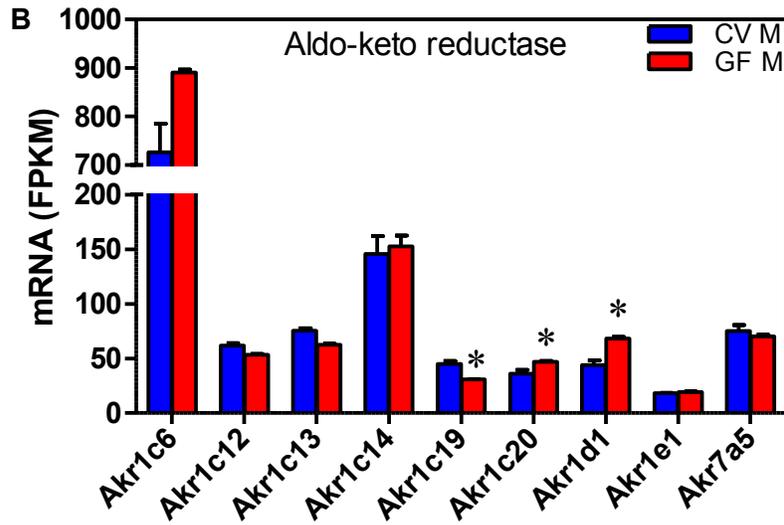
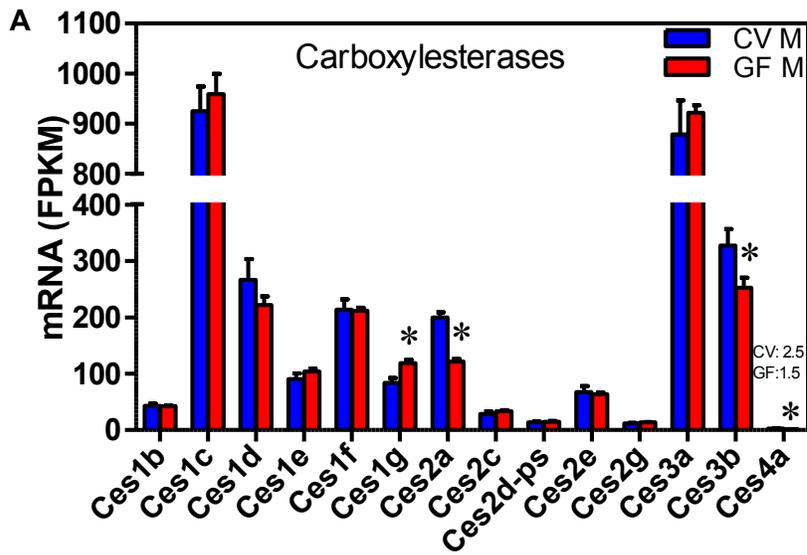
hepatic phase-1 and phase-2 drug metabolizing enzymes in the livers of GF and CV male mice using RNA-Seq. RNA-Seq provides a “true quantification” of transcripts and thus is an unbiased method of quantifying and comparing mRNA abundance of multiple genes (Cui et al., 2012). A physiological effect due to the differences in the levels of hepatic drug metabolizing enzymes was tested in intact animals by performing a pentobarbital sleeping test.

## **II. RESULTS**

### **A. Alterations in mRNA expression of hepatic phase-1 drug metabolizing enzymes in GF mice compared to CV mice.**

Enzymes involved in phase-1 drug metabolism generally catalyze hydrolysis, reduction, and oxidation reactions. In the livers of GF mice, carboxylesterases and cytochrome P450s are the most altered hepatic phase-1 drug metabolizing enzymes compared to CV mice.

**Carboxylesterases (Ces).** Ces are an important family of enzymes that hydrolyze drugs and other xenobiotics. The ester prodrug Cpt-11 (or irinotecan) is hydrolyzed to its active form SN-38 (an inhibitor of topoisomerase-1) by Ces. Ces1c and Ces3a are the Ces with the highest expression levels in the liver of mice.



**Fig. 5.1. Gene expression of (A) Ces, (B) Akr and (C) Aldh.** Total RNA was isolated from livers of adult male conventional (CV) and germ-free (GF) C57BL/6 mice (n = 3 per group). The cDNA libraries were sequenced 50bp paired-end using an Illumina HiSeq2000 sequencer. Approximately 80-100 million reads were generated per sample and over 95% of the reads were mapped to the mouse mm10 genome (Tophat). The transcript abundance was estimated by Cufflinks, and differential expression was determined using Cuffdiff (FDR-BH<0.05). Ces- Carboxylesterase, AKR- Aldo-keto reductase, Aldh-Aldehyde dehydrogenase, FPKM- fragments per kilobase of exon per million reads mapped.

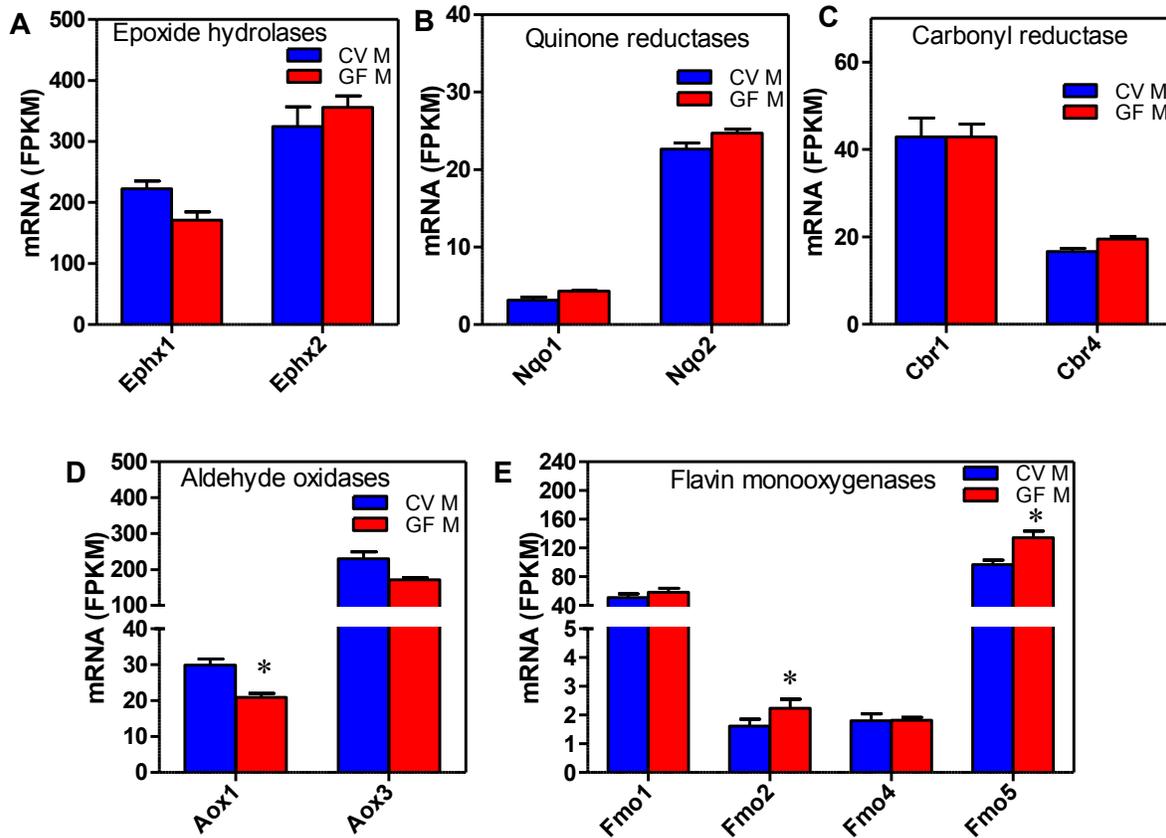
Compared to CV mice, GF mice have decreased levels of Ces2a (39%), Ces3b (23%), and Ces4a (40%) mRNA, and increased levels of Ces1g mRNA (42%). The mRNAs of other Ces (10 out of 14) were similar in the livers of CV and GF mice (Fig. 5.1.A).

**Aldo-keto reductase (Akr):** Akrs are NADPH-dependent oxido-reductase enzymes, which reduce aldehydes to alcohols. Akr1c6 is the highest expressed Akr in the livers of CV mice. GF mice have increased mRNA levels of Akr1c20 (30%) and Akr1d1 (56%), and decreased levels of Akr1c19 mRNA (31%) in liver, compared to CV mice. The mRNAs of the other Akrs (6 out of 9) were quantitatively similar in the livers of both groups of mice (Fig.5.1.B).

**Aldehyde dehydrogenase (Aldh).** Aldh enzymes catalyze the oxidation of aldehydes to carboxylic acids using  $\text{NAD}^+$  as a cofactor. Aldh1a1 and Aldh2 are the most highly expressed Aldh in the livers of mice. Aldh3a2 mRNA is increased (54%) and Aldh1b1 mRNA is decreased (29%) in livers of GF mice compared to CV mice. The mRNAs of other Aldhs (9 out of 11) expressed in liver were similar in CV and GF mice (Fig. 5.1.C).

**Epoxide hydrolase (Ephx), Quinone reductase (Nqo), and Carbonyl reductase (Cbr).** The mRNA levels of different Ephxs, Nqos, and Cbrs were similar in livers of GF and CV mice (Fig. 5.2.A, B and C).

**Aldehyde oxidase (Aox).** Aox is an important class of cytosolic drug metabolizing enzyme with broad substrate specificity in the liver. Aox3 is the highest expressed AOX in the livers of mice, and its mRNA levels are similar in the livers of CV



**Fig. 5.2. Gene expression of (A) Eph, (B) Nqo, (C) Cbr, (D) Aox, and (E) Fmo.** Total RNA was isolated from livers of adult male conventional (CV) and germ-free (GF) C57BL/6 mice (n = 3 per group). The cDNA libraries were sequenced 50bp paired-end using an Illumina HiSeq2000 sequencer. Approximately 80-100 million reads were generated per sample and over 95% of the reads were mapped to the mouse mm10 genome (Tophat). The transcript abundance was estimated by Cufflinks, and differential expression was determined using Cuffdiff (FDR-BH<0.05). Eph- Epoxide hydrolase, Nqo- Quinone reductase, Cbr- Carbonyl reductase, Aox- Aldehyde oxidase, Fmo- Flavin monooxygenase, FPKM- fragments per kilobase of exon per million reads mapped.

and GF mice. Compared to CV mice Aox1 mRNA levels are reduced by about one-third in GF mice (Fig. 5.2.D). Aox1 plays a role in ethanol-induced liver injury (Shaw and Jayatilleke, 1990)

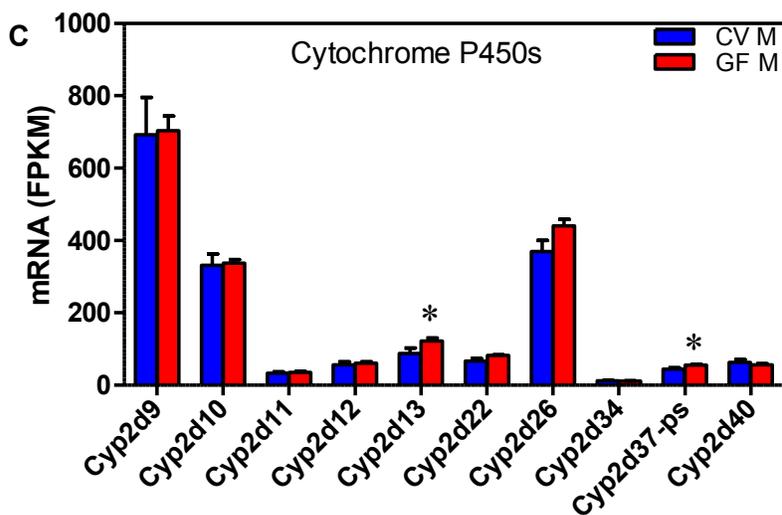
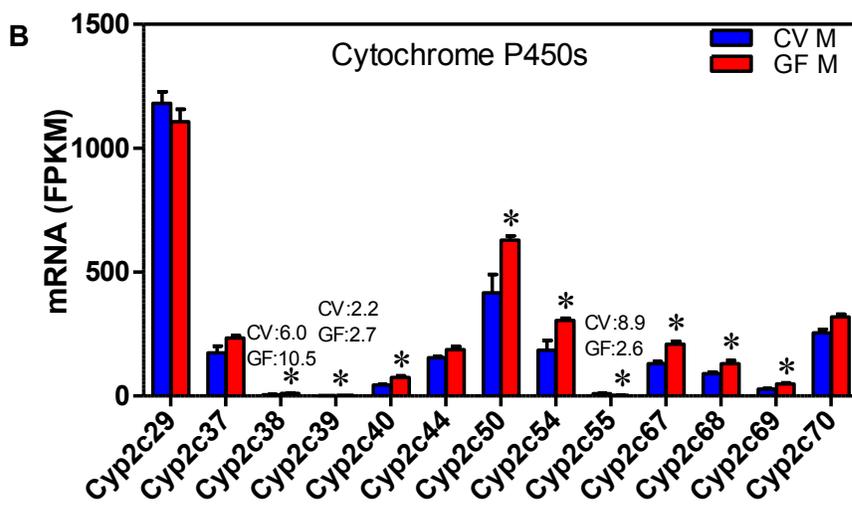
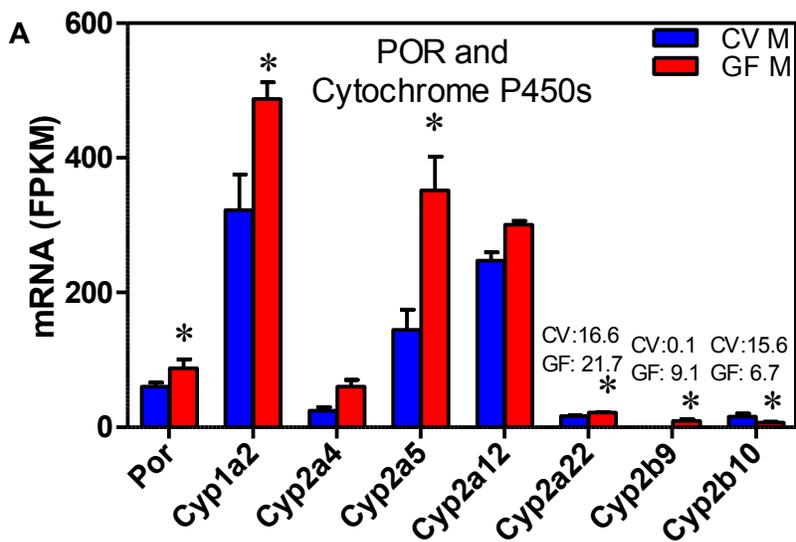
**Flavin monooxygenases (Fmo).** Fmo are a FAD-containing monooxygenases that require NADPH to oxidize nucleophilic nitrogen, sulfur, and phosphorous atoms of a xenobiotic. Fmo1 and Fmo5 are the two most highly expressed Fmos in livers of mice. GF mice have increased Fmo2 (39%) and Fmo5 (38%) mRNA in the liver compared to CV mice, whereas the mRNA levels of Fmo1 and 4 are similar in livers of GF and CV mice (Fig.5.2.E).

**NADPH-cytochrome P450 oxidoreductase (POR).** POR is essential in passing electrons from NADPH to CyPs located in the endoplasmic reticulum. There exists only one POR for the many Cyp enzymes in the liver, and GF mice have increased POR mRNA levels (46%) compared to CV mice (Fig. 5.3.A). This may lead to an overall increase in Cyp activity.

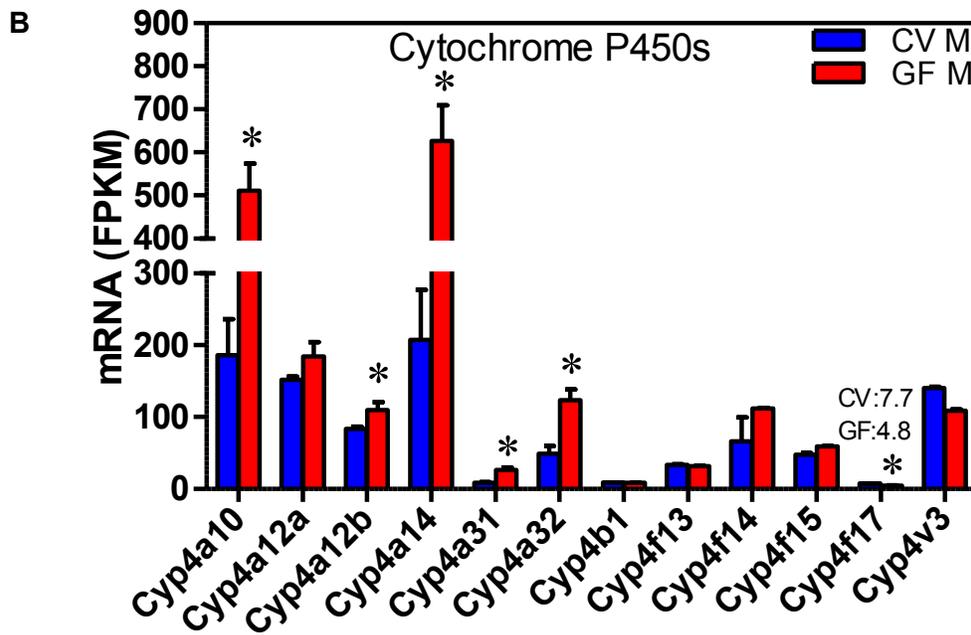
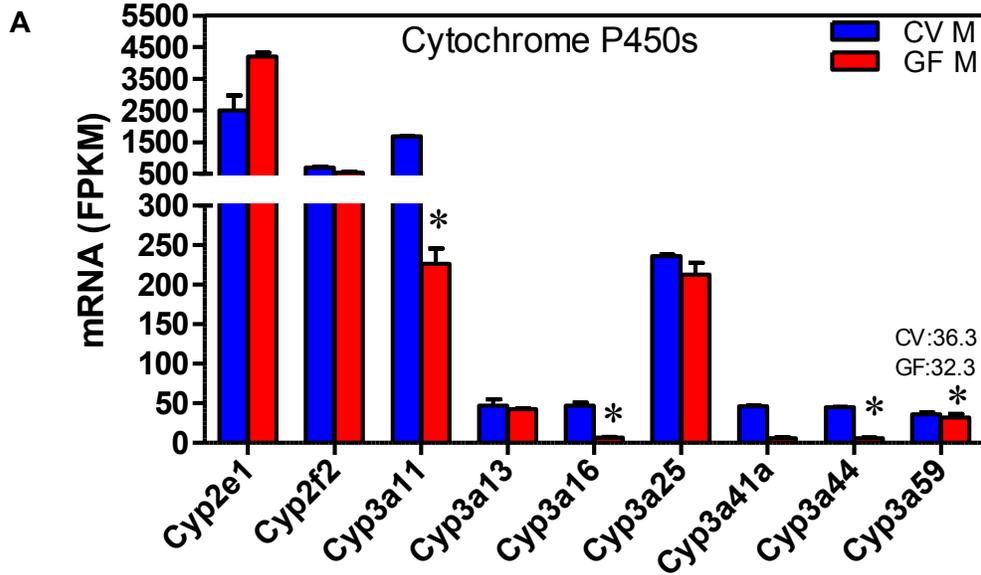
**Cytochrome P450 enzyme (CyPs).** CyPs are the largest family of drug metabolizing enzymes in the liver and are responsible for the majority of hepatic phase-1 drug metabolism. CyPs are heme-containing enzymes that catalyze the monooxygenation of xenobiotics. CyPs are divided into families and subfamilies based on amino acid homology. The first three families, namely Cyp1, Cyp2, and Cyp3, are involved in xenobiotic metabolism. Although, the Cyp4 family is important for  $\omega$ -hydroxylation of fatty acids and prostaglandins, its members also play a role in

xenobiotic metabolism (Hsu et al., 2007). Therefore, the expression of Cyp1, Cyp2, Cyp3, and Cyp4 families in livers of CV and GF mice are described below.

- a) **Cyp1 family-** GF mice have increased Cyp1a2 mRNA levels (51%) in liver compared to CV mice (Fig. 5.3.A).
- b) **Cyp2a subfamily-** Among the Cyp2a subfamily, Cyp2a5 was highly expressed in the livers of mice, and GF mice have increased Cyp2a5 (143%) and Cyp2a22 mRNA levels (33%) in livers compared to CV mice. Cyp2a5 is an Ahr target gene and is responsible for the metabolism of drugs and xenobiotics such as halothane, nicotine, and aflatoxin B1. The mRNA levels of Cyp2a12 and Cyp2a4 are similar (2 of 6) in the livers of CV and GF mice (Fig. 5.3.A).
- c) **Cyp2b subfamily-** Cyp2b subfamily is expressed at low levels in livers of mice. In the livers of GF mice, the mRNA of Cyp2b9 was higher (7454%) but Cyp2b10 mRNA was lower (57%) lower than livers of CV mice (Fig. 5.3.A).
- d) **Cyp2c subfamily-** Cyp2c29 is the highest expressed among the Cyp2c subfamily members in livers of mice. GF mice have higher mRNA levels of the following Cyp2c subfamily members compared to CV mice livers: Cyp2c38, Cyp2c39, Cyp2c40, Cyp2c50, Cyp2c54, Cyp2c67, Cyp2c68, and Cyp2c69. The mRNA of Cyp2c55 was decreased and the mRNA levels of Cyp2c29, Cyp2c37, Cyp2c44, and Cyp2c70 are similar in the livers of CV and GF mice (Fig. 5.3.B).



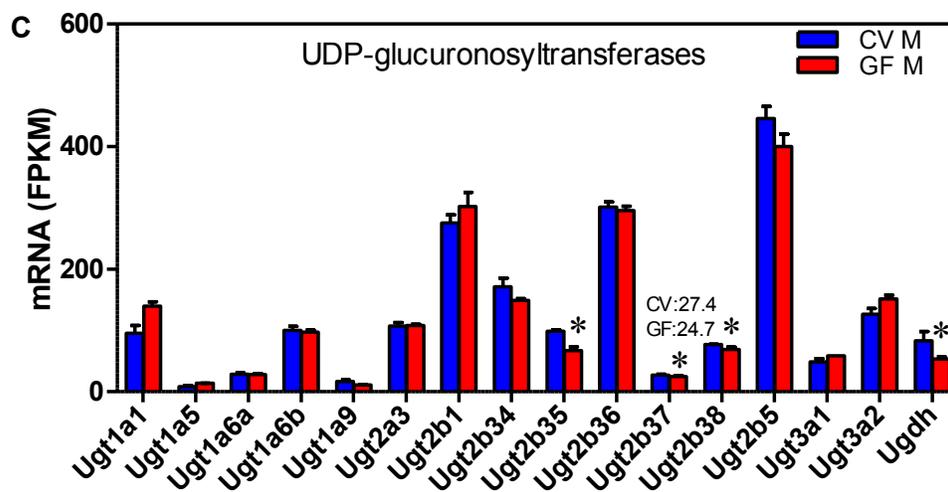
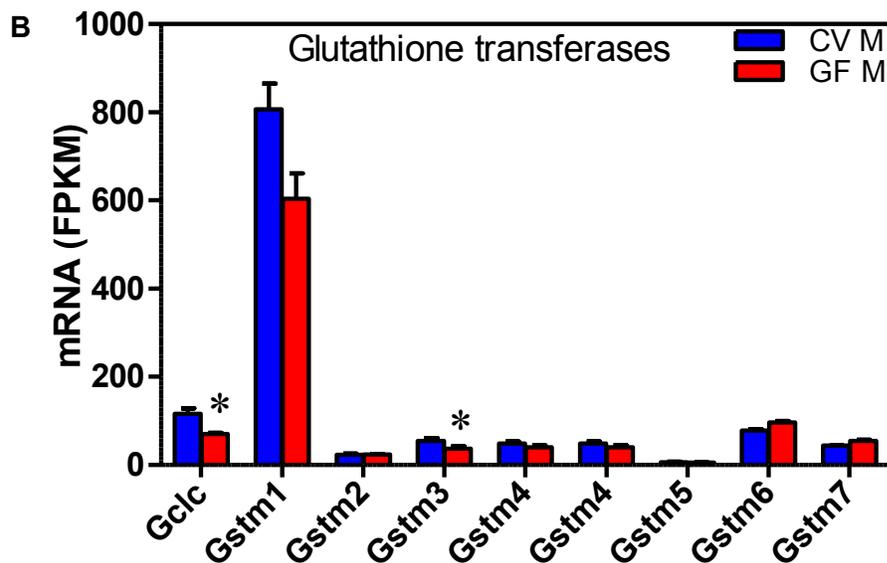
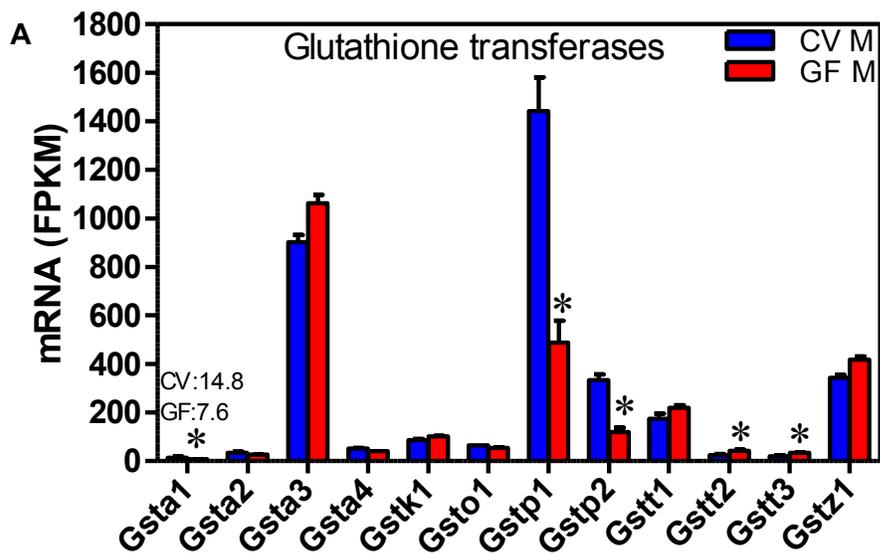
**Fig. 5.3. Gene expression of (A) Por, Cyp1a, 2a and 2b subfamily, (B) Cyp2c subfamily, and (C) Cyp2d subfamily.** Total RNA was isolated from livers of adult male conventional (CV) and germ-free (GF) C57BL/6 mice (n = 3 per group). The cDNA libraries were sequenced 50bp paired-end using an Illumina HiSeq2000 sequencer. Approximately 80-100 million reads were generated per sample and over 95% of the reads were mapped to the mouse mm10 genome (Tophat). The transcript abundance was estimated by Cufflinks, and differential expression was determined using Cuffdiff (FDR-BH<0.05). POR- NADPH-cytochrome P450 oxidoreductase, Cyp- Cytochrome P450, FPKM- fragments per kilobase of exon per million reads mapped.



**Fig. 5.4. Gene expression of (A) Cyp2e, 2f, and 3a subfamily and (B) Cyp4 family.**

Total RNA was isolated from livers of adult male conventional (CV) and germ-free (GF) C57BL/6 mice (n = 3 per group). The cDNA libraries were sequenced 50bp paired-end using an Illumina HiSeq2000 sequencer. Approximately 80-100 million reads were generated per sample and over 95% of the reads were mapped to the mouse mm10 genome (Tophat). The transcript abundance was estimated by Cufflinks, and differential expression was determined using Cuffdiff (FDR-BH<0.05). Cyp- Cytochrome P450, FPKM- fragments per kilobase of exon per million reads mapped.

- e) **Cyp2d subfamily-** Among the Cyp2d subfamily members, Cyp2d9 is the most highly expressed. GF mice have increased Cyp2d13, and Cyp2d37-ps mRNA in the livers compared to CV mice. All other mRNA levels of other eight Cyp2d subfamily members are similar in the livers of CV and GF mice (Fig. 5.3.C).
- f) **Cyp2e and Cyp2f subfamilies-** Cyp2e1 and Cyp2f2 are both highly expressed Cyps in the livers of CV mice and their mRNA levels were similar in GF and CV mice (Fig. 5.4.A).
- g) **Cyp3a subfamily-** Cyp3a11 is the highest expressed member among the Cyp3a subfamily in livers of CV mice and it is the most decreased mRNA in livers of GF mice (87%) compared to CV mice. GF mice also have reduced mRNA levels of Cyp3a16 (86%), Cyp3a44 (87%), and Cyp3a59 (11%). The mRNA levels of Cyp3a13, Cyp3a25, and Cyp3a41a are similar in livers of CV and GF mice (Fig. 5.4.A).
- h) **Cyp4a, 4b, 4f, and 4v subfamilies-** In livers of GF mice, the mRNA of Cyp4a10, and Cyp4a14, Cyp4a31, and Cyp4a32 are increased between 150-200%, whereas mRNA of Cyp4a12b, was increased 31% over CV mice. Cyp4f17 mRNA is decreased (38%) in GF mice, and the mRNA levels of Cyp4a12a, Cyp4b1, Cyp4f13, Cyp4f14, Cyp4f15, and Cyp4v3 are similar in livers of CV and GF mice (Fig. 5.4.B).



**Fig.5.5. Gene expression of Gsts (A), (B) and Ugts (C).** Total RNA was isolated from livers of adult male conventional (CV) and germ-free (GF) C57BL/6 mice (n = 3 per group). The cDNA libraries were sequenced 50bp paired-end using an Illumina HiSeq2000 sequencer. Approximately 80-100 million reads were generated per sample and over 95% of the reads were mapped to the mouse mm10 genome (Tophat). The transcript abundance was estimated by Cufflinks, and differential expression was determined using Cuffdiff (FDR-BH<0.05). Gst- Glutathione transferase, Ugt- uridine diphosphate-glucuronosyltransferase, FPKM- fragments per kilobase of exon per million reads mapped.

## **B. Alterations in mRNA expression of hepatic phase-2 drug metabolizing enzymes in GF mice compared to CV mice.**

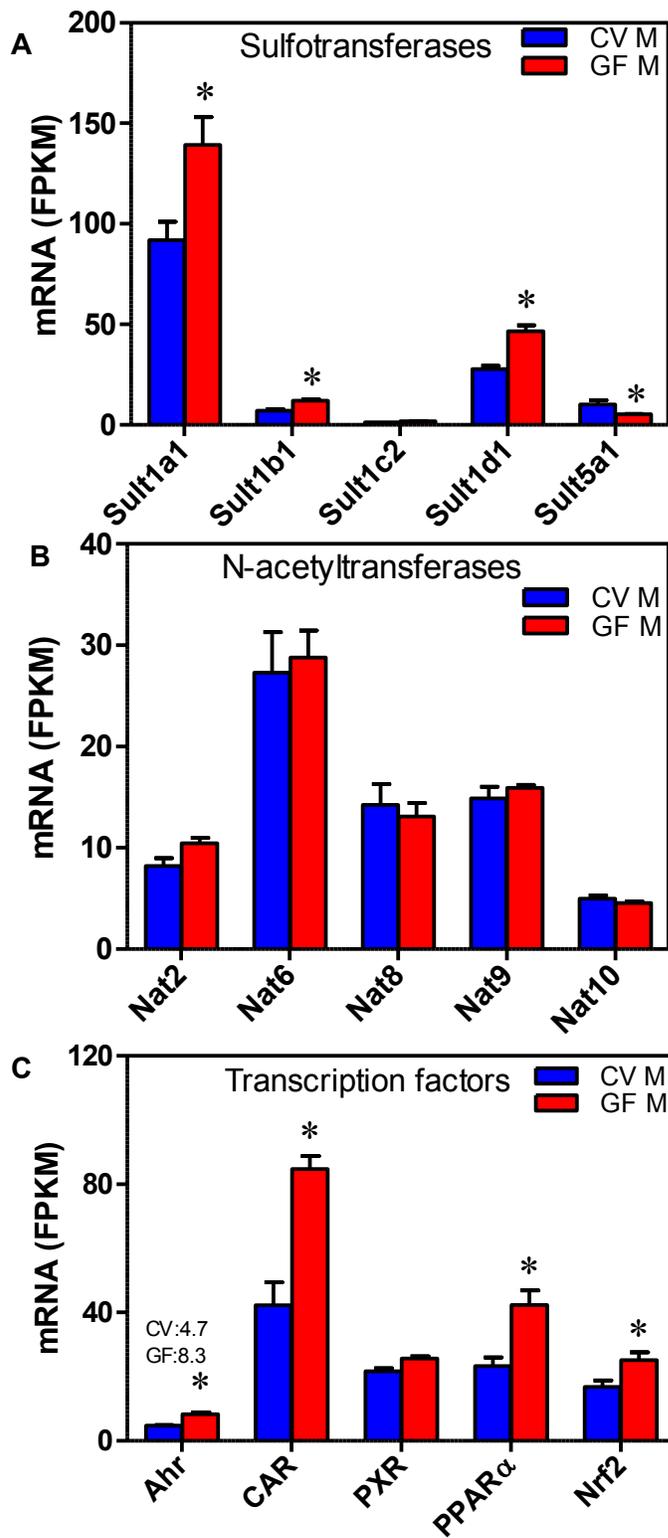
Phase-2 drug metabolizing enzymes are involved in conjugation reactions. Gut bacteria possess enzymes that can deconjugate conjugated xeno- and endobiotics. The glutathione transferases and UDP-glucuronosyltransferases are the most altered phase-2 drug metabolizing enzymes in livers of GF mice compared to CV mice.

**Glutathione transferase (Gst).** Gst enzymes catalyze the transfer of glutathione to the xenobiotic to make it more hydrophilic. Gsts detoxify polycyclic aromatic hydrocarbons and other carcinogens in the diet and tobacco and therefore polymorphisms in Gsts are associated with differences in the susceptibility to carcinogens. Among Gst family members, the highest expressed Gsts in livers of mice include, Gstp1. GF mice have decreased mRNA of Gsta1 (48%), Gstp1 (66%), Gstp2 (64%), and Gstm3 (32%) compared to CV mice. The gene expression of Gstp1 was the second most decreased among the phase-1 and Phase-2 genes in livers of GF mice. Gut bacterial metabolites, the short chain fatty acids, are known to induce the expression of Gstp1 in intestine (Stein et al., 1996), and it appears that they might also increase Gstp1 expression in liver. The mRNA levels of Gstt2 (67%) and Gstt3 (67%) are increased in livers of GF mice compared to CV mice. Other Gsts have similar expression levels in livers of CV and GF mice. The enzyme glutamate-cysteine ligase catalytic subunit (Gclc) is the rate limiting enzyme for glutathione synthesis. Gclc mRNA levels were also decreased (40%) in livers of GF mice compared to CV mice (Fig. 5.5.A and B).

**UDP-glucuronosyltransferase (Ugt).** Ugts are enzymes that catalyze the transfer of glucuronic acid from the co-substrates uridine diphosphate glucuronic acid to the xenobiotic. Among the Ugts, Ugt2b5, Ugt2b36, and Ugt2b1 are the highest expressed in livers of mice. Livers of GF mice have decreased Ugt2b35 (32%), Ugt2b37 (10%), and Ugt2b38 (11%) mRNA compared to CV mice. The mRNA levels of other Ugts are similar in the livers of CV and GF mice (Fig. 5.5.C).

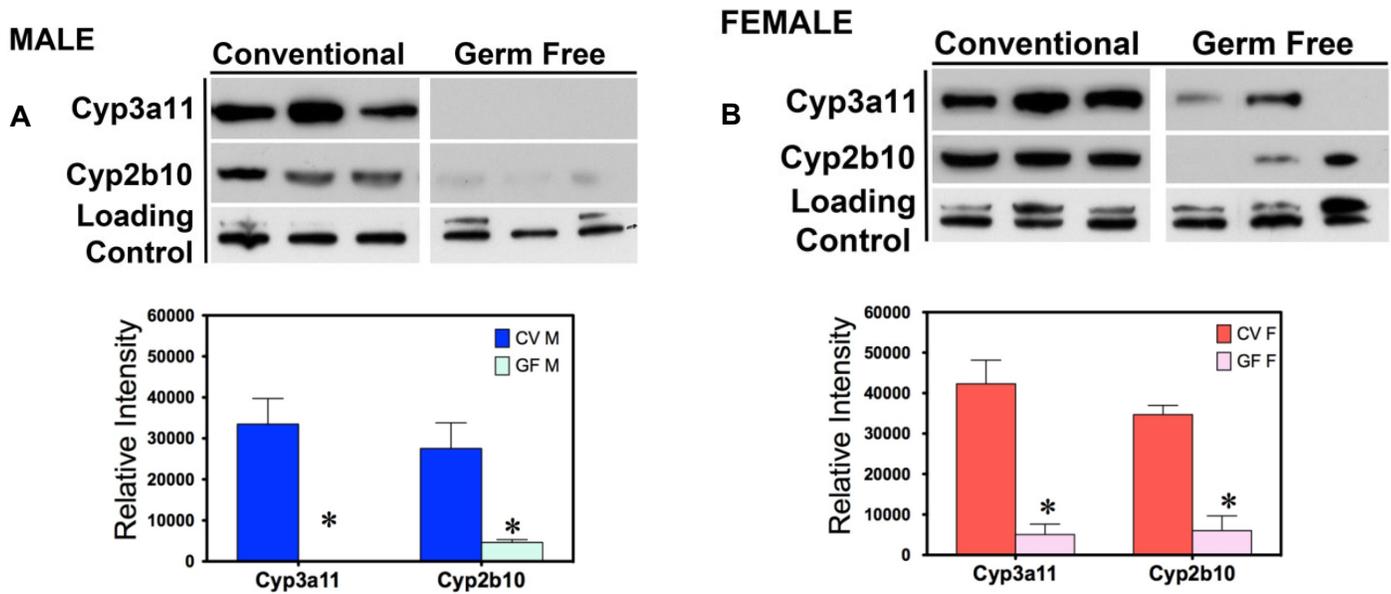
**Sulfotransferase (Sult).** Sults catalyze the transfer of a sulfonic acid group from the co-substrate PAPS (3'-phosphoadenosine-5'-phosphosulfate) to the xenobiotic. Sult1a1 is the highest expressed Sult in the livers of mice. GF mice have increased Sult1a1 (52%), Sult1b2 (70%) and Sult1d1 (68%) mRNA levels compared to CV mice, whereas the mRNA of Sult5a1 is decreased (48%) (Fig. 5.6.A).

**N-acetyl transferase (Nat).** The Nat enzymes catalyze the transfer of an acetyl group from the cofactor acetyl-coenzyme A to an amino group in the xenobiotics. This conjugation makes the xenobiotic less water-soluble unlike other phase-2 drug metabolizing reactions that make them more water soluble. Nat6 is the highest expressed Nat enzyme in the livers of mice. The mRNAs of all Nat enzymes are similar in livers of GF and CV mice (Fig. 5.6.B).

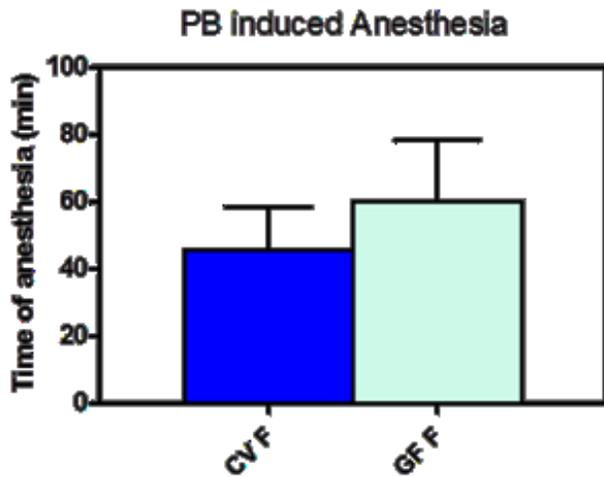


**Fig. 5.6. Gene expression of (A) Sults, (B) Nats, (C) transcription factors in liver.**

Total RNA was isolated from livers of adult male conventional (CV) and germ-free (GF) C57BL/6 mice (n = 3 per group). The cDNA libraries were sequenced 50bp paired-end using an Illumina HiSeq2000 sequencer. Approximately 80-100 million reads were generated per sample and over 95% of the reads were mapped to the mouse mm10 genome (Tophat). The transcript abundance was estimated by Cufflinks, and differential expression was determined using Cuffdiff (FDR-BH<0.05). Sults- Sulfotransferases, Nats- *N*-acetyl transferase, Ahr- Aryl hydrocarbon receptor, CAR- Constitutive androstane receptor, PXR- Pregnane X receptor, PPAR $\alpha$ - Peroxisome proliferator-activated receptor  $\alpha$ , Nrf2- nuclear factor erythroid 2-related factor 2, FPKM- fragments per kilobase of exon per million reads mapped.



**Fig 5.7. Protein expression of Cyp2b10 and Cyp3a11 in (A) Males and (B) Females.** Western blot results of Cyp2b10 and Cyp3a11 in the livers of GF and CV mice. Intensities of protein bands were quantified using Image J software. Asterisks (\*) represent statistically significant differences between CV and GF mice ( $p < 0.05$ ) by Student's *t*-test. Dark blue and light blue bars represent CV and GF male mice; red and pink bars represent CV and GF female mice, respectively. CV- conventional mice. GF- germ-free mice. M- males. F- females.



**Fig 5.8 Pentobarbital induced anesthesia.** Pentobarbital (40 mg/kg) was injected intraperitoneally and the time between losing and subsequently regaining the righting reflex is plotted in the graph as minutes. Data is represented as mean  $\pm$  SEM,  $n = 4$ , female mice per group. CV refers to conventional mice, GF refers to germ-free mice. F refers to females.

### **C. Alterations in mRNA expression of transcription factors in the liver**

Hepatic transcription factors such as, Ahr, CAR, PXR, PPAR $\alpha$ , and Nrf2 act as xenosensors and regulate the expression of hepatic drug metabolizing enzymes. Interestingly, GF mice have higher Ahr, CAR, PPAR $\alpha$ , and Nrf2 mRNA in liver than CV mice. The mRNA of PXR remains the same in the livers of GF and CV mice (Fig. 5.6.C).

### **D. Alterations in protein levels of Cyp enzymes**

The mRNA of the two very important drug metabolizing enzymes, Cyp2b10 and Cyp3a11 were decreased in livers of GF mice compared to CV mice. Therefore, I quantified the protein levels of Cyp3a11 and Cyp2b10 in the livers of CV and GF mice. Similar to their mRNA, both Cyp2b10 and Cyp3a11 protein levels were decreased in livers of GF mice compared to CV mice (Fig. 5.7).

### **E. Pentobarbital sleeping test**

Next I wanted to determine whether differences in mRNA and protein levels of Cyp2b10 and Cyp3a11 resulted in a physiological effect in the GF mice. The pentobarbital sleeping test is an index of hepatic drug metabolism in intact animals. The time between losing and regaining the righting reflex after administration of the anesthetic pentobarbital is an indication of how quickly the pentobarbital is metabolized. The GF mice slept for 60 minutes whereas CV mice slept for only 45 minutes. Thus GF mice slept 33% longer when administered the same dose of pentobarbital intraperitoneally than CV mice (Fig.5.8), suggesting that pentobarbital is metabolized slower in GF mice compared to CV mice.

### III. Discussion

Liver is the major organ for drug metabolism. Moreover, it is known that, antibiotics can alter gut bacteria and decrease the expression of some drug metabolizing enzymes in the liver. However, previous studies, only analyzed a small subgroup of host hepatic drug metabolizing enzymes. Therefore, I analyzed the hepatic transcriptome of the GF and CV mice by RNA-Seq and compared the mRNA levels of phase-1 and phase-2 drug metabolizing enzymes in GF and CV mice. The absence of gut bacteria in mice alters the gene expression of a number of phase-1 and phase-2 drug metabolizing enzymes.

Two groups have earlier performed microarray analysis and described changes in the mRNA of hepatic drug metabolizing enzymes in GF NRMI and IQI mice (Bjorkholm et al., 2009; Toda et al., 2009c). Their results are contradictory to each other and our observations are different from both of their reports, possibly due to differences in the strains of mice used in the study.

Table 5.1 shows the list of hepatic drug metabolizing genes that were altered in GF mice compared to CV mice in our study compared to the two previous microarray studies on GF mice. In my study, the mRNA levels of Cyp2b9 increased markedly (7454%) whereas in the study by Toda et al., the mRNA levels of Cyp2b9 decreased in GF mice compared to controls (Toda et al., 2009c). The mRNA levels of Sult1c2 and Ugt1a1 increase in GF mice in the study by Bjorkholm et al., whereas the mRNA levels were the same in GF and CV mice in my study (Bjorkholm et al., 2009). The gene expression of major xenobiotic-sensing nuclear receptors PXR remained the same in the livers of GF and CV mice in my study, whereas Toda et al. reported a decrease in

PXR mRNA in GF mice (Toda et al., 2009c). The gene expression of other xenobiotic-sensing nuclear receptors Ahr and CAR increase in livers of GF mice compared to CV mice in my study. Toda et al. hypothesize that in GF IQI mice, the decreased concentrations of secondary BAs is the reason for the decrease in gene expression of the nuclear receptor CAR and its target genes (Toda et al., 2009c). Although, Bjorkholm et al. explain that in GF NRMI mice, the mRNA of the nuclear receptor CAR and its target genes increase (Bjorkholm et al., 2009) I do not see an increase in all CAR target genes. In my study, the mRNA of the CAR target gene, Cyp2b9 increases, whereas Cyp2a4 mRNA remains the same and Cyp2b10 mRNA decreases in the livers of GF mice compared to CV mice.

Pentobarbital treated GF mice tended to sleep longer than CV mice, suggesting that metabolism of pentobarbital is slower in GF mice than CV mice, and this agrees with the observation that Cyp2b10 and Cyp3a11 mRNA and protein are lower in GF mice than CV mice.

This study provides a list of drug metabolizing enzymes whose mRNA levels increase or decrease in the absence of gut bacteria (Table 5.2). These genes can be referred to as “gut bacteria target drug metabolizing genes” in the liver of mice as their levels are altered by gut bacteria.

The drug metabolizing enzymes whose mRNA decrease in GF mice are probably increased normally in the presence of gut bacteria to help metabolize gut bacterial metabolites. Therefore, the drug metabolizing enzymes whose mRNA increase in GF mice are probably enzymes whose functions are performed by the gut bacterial

enzymes and therefore in the absence of gut bacteria they have to be induced to compensate for the change.

In conclusion, there are a number of hepatic drug metabolizing enzymes that are target genes for gut bacteria, including the major drug metabolizing enzyme Cyp3a11. These changes may alter the pharmacokinetics and pharmacodynamics of orally administered drugs. The composition of gut bacteria may one day be used to predict a person's response to a drug. It will be important to study the effect of probiotic strains of bacteria on drug metabolizing genes to prevent potential detrimental interactions with a simultaneously ingested drug. Altering gut bacteria might provide a novel approach to modify the drug metabolizing capacity of the liver.

**Table 5.1 Comparison of gene expression of drug processing genes in different strains of GF mice compared to their respective CV mice**

Gene name	Gene Expression In GF mice compared to CV mice (approx.)		
	GF IQI mice (Toda et al)	GF NRMI mice (Bjorkholm et al)	GF C57BL6 mice (this study)
Cyp1a2	50% decrease		51% increase
Cyp2a4	86% decrease	340% increase	No change
Cyp2b9	99% decrease	2325% increase	7457% increase
Cyp2b10			57% decrease
Cyp2c38		140% increase	74% increase
Cyp3a11	90% decrease		87% decrease
Cyp3a13	50% decrease		No change
Cyp3a16	100% decrease		86% decrease
Cyp3a25	20% decrease		No change
Cyp3a41	99% decrease		No change
Cyp3a44	98% decrease		87% decrease
Sult1b1		75% increase	70% increase
Sult1c2		80% increase	No change
Sult1d1	75% decrease	95% increase	68% increase
Gstt3		100% increase	67% increase
Gstp1		110% decrease	66% decrease
Ugt1a1		75% increase	No change
Ahr	52% decrease		50% increase
CAR	80% decrease	100% increase	50% increase
PXR	33% decrease		No change

**Table 5.2 List of genes that are differentially regulated at the transcription level by the presence of gut bacteria.**

	<b>Genes expression changes in GF mice compared to CV mice</b>	
	<b>Decreased in GF mice</b>	<b>Increased in GF mice</b>
<b>Phase-1 drug metabolizing enzymes</b>	<p> <b>Ces2a</b>  <b>Ces3b</b>  <b>Ces4a</b>  <b>Akr1c19</b>  <b>Aldh1b1</b>  <b>Aox1</b>  <b>Cyp2b10</b>  <b>Cyp3a11,16,44,59</b>  <b>Cyp4f17</b> </p>	<p> <b>Ces1g</b>  <b>Akr1c20</b>  <b>Akr1d1</b>  <b>Aldh3a2</b>  <b>Fmo2,5</b>  <b>Cyp1a2</b>  <b>Cyp2a5,22</b>  <b>Cyp2b9</b>  <b>Cyp2c38,39,40,50,54,67,68,69</b>  <b>Cyp4a10,12b,14,31,32</b> </p>
<b>Phase-2 drug metabolizing enzymes</b>	<b>Decreased in GF mice</b>	<b>Increased in GF mice</b>
	<p> <b>Gsta1</b>  <b>Gstp1,2</b>  <b>Gstm3</b>  <b>Ugt2b35,37,38</b>  <b>Sult5a1</b> </p>	<p> <b>Gstt2,3</b>  <b>Sult1a1</b>  <b>Sult1b1</b>  <b>Sult1d1</b> </p>

**CHAPTER 6**  
**DISCUSSION OF DISSERTATION**

## 6.1 Summary and Discussion

Planet earth is not a sterile place, thus all animals that live on earth have microorganisms living on and in them. This must be beneficial for both the animals and the microorganisms as this symbiotic relationship has been selected by nature. Human beings have about 20,000 genes in their genome, but serve as a host to a variety of microorganisms that have about 5-8 million genes in total. The intestinal bacteria interact with the host and thereby, regulate many biochemical processes in the host such as BA and drug metabolism. Gut bacteria vary significantly between individuals and therefore, may be responsible for inter-individual differences in BA concentrations and drug responses. Further, alterations of gut bacteria in diseases or by therapeutic modulation of gut bacteria have the potential to alter host BA signaling and drug responsiveness. Thus, the overall goal of this dissertation was to expand the understanding of the role of gut bacteria in regulating host bile acid homeostasis and hepatic drug metabolism.

Gene-knockout mice are excellent models to understand the function of a gene. Similarly, mice that do not have gut bacteria (GF mice) provide an excellent animal model to understand the function of gut bacteria. Learning how BA profiles and expression of hepatic drug metabolizing enzymes change in GF mice compared to conventional mice, will reveal the regulatory role of gut bacteria in host BA composition, drug metabolism, physiological homeostasis as well as protection against pathological disorders.

Therefore, in **Specific Aim 1**, differences in the BA profile, concentration, and BA signaling in GF mice were compared to CV mice. The BA profile was markedly different

in GF mice compared to CV mice. GF mice have only a few types of BAs (only primary BAs), which suggests that gut bacteria produce differences in the BA profile. It is known that BAs are not equal in their capacity to activate BA receptors, but the role of individual BAs are not yet known. In the absence of gut bacteria, several BAs are missing from circulation, and therefore their functions will be lost. Antibiotic administration might also have the same effect of reducing the BA variety from the BA pool. Therefore, it is very important to discern the function of individual BAs.

GF mice have increased total BAs in all tissue compartments examined, demonstrating that gut bacteria regulate the BA composition and BA pool size which in turn might affect cholesterol homeostasis in the host. T $\beta$ MCA was identified as an FXR antagonist that is able to increase BAs by decreasing the feedback mechanism (Sayin et al., 2013). However, in our study, although T $\beta$ MCA is increased in the ileal tissue, there is no decrease in the mRNA levels of FXR target genes (Fgf15 and SHP), and no increase in Cyp7a1. The reason for this difference is not known, but I suspect there could be a strain difference, since the previous study by Sayin et al. (Sayin et al., 2013) used Swiss webster mice and we used C57BL/6.

The increase in total BAs in all tissue compartments of GF mice is probably at least in part due to an increase in the reabsorption of BAs by the ileal BA uptake transporter (Asbt). GF mice also have decreased gut motility, and thus BAs remain in the intestine longer favoring more BAs to enter the enterohepatic circulation. This would also explain the increased half-life of CA in GF rats reported by others (Gustafsson et al., 1957). Furthermore, taurine conjugation lowers the pK<sub>a</sub> of BAs and increasing their water solubility. The increased concentrations of MCA and UDCA as well as the

increased total taurine conjugated BAs in GF mice makes the GF BA pool more hydrophilic compared to CV mice. This is probably an adaptive response to the increased total BAs, as hydrophobic BAs are known to cause tissue damage.

UDCA, which was thought to be a secondary BA made by gut bacteria, increased in GF mice tissues. This suggested that UDCA is a primary BA in mice. *In vitro* experiments confirmed that UDCA indeed can be synthesized from CDCA by enzymes in mouse liver microsomes. It is important to know the basic biology of UDCA as it is used as an oral drug to dissolve cholesterol gallstones and treat liver diseases. Increased concentrations of UDCA in bile, prevents cholesterol gallstone formation in obese patients who lose weight rapidly (Broomfield et al., 1988).

Although I did not observe alterations in FXR signaling in GF mice, I did demonstrate an increase in TGR5 signaling. TGR5 activation is known to increase gallbladder size, increase GLP-1 secretion, and increase thermogenesis in brown adipose tissue and muscle. In the present study, GF mice displayed increased gallbladder size, increased GLP-1 in serum, and increased mRNA of the enzyme type 2 iodothyronine deiodinase (D2), which increases energy expenditure as heat in brown adipose tissue. This increase in TGR5 signaling could be a reason for the resistance to diet-induced obesity reported in GF mice (Backhed et al., 2007; Rabot et al., 2010).

These alterations in BA composition, BA concentration, and BA signaling in the absence of gut bacteria raise several questions, such as, what are the differences in the BA profile and signaling in dysbiosis-associated illnesses, such as obesity and inflammatory bowel disorder, and do probiotic bacteria alter BA profiles and signaling.

The altered BA profile in GF mice might point to a BA that is a potent TGR5 agonist and that treating with that TGR5 agonist BA could be used to increase TGR5 signaling and increase serum GLP-1 concentration. This might be a good alternative to GLP-1 analogs (eg. liraglutide) and DPP-4 inhibitors (eg. sitagliptin) to treat type-2 diabetes and metabolic disorders. Along the same line, a recent study demonstrated oral administration of the antioxidant tempol to mice decreases *Lactobacillus* species in their gut leading to accumulation of T $\beta$ MCA, inhibition of FXR signaling and resistance to obesity (Li et al., 2013). Although humans do not make T $\beta$ MCA, studies like these might lead to the establishment of T $\beta$ MCA as a therapeutic drug.

**Specific aim 2** determined the alterations in mRNAs of drug metabolizing enzymes in livers of GF mice by RNA-Seq. A number of hepatic phase-1 and phase-2 genes were altered in the absence of gut bacteria. Table 5.2 provides the list of genes that were altered in GF mice compared to CV mice. Because these genes are altered by the absence of gut bacteria, they can be called “gut bacteria target genes”. This is important for two reasons. First, the use of antibiotics, probiotics, or prebiotics may alter the drug metabolizing capacity of the host and thus alter the action and disposition of orally ingested drugs when taken simultaneously. Second, differences in the composition of gut bacteria can lead to variations in the expression of some drug metabolizing enzymes in the liver and this can lead to inter-individual variations in drug response and drug metabolism.

Human CYP3A4 metabolizes more than 60% of all drugs. The mRNA of *Cyp3a11*, the mouse homolog of CYP3A4, decreased 87% in livers of GF C57BL/6 mice compared to CV mice. This suggests that intestinal bacteria play an important role

in regulating this critical drug metabolizing enzyme. The fact that in the pentobarbital sleeping test, GF mice slept longer than CV mice confirmed that the marked changes in the mRNA levels of Cyp3a11 also has consequences at the enzyme activity level which might not be the case for other enzymes where the changes at the mRNA level were smaller. Follow-up experiments investigating the respective enzyme activities and their protein expression levels are needed to clarify this.

Expression levels of several genes decreased in GF mice and it is likely that these genes are involved in metabolizing gut bacterial metabolites and therefore their expression should be higher in the presence of gut bacteria. An example of this is a study that demonstrated that short chain fatty acids can increase the expression of drug metabolizing enzymes in human primary colon cancer cells (Sauer et al., 2007).

Another possibility is that in the presence of gut bacteria the liver upregulates some enzymes such as Ugts, to conjugate drugs and other xeno- and endobiotics to help in their elimination. Subsequently, the conjugated drugs will become deconjugated by the gut bacterial enzymes, such as  $\beta$ -glucuronidases, and the unconjugated drug will enter the enterohepatic circulation to be conjugated by the liver enzymes again. Therefore, in the absence of gut bacteria, these hepatic enzymes are downregulated.

Several genes showed increased expression in GF mice compared to CV mice. The functions of these enzymes might also be performed by gut bacterial enzymes, and therefore, in the presence of gut bacteria these enzymes would be downregulated while they would be increased in the absence of gut bacteria.

Overall, our findings provide additional insights into factors affecting drug metabolism and ways to alter drug metabolism by modulating the number and composition of intestinal bacteria.

## **6.2 Limitations**

a) The major goal of the study described in Chapter 4 was to compare the BA composition and concentration in various tissues of GF C57BL/6 mice compared to CV mice. Although this goal was achieved, BA pool size cannot be calculated from this study because BA concentration of the intestinal contents was not quantified.

b) The biotransformation studies carried out demonstrate that hepatic microsomes can synthesize UDCA from a primary BA, CDCA. The specific enzymes involved in the reaction have not yet been identified.

c) There is evidence suggesting that TGR5 receptor is constitutively activated in GF mice. However, rederivation of TGR5-null mice as GF mice will demonstrate whether these changes seen in GF mice are only due to TGR5 activation.

d) Although GF mice are a unique and useful animal model in evaluating the function of gut bacteria, being germ-free creates fundamental differences in the physiology of the animal that cannot be overlooked. For example, GF mice have an overactive immune system (Olszak et al., 2012) and this could indirectly influence the expression of genes in liver.

e) Further proof that all the changes seen in GF mice are indeed due to the absence of gut bacteria comes when the changes in GF mice are reversed to some extent upon colonization of the GF mice.

In conclusion, this dissertation work has provided the first step towards a detailed roadmap describing the alterations in BA composition, BA signaling, and expression of hepatic drug metabolizing enzymes in GF C57BL/6 mice compared to CV mice.

**CHAPTER 7**  
**FUTURE STUDIES**

## 7.1 BAs and gut bacteria

This dissertation raises important and exciting possibilities of extensive evaluation of the role of gut flora in altering BA profiles thereby promoting health and alleviating metabolic syndrome related pathologies. A few examples of interesting future studies are given below.

- a) Colonize GF mice with intestinal bacteria from conventional mice for different time periods and monitor the microbial composition in the intestine and BA profile in the various BA enterohepatic compartments over that time period. This will provide information about which bacterial population(s) are important for the reappearance of secondary BAs. The GF mice can also be colonized with human intestinal bacteria to generate a humanized microbiome mouse model (Martin et al., 2008; Turnbaugh et al., 2009; Kashyap et al., 2013).
- b) Several probiotic strains of bacteria on the market are known to possess enzymes to metabolize BAs (Tannock et al., 1989; Begley et al., 2006; Ruiz et al., 2013). It would be interesting to colonize GF and CV C57BL/6 mice and study changes in the BA profile that these probiotic bacteria produce in the presence and absence of other bacteria in the intestine. Most of the probiotic preparations possess bile salt hydrolase, the enzyme required for deconjugating BAs. This would result in a decrease in taurine conjugated BAs and potentially in an increase in ileal FXR signaling.
- c) The changes in the BA profile and BA signaling in GF mice compared to CV mice could also be the result of the absence of secondary BAs. Therefore, an

interesting experiment would be to study the effects of that secondary BA feeding will have on the BA profile and BA signaling in GF mice.

## **7.2 Biosynthesis of UDCA**

The observations in this dissertation and previous studies in our laboratory suggest that UDCA is synthesized from CDCA (Zhang and Klaassen, 2010; Song et al., 2011). CDCA biotransformation reactions with specific enzyme inhibitors and inhibitory antibodies would allow to at least narrow down which enzymes are involved.

## **7.3 T-MCA AND T-UDCA as TGR5 agonists**

The increase in the concentration of taurine-conjugated MCA and UDCA correlate with the marked increase in serum GLP1 in GF C57BL/6 mice compared to CV mice. It has been shown that the secondary BAs, DCA and LCA can activate TGR5, which results in secretion of GLP-1 in STC-1 cells (Katsuma et al., 2005). Similar studies need to be conducted with taurine conjugated MCA and UDCA to determine their potency to activate TGR5 signaling.

## **7.4 Hepatic drug metabolizing enzymes and gut bacteria**

The results of Specific aim 2 provide a list of genes, whose gene expression is altered in the absence of gut bacteria. It has been recognized that antibiotics alter gut bacteria and can result in altering hepatic drug metabolizing capacity, however, the effect of probiotic, prebiotic, and antibiotic use on the expression of hepatic drug metabolizing enzymes still remain virtually unknown. Therefore, it would be interesting to feed GF and CV mice probiotics, prebiotics, and antibiotics, and then quantify the expression of drug metabolizing enzymes in liver. To overcome the differences in drug metabolism between rodents and humans a similar study can be performed in humans,

to investigate how taking over-the-counter probiotics or prescription antibiotics can affect the pharmacokinetics and pharmacodynamics of other orally administered drugs.

Another interesting study will be to quantify gene expression of important drug metabolizing enzymes at different post-natal time points during development in both GF and CV mice. This would illustrate the influence of gut bacteria in the regulation of drug metabolizing enzymes during development. Gut bacteria might normally regulate liver maturation by regulating the immune system; therefore, the ontogeny of important drug metabolizing enzymes must be altered in GF mice compared to CV mice.

The above mentioned experiments are only just a few of the many exciting studies that will expand our understanding of the symbiotic relationship between the host and gut bacteria and how perturbations in gut bacteria can influence health and diseases.

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