Importance of bile acid metabolism in the absorption of nutrients in the western-type diet

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Manuscript 1: Brittnee L. Zwicker was involved in the writing of the manuscript. Luis B. Agellon was involved in the writing, revision and submission of the manuscript.

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Abstract

The gut microbiota is a personalized organ and body weight fluctuations affect the innate ratio of bacterial phyla in the microbiome (*Bacteroidetes* and *Firmicutes*) thereby modifying nutrient extraction capability (143, 146). Weight gain inversely influences the ratio resulting in increased energy harvest efficiency, subsequently increasing weight gain leading to obesity (143). As a leading public health concern in industrialized nations, it is imperative to understand the role of the microbiota in relation to obesity. Furthermore, imbalance or damage to the microbiota by bile acid malabsorption has been implicated in the etiology of gastrointestinal disorders such as irritable bowel syndrome and irritable bowel disease (17). ILBP (encoded by *Fabp6*) is a 15 kDa protein expressed in the ileum and is required for the maintenance of bile acid homeostasis within enterohepatic circulation. ILBP intracellularly transports bile acids ensuring their return into enterohepatic circulation. Mice lacking ILBP exhibit high bile acid excretion indicating that bacteria in the lower intestine are exposed to excess bile acids and their cytotoxic effects. These mice represent a model for bile acid malabsorption such as in humans who have undergone ileostomy (182), or suffer from irritable bowel disease or celiac disease. Successful treatment of these diseases by microbiome transplant therapy (bacteriotherapy) has been documented (183).

Resume

Le microbiote intestinal est un organe personnalisé qui joue un rôle majeur dans le métabolisme des nutriments. Les fluctuations du poids corporel affectent le rapport inné de divisions bactériennes du microbiome (*Bacteroidetes*: *Firmicutes*), modifiant ainsi la capacité d'extraction des éléments nutritifs (143, 146). Le gain de poids influe inversement ce rapport et augmente donc l'efficacité de récolte d'énergie. Ceci mène à un gain de poids et conséquemment à l'obésité (143). En tant que problème de santé publique de premier plan dans les pays industrialisés, il est impératif de comprendre le rôle du microbiote par rapport à l'obésité. En outre, un déséquilibre ou dommage à la flore microbienne a été impliqué dans l'étiologie des troubles gastro-intestinaux tels que le syndrome et la maladie du côlon irritable (17). Ilbp (codée par Fabp6) est une protéine de 15k Da exprimée dans l'iléon. Celle-ci est nécessaire pour le maintien de l'homéostasie des acides biliaires dans la circulation entéro-hépatique. Les souris dépourvues de ilbp démontrent une excrétion élevée d'acides biliaires ce qui indique que les bactéries dans l'intestin grêle sont exposés aux effets cytotoxiques des acides biliaires. Ces souris représentent un modèle pour la malabsorption des acides biliaires, telle que témoignée chez les humains ayant subi une iléostomie (182), ou qui souffrent de la maladie du côlon irritable et de la maladie coeliaque. Le succès du traitement de ces maladies par thérapie de transplantation de microbiote (également connu sous le nom bactériothérapie) a été documenté (183).

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List of Abbreviations

ABCB11: Bile salt export pump

ASBT: Apical sodium-dependent bile acid transporter

BA: Bile acids

CA: Cholic acid

DCA: Deoxycholic acid

EE: Energy expenditure

EHC: Enterohepatic circulation

F: Females

FABP: Fatty acid binding proteins

Fabp1: Liver-type fatty acid binding protein

FABP6: Fatty acid binding protein 6

FXRα: Farnesoid-x-receptor α

GDCA: Glycodeoxycholic acid

HDL: High density lipoprotein

HPLC: High performance liquid chromatography

IBS: Irritable bowel syndrome

IBD: Irritable bowel disease

ILBP: Ileal lipid binding protein

LCA: Lithocholic acid

LDL: Low-density lipoprotein

LFD: Standard maintenance low-fat diet

M: Males

NTCP: Sodium/taurocholate co-transporting polypeptide

OSTα: Organic solute transporter α

OSTβ: Organic solute transporter β

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

RQ: Respiratory quotient

SCFA: Short-chain fatty acid

TBA: Total bile acids

TCA: Taurocholic acid

TDCA: Taurodeoxycholic acid

TG: Triglyceride

TMCA: Tauromuricholic acid

VLDL: Very low-density lipoprotein

WD: High-fat western-type diet

UDCA: Ursodeoxycholic acid

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Chapter 1: Introduction

Our current global state of public health is in a dire predicament as the burden of obesity is now a greater cause of chronic disease and death than starvation (1). From a national perspective, the total economic cost of obesity in Canada reached 202 billion dollars at the turn of the millennium with a 25% obesity rate (2, 3). Increased chronic disease and severe economic burden has pushed metabolism to the forefront of science research.

Epidemiologists, geneticists, psychologists, biochemists and molecular biologists among other professionals examine nutrition through different lenses aiming to map and identify the comprehensive role of metabolism. Bile acids are functional molecules that emulsify lipids in preparation for absorption. Absence or mutation of bile acid transporters leads to bile acid malabsorption subsequently affecting nutritional status as this leads to lipid malabsorption (4, 5). Studying the transport of bile acids within the enterohepatic circulation will provide insight to lipid metabolism.

Bile acids are amphipathic biological molecules that facilitate lipid and lipid-soluble molecule digestion by emulsification (6). They are produced in the liver from cholesterol and are one of the major solutes in bile, in addition to unesterified cholesterol, phospholipids, bilirubin, and electrolytes (7, 8). Bile acids are complex molecules, acting as signaling molecules and as nuclear receptor ligands controlling gene transcription of major enzymes involved in their own synthesis and transport (9, 10). These biological molecules are excellent candidates for research because their metabolism is essential for lipid homeostasis (11).

As a major component of lipid metabolism, bile acid transport has been under examination for over 50 years (12). Bile acids are synthesized in the liver then

released after stimulation from lipid containing nutrients, followed by intestinal reabsorption. General bile acid transport within the enterohepatic circulation is established but lack of understanding exists at cellular level transport (7, 8). Fatty acid binding proteins (FABPs) are a family of carrier proteins that orchestrate the transport of their ligands intracellularly and extracellularly (13, 14). *Fabp6* encodes the small cytoplasmic protein ILBP that is expressed in ileocytes (15). ILBP plays a large role in lipid metabolism because after binding to BA, it is responsible for their transport through the enterocyte (16). ILBP null mice exhibit bile acid malabsorption extending the scope of this research to human conditions with bile acid malabsorption such as an ileostomy or gastric bypass surgery (17). The dysregulation of BA metabolism in ILBP-deficient mice is expected to translate into fat malabsorption as well, therefore investigation into the effect of a high fat diet will be presented. In *Fabp6-/-* mice the increased bile acid excretion exposes the lower intestine to increased concentrations of bile acids, which likely have a cytotoxic effect on some species of gut bacteria. Certain species of bacteria metabolize the bile acids and others will succumb to the toxic bile acid exposure changing the profile of bacteria (18, 19). Thus, altering the gut bacteria profile in *Fabp6-/-* mice would alter their energy extraction capability.

Chapter 2: Literature Review (Manuscript 1)

Transport and biological activities of bile acids**

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Abbreviations: ABCB11, bile salt export pump; ASBT, apical sodium-dependent bile acid transporter; EHC, enterohepatic circulation; FABP1, liver-type fatty acid binding protein; FXRα, farnesoid-x-receptor α; ILBP, ileal lipid binding protein; NTCP, sodium/taurocholate co-transporting polypeptide; OSTα, organic solute transporter α; OSTβ, organic solute transporter β

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ABSTRACT

Bile acids have emerged as important biological molecules that support the solubilization of various lipids and lipid-soluble compounds in the gut and the regulation of gene expression and cellular function. Bile acids are synthesized from cholesterol in the liver and eventually released into the small intestine. The majority of bile acids are recovered in the distal end of the small intestine and then returned to the liver for reuse. The components of the mechanism responsible for the recycling of bile acids within the enterohepatic circulation have been identified whereas the mechanism for intracellular transport is less understood. Recently, the ileal lipid binding protein (ILBP; human gene symbol *FABP6*) was shown to be needed for the efficient transport of bile acids from the apical side to the basolateral side of enterocytes in the distal intestine. This review presents an overview of the transport of bile acids between the liver and the gut as well as within hepatocytes and enterocytes. A variety of pathologies is associated with the malfunction of the bile acid transport system.

Keywords: bile acids; enterohepatic circulation; fabp6; gut microbiota; intestine; liver; metabolic syndromes; transporters

1. Introduction

Our view of the biological importance of bile acids has greatly expanded since they were initially purified from bile over 150 years ago. Bile acids are made in the liver from cholesterol, travel to the small intestine and return to the liver for several rounds of reuse. During their journey between the two organs, a small amount of the bile acid pool is continually lost from the body via fecal excretion. As well, a small fraction of bile acids recovered by the intestine evade hepatic extraction and spill over into the general circulation. As a consequence

of their physicochemical and biochemical properties, it is now evident that bile acids have the capacity to affect metabolic function in essentially all the compartments that comprise their itinerary. Factors that modify their normal course and transit within the organism result in changes in physiologic status, some of which promote health and others that lead to pathogenesis of disease.

2. General properties of bile acids

A structural feature common to all bile acids is the steroid nucleus (20). The bile of different organisms contains different species of bile acids that make up a characteristic bile acid profile (21, 22). Cholic acid is a tri-hydroxy bile acid that is found in the bile of many mammalian species (Fig. 1A). Furthermore, bile acids are typically conjugated to amino acids (i.e., *N*-acylamidation) glycine (in mammals and some species of aves) (22) or taurine before being secreted into bile . It is important to note that this modification increases the polarity and therefore the solubility of these molecules. The bile acids produced by the liver through de novo synthesis are referred to as primary bile acids (20-22). In humans, the abundant primary bile acids are glycine conjugates of chenodeoxycholic and cholic acids (23, 24). In rodents, chenodeoxycholic acid is converted to muricholic acid (25, 26). Tauromuricholic and taurocholic acids are the two most abundant bile acid species in murine bile.

3. Known biological activities of bile acids

The classically known function of bile acids is that of a detergent to aid in the absorption of dietary lipids and lipid-soluble nutrients. The ability of bile acids to emulsify lipids and lipid-soluble compounds is attributable to their amphipathic structure, which is a consequence of the hydroxyl groups being oriented towards one side of the steroid nucleus (Fig. 1B). The activity of carboxyl-ester lipase (human gene symbol *CEL*) in the gut lumen is dependent on bile acids and is

important for dietary lipid absorption by neonates (27). Bile acids stimulate bile flow. Bile acids together with biliary phosphatidylcholine maintain the solubility of cholesterol in bile. Since bile acids are synthesized from cholesterol in the liver (6), the formation of bile acids has also been thought of as a pathway for the elimination of cholesterol from the body.

More recently it became apparent that bile acids, as a group of molecules, possess a much wider range of biological activities than initially recognized. Not all bile acids share the same set of biological activities, as the variety of metabolic modifications of their structures augment or diminish their biological properties. It was discovered that bile acids have the ability to regulate gene expression by serving as ligands for several nuclear receptors, namely FXRα (NR1H4) (28, 29), VDR (NR1I1) (30), and PXR (NR1I2) (31). Initially, bile acids were identified as the natural ligands of FXRα (28, 29). The cDNA for this nuclear receptor was originally isolated from a hepatoma cDNA library, and the receptor was thought to be activated by farnesol and its metabolites (32). A structure of FXR in a complex with a bile acid has been determined (33). The role of FXR in regulating the expression of many genes involved in sterol, triacylglycerol and carbohydrate metabolism is now well established (34-36).

It is now well known that bile acids activate various cell signaling pathways, including those involved in cell death and survival (35, 37). Later it was found that bile acids interact with a cell surface receptor known as TGR5 (human gene symbol *GPBAR1*) (38). This receptor exhibits a wider tissue distribution than FXRα, extending to cells found in brown adipose tissue, immune system, skeletal muscle, nervous tissue, and the large intestine, and is linked to signaling pathways involved in energy metabolism and inflammatory response (38-42). Studies using mice suggest that activation of TGR5 by bile acids offer a means to manipulate whole body energy expenditure (39). However, additional studies are needed to resolve conflicting results regarding the importance of plasma bile acids in human energy metabolism (43, 44). In the colon, TGR5 activation by bile acids has been shown to improve glucose metabolism via

induction of glucagon-like peptide-1 and it has been suggested that this mechanism might be useful for controlling or treating diabetes (41, 42, 45).

Some bile acids have the ability to mitigate endoplasmic reticulum stress (46-48). This facet of bile acid action is of significance since the development of many metabolic syndromes may be rooted in the disruption of endoplasmic reticulum function (49).

Finally, bile acids have intrinsic bactericidal properties (50). The antimicrobial activity of bile acids have received renewed attention in recent years upon the discovery that bile acids are capable of inducing the expression of genes involved in the defense of intestinal mucosa from bacterial invasion (51).

4. Bile acid biosynthesis

In mammals, the synthesis of bile acids is accomplished via two distinct pathways. The classical bile acid biosynthetic pathway (also referred to as the neutral pathway) is a well-studied pathway that takes place solely within the liver (20). The main steps of this pathway include hydroxylation of the steroid nucleus as well as its complete desaturation, shortening of the side chain and finally conjugation of the bile acid prior to its transportation out of the hepatocyte into bile. This pathway is controlled by a microsomal enzyme known as cholesterol 7α-hydroxylase (cyp7a1; human gene symbol *CYP7A1*) which displays a high degree of selectivity towards cholesterol as a substrate. Targeted inactivation of the *Cyp7a1* gene in mice (52) clearly demonstrated the importance of this enzyme in the classical pathway and also revealed the existence of an alternative bile acid biosynthetic pathway (also referred to as the acidic pathway). The loss of cyp7a1 in mice caused increased incidence of neonatal lethality due mainly to inability to absorb dietary lipids. In humans, cyp7a1 deficiency has been observed but this mutation is rare (53). Affected individuals exhibit increased low density lipoprotein cholesterol concentration. A polymorphism in the human *CYP7A1* gene promoter has also been shown to be associated with increased

low density lipoprotein cholesterol concentrations (54). The classical pathway is subject to feedback inhibition involving SHP-mediated repression of the Cyp7a1 gene following the activation of hepatic FXRα function by bile acids. There is evidence indicating that the human and murine Cyp7a1 gene promoters are subject to distinct regulatory mechanisms (55-59).

Oxysterols that are enzymatically formed in extrahepatic tissues can be converted into bile acids in the liver via the alternate pathway (20) and oxysterol 7α-hydroxylases (cyp7b1, human gene symbol *CYP7B1;* cyp39a1, human gene symbol *CYP39A1*) are the key enzymes allowing entry of these compounds into this pathway (52, 60). Oxysterol 7α-hydroxylases, unlike cyp7a1, appear to be constitutively expressed and not sensitive to bile acid or cholesterol status (60, 61). Compared to mice, humans may have greater reliance on the alternative bile acid biosynthetic pathway for disposal of oxysterols from the liver (62). The amount of bile acids synthesized by the human liver is generally equal to the amount that is lost in stool; however some species may use bile acids as a means to rid the body of excess cholesterol by increasing the rate of bile acid synthesis via the classical pathway and reducing the recovery of intestinal bile acids. Sex dimorphism in the expression and regulation of some genes involved in bile acid synthesis is evident (4, 60, 63-65).

5 Transport of bile acids

Under normal physiological conditions, bile acids follow a defined route of transport within the body. The bile acids are normally stored in the gallbladder and cycle between the intestines and the liver via the enterohepatic circulation (EHC) (Fig. 2). Conjugation of bile acids to glycine or taurine renders them less likely to undergo passive transport, thus requiring membrane transporters for cellular uptake and secretion, which occur at opposite poles of the cells. The EHC represents an important conduit between liver and intestine, and is needed for normal physiologic function.

5.1. Inter-organ transport

The transporters responsible for the major trafficking of bile acids between organs have been mostly elucidated in mice through the use of targeted gene inactivation (66-69) and this process is now well described (8, 70). The bulk of the bile acids in the body are normally stored in the gallbladder, and are released into the small intestine during a meal where they form mixed micelles with diet-derived lipids as well other biliary lipids. A small proportion of the bile acids are deconjugated as they travel down towards the ileum. Unconjugated bile acids are thought to be reabsorbed passively throughout the small and large intestines whereas the bulk of conjugated bile acids are recovered mainly by ileal enterocytes by active transport involving the apical sodium-dependent bile acid transporter (ASBT; human gene symbol *SLC10A2*) and then secreted into portal blood via a heterodimeric transporter composed of OSTα (human gene symbol *SLC51A*) and OSTβ (human gene symbol *SLC51B*) (OSTα/OSTβ). In mice, inactivation of the gene for ASBT eliminates intestinal absorption of taurocholic acid (67) and deletion of the gene for OSTα drastically decreases (>80 %) the basolateral secretion of taurocholic acid by ileal enterocytes (69).

The major carrier of bile acids in the blood is serum albumin (71, 72). In humans, the physiological bile acid concentration ranges from <5 μM (fasting) and 3-7 μM (postprandial) in systemic blood and 4-27 μM (fasting) and 22-55 μM (postprandial) in portal blood (73-75).

In the liver, the bile acids are absorbed by the sodium/taurocholate co-transporting polypeptide (NTCP; human gene symbol *SLC10A1*) and resecreted to bile along with newly synthesized bile acids via ABCB11 (also referred to as bile salt export pump; human gene symbol *ABCB11*). NTCP and ABCB11 are expressed mainly by hepatocytes, and show preference for conjugated bile acids. In mice lacking ABCB11, secretion of taurocholic acid into bile by hepatocytes is reduced by >93 % (66). The unconjugated bile acids are taken up by

hepatocytes via organic anion transporting polypeptides (encoded by the *SLCO* genes in humans). Entry of unconjugated bile acids into cells by simple diffusion is not efficient but may occur if there is a steep concentration gradient (76).

The transporters discussed above are the key mediators of cellular import and export of bile acids within the EHC, although other membrane ion transporters with relaxed specificities have also been found to account for a minor proportion of bile acid transport (8, 36, 77). A small amount of bile acids spills into the general circulation and is the source of bile acids that interact with TGR5 in peripheral tissues (see Section 3). Small amounts of bile acids have been detected in the ovaries, heart and brain (78-80) although it is not yet clear what purpose they serve in these organs. Renal cells are capable of taking up bile acids in general circulation via ASBT, which are then excreted into urine via OSTα/OSTβ.

Bile acids themselves play a prominent role in regulating the expression of the genes involved in their uptake and secretion within the enterohepatic circulation. Not surprisingly, these genes are responsive to the same transcription factors that regulate bile acid synthesis (36, 77, 81). Sexual dimorphism in the expression of genes encoding bile acid transporters in mice has been noted (77).

5.2. Intracellular transport

Bile acids enter and exit bile acid-transporting cells through opposite poles of the cell. It has been proposed that intracellular transport of bile acids can occur via vesicular and protein-mediated transport. In this regard, fluorescent bile acid analogs have been useful tools for visualizing the movement of bile acids within the cell. Data obtained using these tracers requires careful interpretation as some of these compounds apparently do not follow the same route of transit within the cell as do natural bile acids (82, 83). A potential improvement in the imaging of intracellular bile acid distribution is the newly

developed synthetic biological bile acid sensor (84); however this requires that bile acids not be sequestered in order to allow physical interaction with the bile acid sensor. In general, compelling evidence supporting vesicular transport of bile acids has not yet been obtained (85) suggesting that the intracellular movement of bile acids is mediated by proteins. The enzymes involved in the synthesis and metabolism of bile acids, such as enzymes involved in further enzymatic modification of bile acid structure or in the reconjugation of unconjugated bile acids recovered from blood (see Section 6.1), or members of the fatty acid binding protein family (86, 87), namely FABP1 (also called L-FABP; human gene symbol *FABP1*) and ILBP (also called IBABP), are likely involved in this process.

Fatty acid binding proteins are soluble and small molecular weight proteins that are known to bind a variety of hydrophobic molecules (86, 87). These structurally related proteins are encoded by separate genes and different fatty acid binding protein family members are distributed in various tissues and cell types within the body (86, 87). Although both ILBP and FABP1 have been shown to bind bile acids and fatty acids, each of these proteins displays a distinct ligand preference: ILBP exhibits greater affinity for bile acids whereas FABP1 prefers fatty acids (15, 88, 89).

5.3 Liver-type fatty acid binding protein

FABP1 is an abundant protein found in the liver and in the small intestine. It is also detectable in the human colon (90, 91). In the small intestine, FABP1 is asymmetrically distributed along the proximal-to-distal axis with the maximum abundance occurring slightly distal to the midpoint of the organ. Although FABP1 has been seen to bind bile acids, it shows a clear preference for fatty acids. Moreover, the pattern of FABP1 distribution in the small intestine does not coincide with the distribution of both ASBT and OSTα/OSTβ and hence it has not generally been regarded as a major intracellular transporter of bile acids.

In vivo studies using mice have implicated the involvement of FABP1 in bile acid metabolism. Mice lacking FABP1 display disruption in bile acid homeostasis and increased susceptibility to gallstone formation on a lithogenic diet (92, 93). The phenotype of genetic FABP1 deficiency may be sex-dependent as male mice exhibit larger disturbances in bile acid metabolism (92). Direct involvement of FABP1 in bile acid transport has not yet been demonstrated; however, it is of interest to note that the presence of FABP1 attenuates the availability of bile acids to an engineered biological bile acid sensor in cells (84), supporting the idea that FABP1 is capable of binding bile acids in vivo.

5.4 Ileal lipid binding protein

ILBP is another member of the fatty acid binding protein family and is most related to FABP1 (13, 87). The tertiary structure of ILBP from different species has been solved (Fig. 3) (94-97). Primary structure alignment of ILBPs from various species reveals an exceptionally high degree (>90 %) of amino acid sequence conservation through evolution. Despite the high degree of relatedness, it is still possible to segregate ILBP protein sequences according to taxonomic class (Fig. 4). Interestingly, the phylogeny of ILBP proteins appears to coincide with the evolution of bile acid chemical structure and bile composition in vertebrates (21, 22), raising the possibility that ILBP protein structure evolved tailored to the characteristic or abundant bile acid species found in the bile of different animals.

ILBP has been shown to bind both bile acids and fatty acids, although it shows a clear preference for conjugated bile acids (89, 98). ILBP is co-expressed with ASBT and OSTα/OSTβ along the longitudinal axis of the small intestine (76, 99). Although ILBP is predominantly expressed in ileal enterocytes, it has been detected in other cell types notably cholangiocytes and ovarian cells (100-103).

A role for ILBP in intracellular trafficking of bile acids has long been postulated based on its affinity for bile acids, its tissue distribution and colocalization with known membrane transporters of bile acids in the small intestine. This is further supported by the finding that the activation of FXR function can be enhanced by the presence of ILBP (104) in a manner similar to the activation of nucleus-localized peroxisome proliferator-activated receptors by FABP1 (105, 106). The role for ILBP in bile acid transport was initially examined in vivo using mice with global FXR deficiency which are also deficient in ILBP (107). The findings of this study suggested that ILBP is not required for the enterohepatic circulation of bile acids; however, the exact role of ILBP in bile acid transport and metabolism could not be properly assessed because FXR is needed for appropriate expression of many genes involved in bile acid transport and metabolism (20, 36, 81).

Recently, our laboratory described the creation of a mouse line that specifically lacks ILBP (16). A prominent feature of genetic ILBP deficiency is the disruption of bile acid homeostasis characterized by enhanced fecal excretion of bile acids particularly by female mice. The analysis of bile acid transport in the EHC of ILBP-deficient mice using radiolabeled taurocholic acid tracer introduced by oral gavage uncovered a defect in the transport of the tracer from intestinal lumen to portal blood. Experiments using everted gut sacs revealed impaired luminal to serosal transport of radiolabeled taurocholic acid in the absence of ILBP.

Although these studies provided the strongest evidence yet for ILBP serving as the intracellular transporter of bile acids in ileal enterocytes, the findings also raised several intriguing features of bile acid transport in the intestine. Accumulation of bile acids was evident in the intestine of male but not female ILBP-deficient mice. This sex dimorphic phenotype, along with sex dimorphic expression of genes encoding bile acid transporters (77) suggests the possibility that there may be differences in bile acid transport mechanisms, at least in mice, between sexes. Additionally, intestinal bile acid transport was not

abolished by the loss of ILBP, indicating that additional mechanisms exist to support the movement of bile acids within enterocytes. FABP1 may provide a compensatory role in this regard. Future studies should help clarify these issues.

6. Metabolism and biotransformation of bile acids

Bile acids cycle through the EHC several times and accumulate structural modifications during their journey until they are finally excreted in stool (Fig. 2). These modified bile acids are referred to as secondary and tertiary bile acids (21, 22, 108). Some of the modifications are introduced by hepatic enzymes, which generally produce more hydrophilic derivatives, whereas others are made by gut bacteria which generally convert the bile acids to more hydrophobic derivatives. It is known that gut microflora contributes significantly to the metabolism of bile acids (80, 109, 110). In general, taurine conjugates of cholic and muricholic acids feature prominently in the bile acid pool mice and rats, and taurocholic and tauromuricholic acids are present in ≥2:1 ratio in non-germfree animals as compared to ~1:1 ratio in germfree animals (80, 110-112). In addition, the bile acid pool of conventional animals shows far greater diversity of bile acid species compared to germfree animals (80, 110, 112)

6.1. Modification of bile acids by hepatic enzymes

Bacterial metabolism of bile acids in the gut produces unconjugated bile acids (see Section 6.2), some of which are toxic to host cells. In the liver, reclaimed unconjugated bile acids are reconjugated to glycine or taurine along with newly synthesized primary bile acids, a process started by bile acid:coenzyme A ligase (human gene symbol *SLC27A5*) and completed by bile acid-CoA:amino acid N-acyltransferase (human gene symbol *BAAT*) (20). In addition, detoxification systems act on the recovered secondary bile acids to reduce their cytotoxicity. Conjugation to glucuronic acid is catalyzed by UDP-

glucuronosyltransferases (human gene symbols *UGTs*) (113). Sulfonation of bile acids is carried out by steroid/bile acid-sulfotransferase (also called dehydroepiandrosterone sulfotransferase; human gene symbol *SULT2A1*) (114). Glucuronidated and sulfated bile acids are poorly transported by the major bile acid transporters and therefore are excreted out of the body.

A recent study reported that small amounts of glutathione conjugates of bile acids are detectable in the biliary bile of human infants (115). However, glutathione-conjugated bile acids are not detectable in the bile of human adults indicating that this type of modification is of minor importance in the metabolism of bile acids in human adults.

6.2. Modification of bile acids by gut bacteria

Given the importance of gut bacteria in the metabolism of bile acids, the ability of bile acids to modulate bacterial growth and shape the intestinal microbiome has important ramifications in determining the health status of the host organism (116). Bactericidal activity of bile acids is attributable in part to their detergent properties (50). Recently, it was found that conjugated bile acids also participate in assisting the host in controlling overgrowth within the gut microflora through FXR by inducing the expression of genes (encoding angiogenin, inducible nitric oxide synthase, interleukin 18) in the ileum that are known to have antimicrobial effects (51).

Conversely, gut bacteria metabolize conjugated bile acids to decrease their bactericidal activity. A small fraction of the conjugated bile acids released into the small intestine is deconjugated by gut bacteria in this part of the digestive tract, and the conjugated bile acids that escape ileal recovery undergo deconjugation in the large intestine. This reaction is catalyzed by bile acid hydrolases made by gut bacteria, and enable bacteria to tolerate the presence of bile acids in the gut lumen. The ability to deconjugate bile acids appears to be present among the major bacterial divisions of the gut microbiota (117).

Deconjugated bile acids undergo further modification, namely 7α-dehydroxylation, which further decreases the toxicity of bile acids to gut bacteria. Dehydroxylation can only occur after deconjugation due to inaccessibility of the carbon-7 hydroxyl group (109, 118). The dehydroxylation of chenodeoxycholic acid generates lithocholic acid (a secondary bile acid containing only one hydroxyl group) which is highly toxic to liver cells and has been implicated in the development of colorectal cancer (108). Desulfation (a form of chemical deconjugation) of bile acids by the gut microflora, in particular the Clostridium strains, has been seen to accelerate the clearance of sulfated bile acids in gnotobiotic rats as compared to germ-free and conventional rats (119). Clostridium strains, as well as other bacteria belonging to *Firmicutes*, also have the capacity to epimerize the 7α-hydroxyl group of chenodeoxycholic acid to produce ursodeoxycholic acid (120-122), a non-toxic form of bile acids that appears to be desirable to both the host and gut bacteria (123, 124). The beneficial activities of ursodeoxycholic acid (and its taurine conjugated form) have been known for some time (125); but the molecular mechanisms of their mode of action on cellular function are becoming apparent only recently. Tauroursodeoxycholic and ursodeoxycholic acids have been shown to prevent calcium-mediated apoptosis and to inhibit the activation of the unfolded protein response (35, 46-48).

7. Disorders caused by impaired activities of the major bile acid transporters

The proper movement of bile acids in the EHC is essential for normal physiologic function. The interruption of bile acid movement within the EHC leads to a variety of clinical conditions. The disorder manifested and extent of severity may differ depending on whether bile acid transport is interrupted in the liver or in the intestines. Also, gut bacteria have differential sensitivity and ability to metabolize bile acids (109) , and thus exposure of gut bacteria to high levels of

bile acids can alter the density and diversity of the gut microflora.

In humans, non-synonymous substitutions in ASBT amino acid sequence that cause loss of function result in primary bile acid malabsorption which is associated with diarrhea and steatorrhea (8, 126). However, these conditions are not seen in mice lacking ASBT thus pointing to species differences,such as differences in bile acid composition or regulation of bile acid metabolism, as possible modifiers of disease susceptibility. In this regard, the use of ASBT inhibitors to stimulate colonic TGR5 activity as a possible treatment for diabetes, as suggested by studies done in rodent species, may not be well-suited for clinical application in humans.

The *SLC10A2* gene (encodes ASBT) was also identified as a possible gallstone susceptibility gene (127, 128). Other variants of ASBT and reduced expression of the *SLC10A2* gene have been observed among gallstone carriers (129, 130) However gallstone disease is a multi-factorial disorder and it is likely that defects in other bile acid carriers and transporters contribute to susceptibility. Even though *Fapb1* has been identified as a possible gallstone susceptibility gene in mice (127, 128) and loss of FABP1 was found to increase susceptibility to diet-induced gallstones (93), no association between FABP1 variants and gallstone disease has been described in humans. Similarly, *Fabp6* is also a suspected gallstone susceptibility gene (127, 128). Decreased expression of *FABP6* was found in some gallstone carriers (129). The consequence of ILBP deficiency on gallstone formation in the mouse has not yet been studied.

Unlike ASBT, no inborn defects in bile acid transport based on NTCP have been described. Genetic polymorphisms in NTCP have been identified among Asians but the clinical significance of these variations is not yet apparent (131).

Cessation or impairment of bile flow in the liver is a serious condition that leads to hypercholemia, pruritus, hyperlipidemia, pancreatitis, jaundice, and liver failure. In humans, the disorder called progressive familial intrahepatic cholestasis type 2 (PFIC2) is caused by a defective canalicular export of bile acids due to mutations in the *ABCB11* gene (132). Other functional variants of

ABCB11 are associated with a less severe form of the disorder referred to as benign recurrent intrahepatic cholestasis type 2. The clinical features of human PFIC2 disorder are not reproduced in ABCB11-deficient mice and this is due to differences in the composition of the bile acid pool between the two species, as murine bile contains bile acids that are more hydrophilic than those found in human bile (66), and compensatory action by transporters encoded by *Abcb1a* and *Abcb1b* in the murine liver (133). Variants of ABCB11 with reduced function are associated with intrahepatic cholestasis of pregnancy (134, 135).

The expanding knowledge of mechanisms of transport and biological activities of bile acids has offered insights into other possible metabolic disorders caused by impaired bile acid metabolism. A case of familial hypertriglyceridemia was found to be associated with a loss-of-function *SLC10A2* gene variant characterized by a 646insG frame-shift mutation (136). Earlier it was found that chenodeoxycholic acid therapy decreased plasma triglyceride concentrations in patients with hypertriglyceridemia (137). These observations are in keeping with the later discovery that bile acids inhibit triglyceride synthesis as well as regulate glucose homeostasis through FXR (5, 138, 139). A variant of ILBP, termed Fabp6-T79M (see Fig. 3), has been found to be associated with resistance to development of diabetes in obese individuals (140, 141). The amino acid change occurs near the portal region of ILBP and is predicted to affect protein stability and bile acid binding. The influence of this variant on the function of ILBP, or on ileal bile acid recovery, has not yet been tested experimentally. However, based on the phenotype of ILBP-deficient mice (16), defective bile acid binding activity of Fabp6-T79M would cause malabsorption of bile acids in the small intestine. Thus one explanation that could account for the resistance of obese individuals with the Fabp6-T79M allele to diabetes might be the stimulation of colonic TGR5 due to increased bile acid concentration in the colon. It is also likely that the character of the gut microbiota is influenced by the composition of bile acids in the EHC. Alteration of the gut microbiome can have a significant effect on the ability of the host to harvest nutrients and therefore influence the development of

acquired metabolic syndromes such as obesity and diabetes.

8. Summary

There is now ample evidence demonstrating the diverse and versatile biological activities of bile acids in normal physiology. Bile acids originate from the liver and find their way throughout the body. Through their journey, bile acids have the capacity of modulating gene expression and cellular function in various cells and tissues. An intriguing feature of bile acid transport is the departure of bile acids from the body through excretion into the lumen of the intestine via bile, and their return to the body through reabsorption in the ileum by ileocytes, a directional process that is facilitated by distinct apical and basolateral membrane transporters and a soluble intracellular bile acid binding protein. The concept that is emerging from many studies is the prominent role that bile acids play in energy homeostasis spanning the acquisition, assimilation and utilization of dietary macronutrients, namely fats and carbohydrates.

Conflict of interest

None declared.

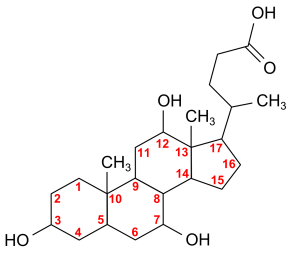
Acknowledgements

Our research is funded by grants from the Natural Sciences and Engineering Research Council, Canadian Institutes of Health Research and Canada Foundation for Innovation. LBA holds the Canada Research Chair in Biochemistry and Molecular Biology of Nutrition.

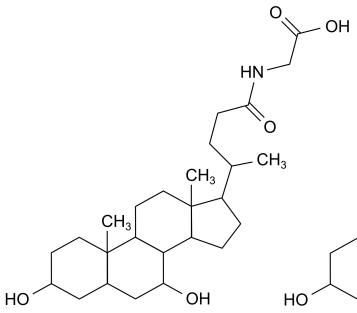
A

Unconjugated bile acid

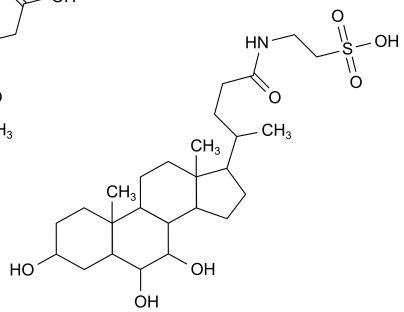
Conjugated bile acids



Cholic acid

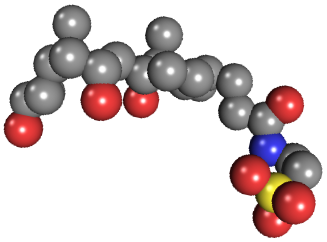


Glycochenodeoxycholic acid



Tauromuricholic acid

B



Taurocholic acid

Figure 1. Structures of bile acids. A. Cholic acid is an unconjugated bile acid and carries three hydroxyl groups at carbon-3, carbon-7 and carbon-12 of its steroid moiety. The carbon atoms that make up the 4 rings of the steroid moiety are numbered. Glycochenodeoxycholic and tauromuricholic acids are conjugated bile acids. Glycochenodeoxycholic acid is typically found in human bile. The steroid moiety of this bile acid contains two hydroxyl groups at carbon-3 and carbon-7, and is conjugated to glycine. Tauromuricholic acid represents about half of the bile acids in murine bile. The steroid moiety of this bile acid contains three hydroxyl groups at carbon-3, carbon-6 and carbon-7, and is conjugated to taurine. B. A space filling model of taurocholic acid generated by the Pc3D viewer (available at http://pubchem.ncbi.nlm.nih.gov) showing the orientation of polar and ionizable functional groups relative to the steroid nucleus which is oriented parallel to the horizontal plane. The carbon (grey), oxygen (red), nitrogen (blue) and sulphur (yellow) atoms are shown.

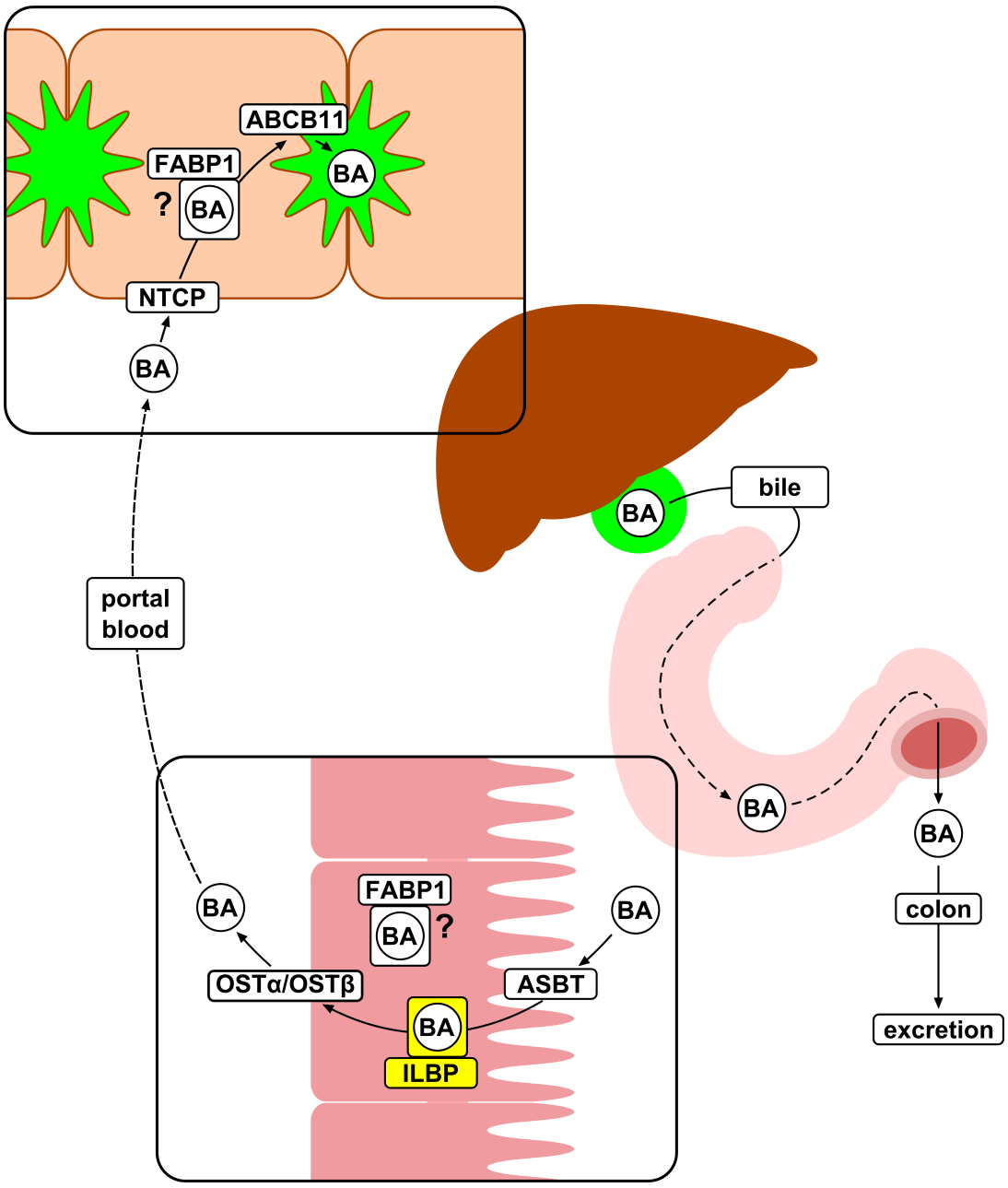
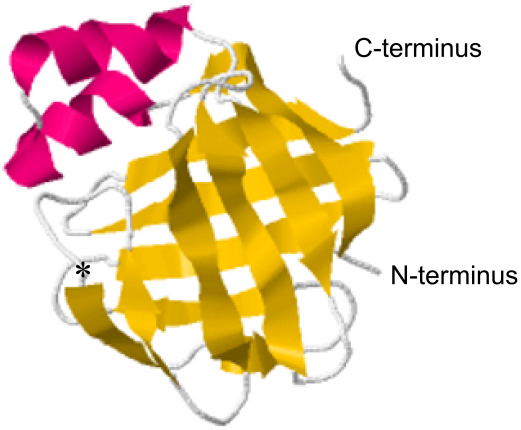

ABCB11
FABP1
?
BA
BA
NTCP
BA
BA
bile
portal
blood
BA
BA
colon
excretion
FABP1
BA
?
BA
BA
OSTα/OSTβ
ASBT
BA
ILBP

Figure 2. Transport of bile acids within the enterohepatic circulation and excretion out of the body. Bile acids are synthesized by the liver and released into the lumen of the small intestine via bile. The majority of the conjugated bile acids are recovered in the distal portion of the small intestine via an active process involving ASBT. Unabsorbed bile acids travel to the colon where they are further modified by gut bacteria. Bile acids that are not reabsorbed by the colon are excreted out of the body. ILBP is the principal intracellular transporter of reabsorbed conjugated bile acids in ileal enterocytes. FABP1 may also participate in the transport of bile acids. Bile acids are excreted out of ileal enterocytes via the heterodimeric transporter OSTα/OSTβ, and carried back to the liver via portal blood. The NTCP located on the basolateral (sinusoidal) membrane of liver hepatocytes reabsorbs the bile acids from portal blood. FABP1 may be involved in the transport of bile acids to the apical (canalicular) membrane where ABCB11 facilitates the secretion of newly synthesized and recovered bile acids to bile.


C-terminus
N-terminus
*

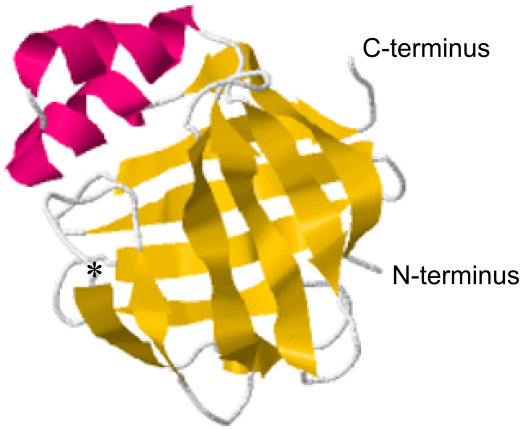

C-terminus
N-terminus
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Figure 3. Stereo drawing of the human ILBP 3D structure without its ligand. The structure shown was generated using data (PDB ID: 1O1U) (95) and software available at the RCSB Protein Data Bank website (http://www.rcsb.org/pdb/home/home.do). The tertiary structure of the protein features a β-clam motif that makes up the ligand binding cavity and two α-helices that form a lid over the portal region of the protein. The asterisk indicates the location of the T79M variation on the protein structure.

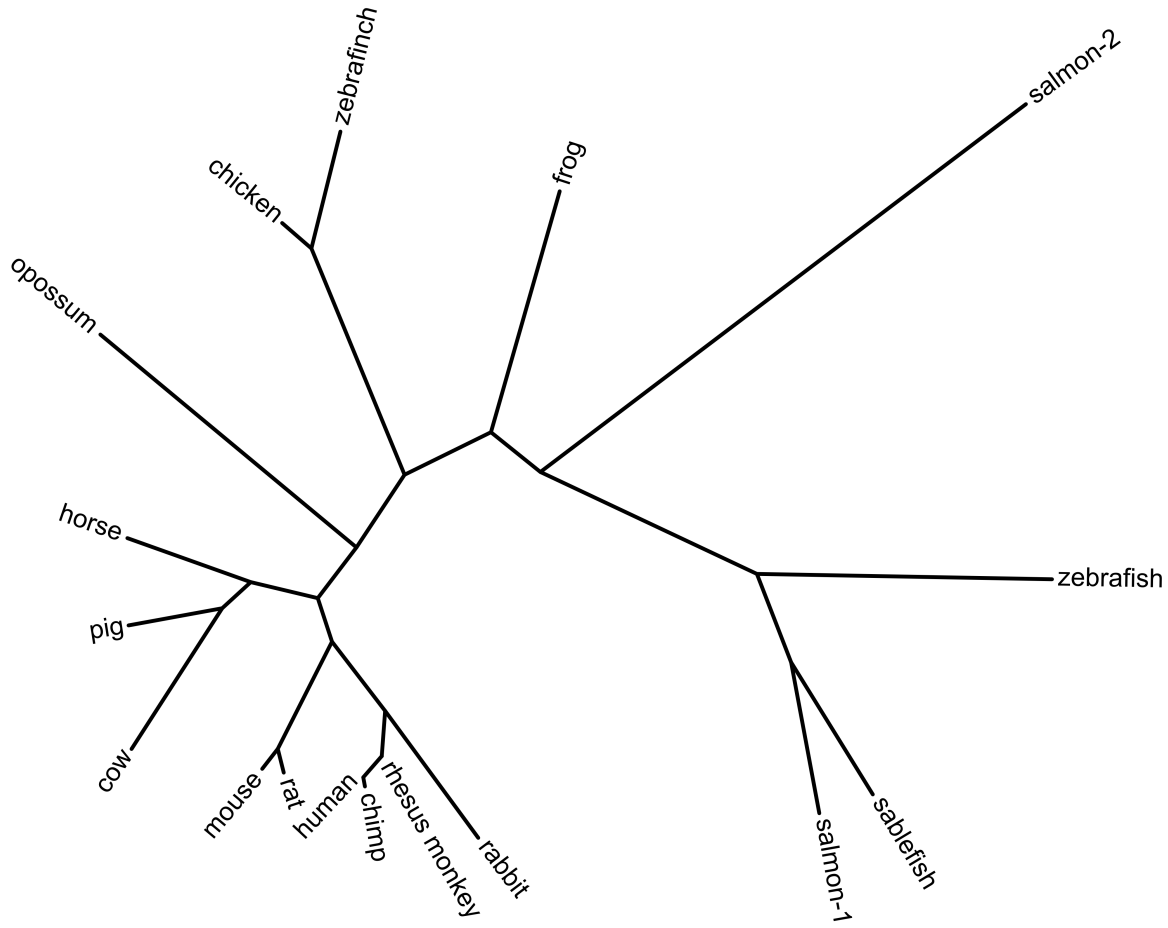
zebrafinch
chicken
opossum
horse
pig
cow
mouse
rat
human
chimp
rhesus monkey
rabbit
frog
salmon-2
zebrafish
salmon-1
sablefish

Figure 4. Evolutionary relationship of ILBP structure from various vertebrate species. The unrooted tree was constructed using PhyML (using software available at http://www.phylogeny.fr/). The ILBP amino acid sequences used to generate the tree were from the following species: chicken (*G. gallus*; XP_414486.1), chimpanzee (*P. troglodytes*; XM_001141744), cow (*B. Taurus*; BC102199), horse (*E. caballus*; XP_001503514), human (P51161), mouse (*M. musculus*; NM_008375), opossum (*Monodelphis domestica*; XM_001369808), pig (*S. scrofa*; P10289), rat (*R. norvegicus*; NM_017098), rabbit (*O. cuniculus*; P50119), rhesus monkey (*M. mulatta*; XM_001083748), sablefish (*A. fimbria*; BT082617), salmon (*S. salar*; BT048267 and BT049284), African clawed frog (*X. laevis*; Q4KL99), zebra finch (*T. guttata*; XP_002194045.1), zebrafish (*D. rerio*; EU665314).

Bridge section between chapter 1 & 2

Chapter 2 comprehensively reviewed the cellular and intracellular transport of bile acids (BA) in the enterohepatic circulation and highlighted evidence indicating that ILBP is their ileal intracellular transporter. ILBP, a member of the fatty acid binding protein family (FABP), is the most attractive candidate for ileal intracellular transport largely due to its affinity for conjugated bile acids (BA), primary tissue distribution in the ileum and the colocalization with other transporters on the longitudinal axis of the small intestine (see chapter 2).

An in vivo study by Kok et al (107) suggested that the absence of ILBP does not cause complete arrest of intracellular BA transport and that some BA still have the means to circulate. Our lab demonstrated, with the strongest evidence to date, that ILBP ablation results in decreased BA absorption (16). It is more pronounced in females than males, indicating the presence of a dimorphic phenotype in BA absorption. The function of BA is to emulsify lipids in preparation for absorption. ASBT mutations in humans results in BA malabsorption and subsequently fat malabsorption (71, 126, 142). Since ILBP deficiency results in BA malabsorption it was investigated if lipid absorption would be impacted. And, whether altered BA absorption will modify the composition of the gut microbiome due to the bacteriostatic and bactericidal effects of BA. Elucidating the influence of BA malabsorption on fat absorption and the gut microbiota is of significance because it mirrors human disease states such as irritable bowel syndrome (IBS), irritable bowel disease (IBD) and Crohn's disease and therefore extends the scope of the project.

Biotransformation of BA occurs by enzyme modifications exerted by the gut microflora. In turn, highly polar bile acids are bactericidal to specific species within the gut microflora and influence the expression of antimicrobial genes in the intestine by controlling the activity of FXRα (124). The colon is highly

concentrated with bacteria and exposure to excess BA will have a bactericidal effect on the density and diversity of the gut bacteria. In turn, certain species of bacteria are able to metabolize and modify BA; thriving in an environment other gut bacterial species cannot survive in, thus altering the gut microbiome profile.

The gut microbiota is a major determinant of energy harvest (143). Alterations in the gut microbiota profile due to dysregulation of BA homeostasis would alter the nutrient extraction capability of the host and be reflected in weight change. Increased energy extraction capability leads to weight gain and obesity, thus, it is associated with chronic disease in humans.

The gut microbiome is regulated by modifiable factors and non-modifiable factors (144). Modifiable factors include environmental influences such as diet and nutritional status (145, 146). Non-modifiable factors include genetics, sex and age (18) of which genetic background is primary factor. Familial similarity has been established from human twin studies and the “core microbiome” denotes the similarity between siblings (147-149). Bacterial colonization of the previously germfree infant gut occurs during birth and through initial feedings (148). Although individual differences within bacterial classes exist, the overall patterns of the gut microbiota profiles have familial resemblances (148).

The genetic component that determines our gut microbiome profile cannot be reset, and thus focus should be placed on the modifiable factors, such as diet and weight. Alteration of the gut microbiota by diet is recognized as having an affect on the ratio of two major bacterial phyla (*Bacteroidetes* and *Firmicutes*). A high- fat diet leading to weight gain has been demonstrated to increase the ration of Firmicutes to Bacteriodetes in humans and in mice (148). Therefore, intake of a high-fat western-type diet (WD) may cause alterations in factors such as anthropometrics (increase in weight) and in metabolic traits (respiration quotient)

of the model. The relationship between diet, weight and the gut microbiome is significant in solving the obesity epidemic.

In this study, the idea that ILBP deficiency will result in BA malabsorption and subsequently lipid malabsorption will be tested by using an internal manipulation of ILBP ablation in mice and an external manipulation of a WD. Introducing a WD to ILBP null mice will reveal how BA malabsorption affects their ability to cope with the metabolic stress of a lipid-rich diet. The WD is used in this methodology because the nutrient profile resembles the average macronutrient distribution commonly consumed in Westernized nations (146, 150). The composition of the experimental WD was first described by Plump et al. (151). Since then it has been used in numerous studies to investigate the effects of the human WD macronutrient profile on gene expression, metabolism and disease progression in mice (152-159). Secondly, the effect of the WD on the gut microbiota profile of ILBP mice will be examined. Understanding the effects of BA malabsorption merged with a WD extends the implications to humans with conditions exhibiting BA malabsorption that consume a high-fat diet. Our lab will examine the model from a multi-systems perspective to determine the effects of increased BA exposure on lipid absorption and the subsequent effect on the gut microbiota profile.

Chapter 3: Manuscript 2

Bile acid malabsorption due to ILBP deficiency exacerbates body weight gain in response to a western-type diet

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Abbreviations: BA, bile acids; EHC, enterohepatic circulation; Fabp1, liver-type fatty acid binding protein; FXRα, farnesoid-x-receptor α; ILBP, ileal lipid binding protein; FABP6, fatty acid binding protein 6; LFD, standard maintenance low fat diet; WD, high fat western diet

** A version of this manuscript is being prepared for submission.

Abstract

Background/Aims: *Fabp6-/-* mice provide a model of bile acid (BA) malabsorption as they exhibit increased excretion rates of BA. BA are required for absorption of fat-soluble molecules. The aim is to determine how ILBP deficiency alters the capacity of mice to withstand a lipid-rich diet such as the common .western-type high fat diet (WD) (Research Diets, D12079B) and whether loss of ILBP modifies the gut microbiome. ***Methods:*** C57BL/6J *(Fabp6+/+)* and *Fabp6-/-* sibling mice were placed into age and sex-matched groups (n=8), fed either the standard laboratory maintenance low fat diet (LFD, LabDiet 5001) or the WD for six weeks and assessed for biometric, biochemical and physiological parameters. Genomic analysis of gut microbiome by qualitative PCR (qPCR) focused on three phylum (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*) to represent the murine microbiota in totality. ***Results:*** Introduction of WD to ILBP null mice resulted in an unprecedented obesogenic metabolic phenotype despite BA malabsorption. ILBP null mice on the WD demonstrated increased adiposity compared to *Fabp6+/+* mice and increased lipid excretion in male ILBP null mice ($p < 0.05$) on the same diet. BA exhibited bacteriostatic activity when tested in bacterial cultures using *E. coli* as a model gut bacterium. Thus, gut microbiome modifications resulted from bacteria in the colon being exposed to increased BA. The gut microbiome profile showed increased *Bacteroidetes* to *Firmicutes* in WD-fed *Fabp6-/-* mice whereas *Fabp6+/+* mice showed decreased *Bacteroidetes* to *Firmicutes* on the same diet. Gallbladder BA profile for female *Fabp6-/-* mice on the WD consisted of primary and secondary BA, indicating interplay between BA and the microbiota. ***Conclusion:*** Excess BA excretion from ILBP deficiency resulted in altered tolerance to WD diet and increased energy extraction capability through modification of the gut microbiota profile. Increased ability for carbohydrate fermentation by the gut microbiome of ILBP mice on the WD is likely responsible for increased adiposity and increased total triglycerides in the liver.

Introduction

Fabp6 encodes the ILBP, a 15 kDa cytoplasmic protein that is expressed in the enterocytes of the ileum. ILBP binds fatty acids but preferentially binds BA. ILBP is an ileal intracellular transporter that shuttles bile acids through the enterocyte then passes them to the organic solute transporting proteins (OST) which then enter enterohepatic circulation (EHC) in the portal blood (7, 16, 76). As detergents, BA are toxic to bacteria and their potency is defined by hydrophobicity, polarity and conjugation, as discussed in chapter two. Lithocholic acid (LCA) and deoxycholic acid (DCA) are most hydrophobic and induce apoptosis, whereas ursodeoycholic acid (UDCA) is the least hydrophobic and does not induce apoptosis.

Humans with ASBT mutations exhibit BA malabsorption and fat malabsorption (142, 143). However, according to current knowledge, ASBT deficient mice do not exhibit BA malabsorption on a LFD, but have not been tested on a high fat diet (62). Therefore, ILBP null mice are excellent models for this study because they exhibit BA malabsorption. Male and female ILBP null mice have increased BA excretion rates, exposing the ileum and colon to an increased concentration of BA. Thus, examining ILBP null mice on a lipid-rich WD provides insight on the effect of BA malabsorption on dietary lipid absorption. The WD is selected in place of any high fat diet to ensure this study is relevant to current literature (152-158,168).

Obesity is a prime public health concern in industrialized nations and it is therefore important to understand the adaptive role of the gut microbiota on a WD. The gut microbiome is defined as the totality of microbe genomes that inhabit the intestines (143,168). It is a personalized organ that modulates nutrient metabolism of multiple organ systems through host-microbial co-metabolism

(144). With an estimated 1000-1150 bacterial species present in the gut, totaling 10^{13}-10^{14} colony forming units (144), it is an extensive network of microbial species. The greatest density and diversity of bacterial species reside in the colon, and this is where the gut microbiota becomes exposed to excess BA. Over 90% of gut microbes are from the *Bacteroidetes* and *Firmicutes* phyla. The ratio between these phyla is recognized as an indication of energy extraction capability, which impacts body weight (168). Humans of a normal, healthy weight (BMI of 18.5-24.9) have an increased proportion of *Bacteroidetes* relative to *Firmicutes* (143). In overweight/obese individuals this ratio is reversed. A greater proportion of *Firmicutes* increases the energy extraction capability and absorption of carbohydrates, and promotes the absorption of dietary fats within the gut (160).

In the EHC, interplay exits between BA and gut bacteria. When BA enter the EHC (ileum), certain anaerobic gut bacteria modify BA by deconjugation and dehydroxylation (see chapter two) to avoid cell death or arrested growth. BA can alter the gut microbiota profile because they are toxic detergents. Therefore modification of the gut profile is expected in ILBP null mice with BA malabsorption. Thus, *Fabp6-/-* mice will likely display an altered gut microbiome with different energy extraction capabilities compared to *Fabp6+/+* mice, changing their response to a WD. The primary aim is to determine if mice with an ILBP deficiency will malabsorb fat. The secondary aim is to determine if ILBP null mice have an altered capacity to withstand a lipid-rich diet and whether BA malabsorption affects the composition of the gut microbiota profile.

Methods

Animals and diet

Wild-type C57BL/6J (*Fabp6+/+*) mice were procured from Jackson Laboratory (Bar Harbor, Maine, USA). *Fabp6 -/-* mice (161) were backcrossed to C57BL/6J mice to produce an F1 generation of heterozygotes which were interbred to yield 30 *Fabp6 +/+* and 30 *Fabp6 -/-* mice that harbor the same familial gut microbiome. The mice used in this study ranged in age from 14-28 weeks old and were maintained on either a LFD or fed a WD for six weeks. The institutional animal welfare and policy committee under McGill AUP 5350 approved use of animals for this study.

Mouse genotyping

The mice were genotyped by multiplex polymerase chain reaction (PCR) to determine the presence of the wildtype or disrupted *Fabp6* alleles. The extracted genomic DNA was mixed with 2.5 units of DNA Taq polymerase, 250 uM each of dATP, dTTP, dCTP and dGTP, 50 mM tris-HCl (pH 8.3), 0.1% ficoll, 0.25 mg/ml BSA, 0.10 mM tartrazine, 1.5 mM MgCl2 with 1.25 uM of each primer in the multiplex (P-1F/R, P-2F/R, P-3F/R). The primers used were: Primer-1F, 5'-ACGATAGTTCATGAAGCGC-3'; Primer-2R, 5'-GCAGAGGATCAGGAGATTCAG, Primer-3R, 5'-GCGCATGCTCCAGACTGCCTT. Primer-1F and Primer-2R were designed to yield an 1134 base pair amplicon representing the wildtype *Fabp6* allele. Primer-1F and Primer-3R were designed to yield a 454 base pair amplicon representing the disrupted *Fabp6* allele.

Controlled diet feeding study

Age and sex matched sibling mice placed into eight groups: 4 groups (male *Fabp6+/+,* male *Fabp6-/-,* female *Fabp6+/+* and female *Fabp6-/-*) were put on the LFD and 4 analogous groups were placed on the WD for a six-week period. The LFD (162) contained 23.9 % protein (amino acid cocktail), 10.7 % fat (cholesterol, essential fatty acids, monounsaturated fats, saturated fats) and 48.7 % carbohydrates (starch, fiber, glucose, fructose, sucrose, lactose) for a total of 3.02 kcal/gram of utilizable energy. The open source purified WD (163) was composed of 20 % protein (casein, DL-methionine), 50 % carbohydrate (cornstarch, maltodextrin 10, sucrose, cellulose) and 21 % fat (milk fat, corn oil, cholesterol) with 4.7 kcal/gram of utilizable energy. Each of the 8 groups was housed separately (n = 5-8 per group). During the diet trial, mice were housed at 22-24°C with a 12 hr light/dark cycle, the dark cycle begins at 6h00. Mice were given ad libitum access to food and water, except during the last light cycle preceding termination when only water was provided.

Metabolic profile of *Fabp6+/+* and *Fabp6-/-* mice

Respiratory metabolism, food intake and activity were recorded using the Oxylet system (Panlab-Harvard Apparatus, BCN, Spain). Mice (n = 3) from each group were placed in individual metabolic cages for 72 h at week one and six. Respiratory quotient (RQ: O_2/CO_2), energy expenditure (EE: kcal/day/kg^0.75), food intake (g) and activity in the x and y planes (beam breaks) were collected in 24 h blocks and stool samples were collected at the end of each 24 h period.

Biochemical analysis

Total triglyceride and cholesterol concentrations were determined in vitro using commercial clinical kits (MP Biomedicals, CA, USA; Genzyme Diagnostics, MA,

USA). Plasma glutamate-pyruvate transaminase (ALT) and glutamate-oxaloacetate transaminase (AST) activity assays were adapted from commercial kits for smaller volumes (Biotron Diagnostics, CA, USA). Liver tissue homogenate samples were analyzed for protein content (Product # 23225, Thermo Scientific Pierce, ON, CA) and tested for total triglyceride content (164) (Percent Fat Saftest, MP Biomedicals, OH, USA). The plasma lipid profile was determined by analyzing fresh plasma samples through size exclusion chromatography with high performance liquid chromatography (HPLC) (Beckman System Gold, MN, USA). Fractions (100 ul) of column eluate were collected between 5-35 minutes then assayed for total triglyceride and cholesterol concentration (Genzyme). In this system, large lipoproteins elute out of the column before smaller lipoproteins.

Analysis of bile acids

Total bile acids (TBA) in plasma were determined by using the commercial total bile acids assay kit (DZ042A-KB1, Diazyme, CA, USA). TBA excretion was determined using homogenized stool sample (Mini-Bead Beater, Biospec, OK, USA) matter reconstituted in 1 ml of 50% t-butanol, and was analyzed with commercial TBA assay kit. The speciation of BA in the gallbladder was determined by HPLC (Agilent, 1200 Series, CA, USA) as described previously (76).

Analysis of DNA in stool

Stool samples collected from each mouse were dried then mechanically pulverized into powder using stainless steel beads in the Mini-BeadBeater (BioSpec). DNA extraction (50 mg of stool powder/sample) was completed by using a method previously described by Kocheringskara et al., that was adapted to smaller volumes (165). DNA concentration was determined using the Qubit

assay for DNA (Invitrogen, CA, USA). The representation of bacterial classes in stool DNA was estimated by qPCR. The extracted genomic DNA was mixed with 2.5 units of DNA Taq polymerase, 250 uM each of dATP, dTTP, dCTP and dGTP, 50 mM tris-HCl (pH 8.3), 0.1% ficoll, 0.25 mg/ml BSA, 0.10 mM tartrazine, 1.5 mM MgCl2 with 1.25 uM of each primer. The primer sequences used were: *Bacteroidetes* (phylum specific) Bact-F285: GGTTCTGAGAGGAGGTCCC and reverse primer Univ-R338: GCTGCCTCCCGTAGGAGT; *Firmicutes* (*Enterococcus* and *C. leptum*) Ent-F:CATTGACGTTACCCGCAGAAGAA) and Ent-R: CGCTTGCACCCTCCGTATTA; *Firmicutes* (*C. leptum*), C-LeptF: GTTGACAAAACGGAGGAAGG and C-LeptR: GACGGGCGGTGTGTACAA; *Actinobacteria* (phylum specific) Bifido-F143: CTCCTGGAAACGGGTGGT and Univ R338: GCTGCCTCCCGTAGGAG (166). The copy number of the murine c-mos promoter sequence amplified by murine DNA specific primers UMSA-F: TCAGATTTGTGCATACACAGTGACT and UMSB-R: GTAAACATTTTTCGGGAATAAAAGTTGAGT was used to estimate the amount of mouse genomic DNA in the stool samples. To determine overall diversity of the gut microbiome, universal primers were used to amplify the 16S gene sequence of bacterial genomes, 341-F: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG and reverse primer 534-R: ATTACCGCGGCTGCTGG (165).

Statistics

All statistical calculations were performed using GraphPad Prism5 (GraphPad Software Inc., CA, USA). Statistical tests used included two-way ANOVA and t-tests where appropriate. Differences were considered significant when $P < 0.05$.

Results

WD resulted in increased adiposity in *Fabp6-/-* mice

Figure 1 demonstrates that male and female mice on LFD have significantly lower fat pad mass ($P < 0.01$, $P < 0.05$) than mice on the WD. Total fat pads in *Fabp6+/+* and *Fabp6-/-* mice on LFD were similar (Figure 1). Both male and female *Fabp6-/-* mice on WD have greater fat pads ($P < 0.001$, $P < 0.05$) than *Fabp6+/+* mice on the same diet (Figure 1) thereby increasing their weight (Table 1) and mBMI (Figure 1). Mice on the LFD have normal mBMIs (mBMIs: see Appendix 1). These results indicate that feeding the WD to mice lacking ILBP increases body weight gain due to adiposity.

Analysis of plasma chemistry demonstrated that cholesterol and glucose concentrations increased after six weeks on the WD in all mice (Table 2). Interestingly, plasma triglyceride concentration decreased in mice fed the WD, except in male *Fabp6+/+* mice ($P < 0.01$) (Table 2). Lipoprotein profiles determined by HPLC demonstrated that loss of ILBP did significantly change the distribution of lipoproteins on the LFD. Conversely, the WD increased cholesterol in the high-density lipoprotein (HDL) fraction and the triglyceride in the very-low density lipoprotein (VLDL) fraction in all mice (Figure 2). Interestingly, male ILBP null mice on the WD demonstrated an increase of cholesterol in both the low-density lipoprotein (LDL) and HDL fractions (Figure 2). ILBP null mice on the WD display a magnified triglyceride and cholesterol concentration in the VLDL and HDL fractions, respectively, compared to *Fabp6+/+* mice on the same diet.

All mice on WD had greater ALT and AST levels which is indicative of liver dysfunction (Table 2). Liver morphology observed during necropsy indicated that male mice on WD had light pink, enlarged livers, inferring fatty liver infiltration. Liver weights of male and female mice ILBP null mice on the WD had a mean of

2.42 ± 0.46 and 1.49 ± 0.11, respectively, ($P < 0.05$) compared to *Fabp6-/-* mice (Table 2). No differences in the triglyceride content of liver on the LFD existed. Conversely, the triglyceride concentration of the liver of all male mice on WD demonstrated significantly higher levels than their counterparts on LFD ($P < 0.01$) (Figure 3). Female ILBP null mice demonstrated increased liver triglycerides compared to their counterparts on a LFD ($P < 0.01$) and decreased liver triglycerides compared to female *Fabp6+/+* mice on the WD ($P < 0.01$). The WD induced hepatomegaly and decreased liver enzyme function.

On LFD, stool output (dry weight) was 10 % and 8 % of food intake (wet weight) for male *Fabp6+/+* and *Fabp6-/-* mice, compared to 20 % and 10 % for female *Fabp6+/+* and *Fabp6-/-* mice. The greatest kilocalorie intake difference was a 22% decrease in female *Fabp6-/-* mice on the WD compared to female *Fabp6-/-* mice on the LFD (Figure 5a). Despite decreased intake, female *Fabp6-/-* mice meet energy and macronutrient requirements (167). Male *Fabp6-/-* mice demonstrated no difference in total kilocalorie intake due to the high density (4.7kcal/g) of the WD (Figure 5a). The WD decreased stool output in both male *Fabp6+/+* and *Fabp6-/-* mice to 4 % and 5 %, respectively, while it was 9% for both female *Fabp6+/+* and *Fabp6-/-* mice. TBA excretion was increased in ILBP null female mice on the LFD ($P < 0.01$) (Figure 3). On the WD, all ILBP mice demonstrated increased TBA excretion in comparison to Fabp6+/+ mice ($P < 0.05$, $P < 0.01$) (Figure 3). Total triglyceride excretion is significantly increased in male and female ILBP null mice compared to *Fabp6+/+* mice on the same diet ($P < 0.001$, $P < 0.01$). ILBP female mice also have greater ($P < 0.01$) triglyceride excretion than their counterparts on the WD (Figure 3). Cholesterol excretion increased in ILBP males on the WD compared to the LFD ($P < 0.01$). In female ILBP null mice, cholesterol excretion increased compared to *Fabp6+/+* female mice on the WD ($P < 0.01$) (Figure 3).

Metabolic profile

Fat accumulation within the liver and the body (adiposity) was unexpected as ILBP null mice have increased triglyceride excretion (males, $P < 0.05$) compared to *Fabp6+/+* mice on the WD. To determine if food intake can account for the observed difference in body weight gain the metabolic profile was examined. Uniquely, *Fabp6-/-* mice consumed 0.5-1.0 grams more of the LFD than Wild type; yet demonstrate no difference in mBMI (Figure 1). Also, food intake by male *Fabp6-/-* mice on the LFD and male *Fabp6+/+* mice on the WD had 50 % greater intake than male *Fabp6-/-* mice on the WD. ($P < 0.05$) (Figure 5). Total food intake on the WD was inversely associated with mBMI. *Fabp6-/-* mice on the WD displayed the lowest intake, greatest adiposity and highest level of activity during the dark cycle in comparison to *Fabp6+/+* mice (Figure 1,5). Surprisingly, energy intake and activity does not correlate with body weight gain or loss. Male *Fabp6-/-* mice on the LFD demonstrated a consistent RQ of ~0.8 during the six week diet trial. In comparison, after six weeks on the WD male ILBP null mice decreased in RQ to ~0.7 (Figure 6). Similarly, female *Fabp6-/-* mice on the LFD for six weeks had a consistent RQ of ~0.85. However, after consuming a WD for the trial female ILBP null mice decreased their RQ to ~0.75. Male ILBP null mice demonstrate increased energy expenditure (EE, ~90k kcal/day/kg^0.75), which correlates to their higher activity levels, compared to EE of male *Fabp6+/+* on the WD (~80 kcal/day/kg^0.75) (Figure 7). Conversely, female ILBP null mice demonstrate decreased EE (~70 kcal/day/kg^0.75) versus female *Fabp6+/+* (~80 kcal/day/kg^0.75) on the WD (Figure 7). Thus, the metabolic profile and activity level was modified and the differential weight gain in ILBP null mice on the WD cannot be accounted for by activity level.

BA metabolism in *Fabp6-/-* mice

A previous study done by our laboratory established that loss of *Fabp6-/-* disrupts BA homeostasis (16). As shown in Figure 3 the increased BA excretion on LFD diet was confirmed and the same feature is observed in mice on the WD. Also, there are no changes in plasma bile acid concentration (Table 1) and this finding is consistent with defective bile acid reabsorption.

To determine if the WD further alters BA metabolism in *Fabp6-/-* mice, the gallbladder BA profile was analyzed. HPLC analysis revealed that all male mice on LFD have similar profiles (Table 3) and females differ from each other, and males because they contain secondary BA. ILBP null mice on the WD have three common BA species in the gallbladder: CA, TCA and TMCA. Differences occur in female ILBP null mice as they have secondary BA, DCA. Conversely, their male counterparts have TDCA (taurine conjugated secondary BA) in their gallbladder BA profile. ILBP null male mice on the WD tended to display an increase in the TCA to TMCA ratio compared ILBP null male mice on the LFD (Figure 4).

Remodeling of the gut microbiome

The gut microbiota was assessed by quantitative PCR. Three phylum-specific validated primers were chosen for analysis, representing the majority of the murine microbiome (166). Phyla analyzed were *Bacteroidetes*, *Firmicutes* and *Actinobacteria*.

In male and female *Fabp6+/+* mice the *Bacteroidetes* composes the greatest proportion (M=40 %, F=40 %), although males have more *Firmicutes* than females (M=30 %, F=25 %) and females have more *Actinobacteria* than males (M=30 %, F=35 %) of the profile analysis. Loss of ILBP alters the proportion of the three phyla in similar ways in both sexes. In general, loss of ILBP increases

the proportion of *Firmicutes* (M & F=33 %) at the expense of *Bacteroidetes* (M & F=37 %), in both males and females, but *Bacteroidetes* remain the dominant phylum. Introduction of the WD further alters the ratio of the three phyla characterized by a decrease in *Firmicutes* (M=25 %, F=30 %) and an increase in *Bacteroidetes* (M=45 %, F=40 %) with no significant change in *Actinobacteria* (M/F=30 %).

Preliminary analysis demonstrated that male *Fabp6+/+* showed greater bacterial density than female *Fabp6+/+* on the LFD. Loss of ILBP increases the density in both sexes on the same diet (M=12 %, F=17 %). Introduction of a WD to *Fabp6+/+* male mice decreases bacterial density with no change in females (M= 32%). Conversely, introduction of a WD to ILBP null mice increases the density in male mice with no effect on females (M=28 %). WD decreases the total diversity of the gut microbiome in male *Fabp6+/+* but increases the diversity in male *Fabp6-/-* mice. Therefore, loss of ILBP induced that *Fabp6+/+* mice and ILBP null mice already have different gut microbiome profiles, that can be dimorphic, and introduction of a WD exacerbates these differences.

Discussion

In humans, ASBT deficiency results in primary BA malabsorption that is accompanied by steatorrhea (8, 126). Interestingly, ASBT deficiency is not associated with steatorrhea on diet with low fat content (67), but this could be due to the minimal fat content of a LFD and has not been tested in the context of a lipid-rich diet. In the present study, mice deficient in ILBP, which also show BA malabsorption, were used to evaluate dietary fat absorption when challenged with WD, which contains a high dietary fat content (6, 16). Introduction of WD to ILBP null mice resulted in an unprecedented obesogenic metabolic phenotype despite BA malabsorption and increased triglyceride and cholesterol excretion. The analysis of mice on the WD revealed the interplay between BA metabolism,

fat malabsorption and the gut microbiome. Unexpectedly, ILBP null mice on the WD demonstrated increased adiposity despite increased lipid excretion.

As expected, the profile of *Fabp6+/+* mice on the WD is obesogenic (143, 168). However, the excessive adiposity of *Fabp6-/-* mice could not be explained by food intake, physical activity and EE. Moreover, after examining the metabolic profile the RQ indicated that *Fabp6-/-* mice utilized fat as an energy substrate. A possible explanation is that since the WD is abundant in refined carbohydrates (sucrose) (163) the ILBP null mice were using sucrose as an energy source. Increased ability for carbohydrate fermentation appears to take precedence over a high fat content in ILBP mice on the WD. The WD increased saccharolytic fermentation leading to production of SFCA that are stored within the liver and subcutaneously. Increased SCFA production of butyric, acetic and propionic acid may also explain the increased activity and energy expenditure in *Fabp6-/-* mice. Hyperactivity during the dark cycle could be due to the production of propionic acid, a bi-product of saccharolytic fermentation by the gut microbiome, which may be a neurological stimulant that increases agitation (169). Therefore, increased energy extraction of carbohydrates (112, 143) is responsible for excessive adiposity, hepatomegaly, fatty liver infiltration and liver dysfunction, especially the male ILBP null mice.

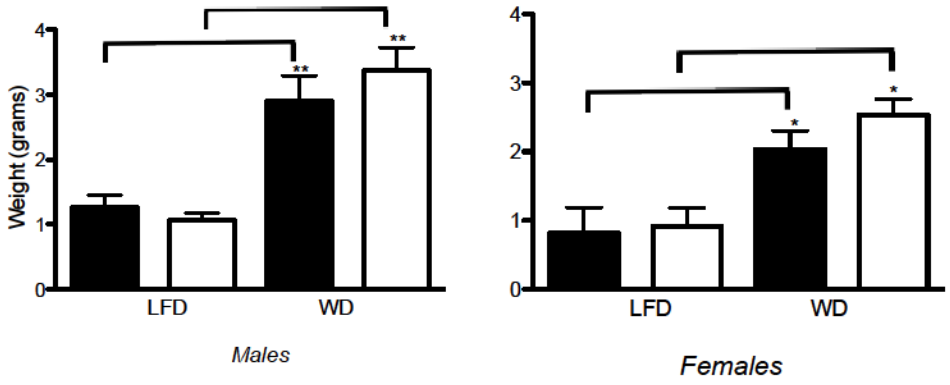
The G-coupled bile acid protein receptor (TGR5) is a cell surface receptor expressed widely in tissues that bind their natural ligand, BA, and has been implicated in energy homeostasis (40, 170). TGR5 is associated with the activation of the protein kinase B and extracellular signal-regulated kinases signaling pathways in vivo and in vitro (171, 172). Recently, it was demonstrated in mice and rats that activation of TGR5 by BA in the colon improves glucose control. TGR5 stimulates the release of GLP-1, which is believed to exert an anti-diabetic effect. Individuals with the T79M mutation of ILBP are resistant to diabetes (140, 141). The T79M variation may decrease BA binding, promoting

BA malabsorption similar to that shown by ILBP mice. Resistance to diabetes could be attributable to TGR5 activation. In the present study, *Fabp6+/+* and ILBP null mice on the WD both exhibited hyperglycemia. TGR5 function and plasma GLP-1 levels should be examined to determine their potential contribution to glucose homeostasis.

ILBP null mice demonstrated a modified gut microbiome profile compared to *Fabp6+/+* and introduction of a WD amplified the difference (4). BA extend their antimicrobial effect by regulating FXRα (173) which can further modify the profile of the gut microbiome. All mice in the study were siblings and are thereby comparable to each other based on the principle of a core gut microbiome (148). Investigation of species detected by the primer sets used in the analysis (166) revealed that *Fabp6+/+* mice on the WD displayed decreased *Bacteroidetes* relative to *Firmicutes*, as expected (143). Interestingly, ILBP null mice demonstrated the overall phenotype associated with a normal weight (increased *Bacteroidetes* relative to *Firmicutes*); however, the proportion of *Firmicutes* was (174) marker for obesity (109). Conversely, after six weeks on the WD the proportion of *Bacteroidetes* was greater relative to *Firmicutes* in WD-fed *Fabp6-/-* mice. To further investigate the interplay between BA and the microbiota, the gallbladder BA speciation revealed that ILBP null mice on the WD had primary and secondary BA. The appearance of unconjugated bile acids in gallbladder bile suggest defective N-acylamidation of bile acid in the liver. It has been clearly demonstrated that the WD decreases diversity in humanized gnotobiotic mice (146, 149, 152); however ILBP null mice on the WD demonstrate increased overall diversity of the gut microbiome when compared to their *Fabp6+/+* counterparts. This body of work strengthens the idea that ILBP null mice manage nutrient metabolism through different nutrient-gene interactions regulated by the gut microbiome. Loss of ILBP therefore alters the gut microbiome profile and subsequently affects energy extraction capability that is exacerbated by a WD.

In summary, bile acid malabsorption did cause fat malabsorption in male and female ILBP null mice on the WD. In the presence of bile acid malabsorption the gut microbiome is altered. The feeding of the WD resulted in an unforeseen obesogenic effect on *Fabp6-/-* mice, especially males, due to a hypothesized modification of the gut microbiome by exposure to excess BA. Unexpectedly, the sucrose content (34%) (163) of the WD likely took precedence over fat, stimulating increased energy extraction capability and greater adiposity in ILBP null mice. Male and female differences were seen underscoring the existence of sex differences in the metabolism of nutrients. This study demonstrated that ILBP deficiency altered the capacity of mice to withstand a lipid-rich diet through alteration of the gut microbiome.

A


Weight (grams)
0
1
2
3
4
**
**
LFD
WD
Males
0
1
2
3
4
*
*
LFD
WD
Females

B

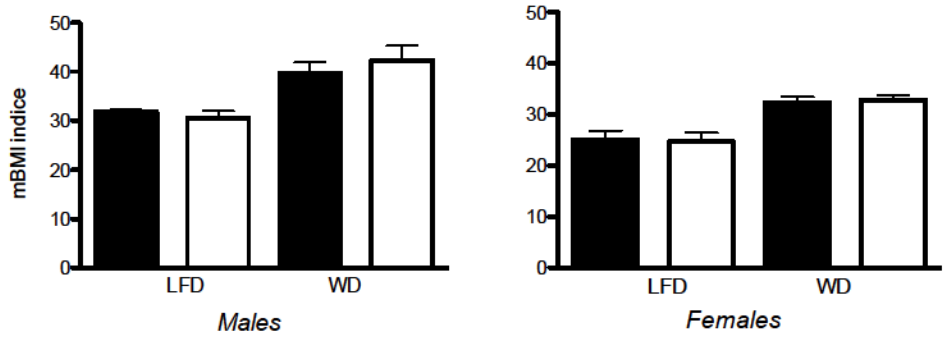
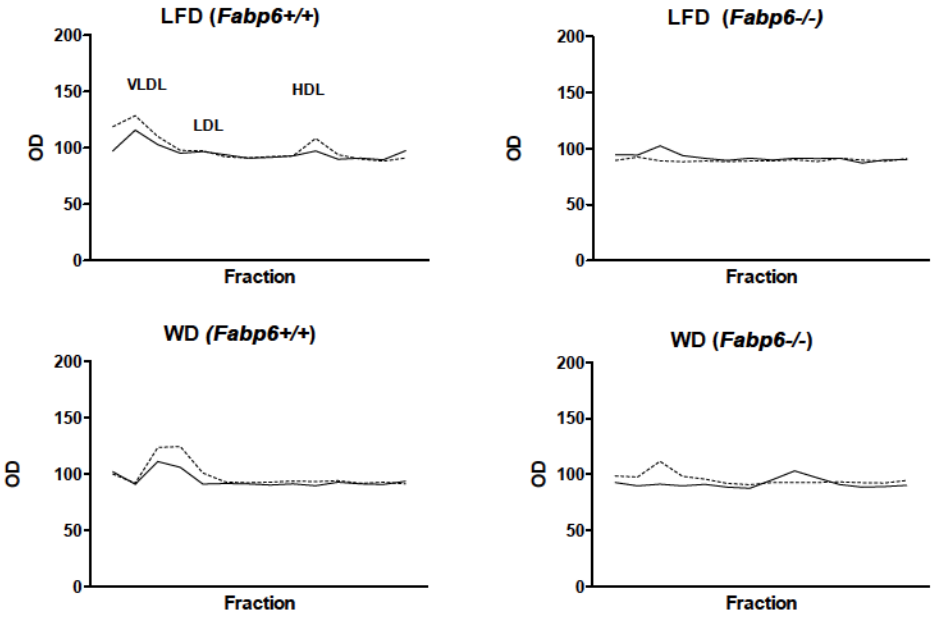

mBMI indice
0
10
20
30
40
50
LFD
WD
Males
0
10
20
30
40
50
LFD
WD
Females

Figure 1.
Effect of WD on adiposity in *Fabp6+/+* and *Fabp6-/-* mice. Data shown is the mean $\pm$ SEM (n=5-8 mice per group). *P <0.05, **P <0.01. (A) Effect of WD on total fat pads (mesentery, renal, gonadal). For total fat pads, black and white bars indicate *Fabp6+/+* and *Fabp6-/-* mice respectively. (B) For the mBMI, black and white bars indicate *Fabp6+/+* and *Fabp6-/-* mice respectively, see Appendix 1 for formula used to calculate the mBMI values.

A - Triglyceride



B - Cholesterol

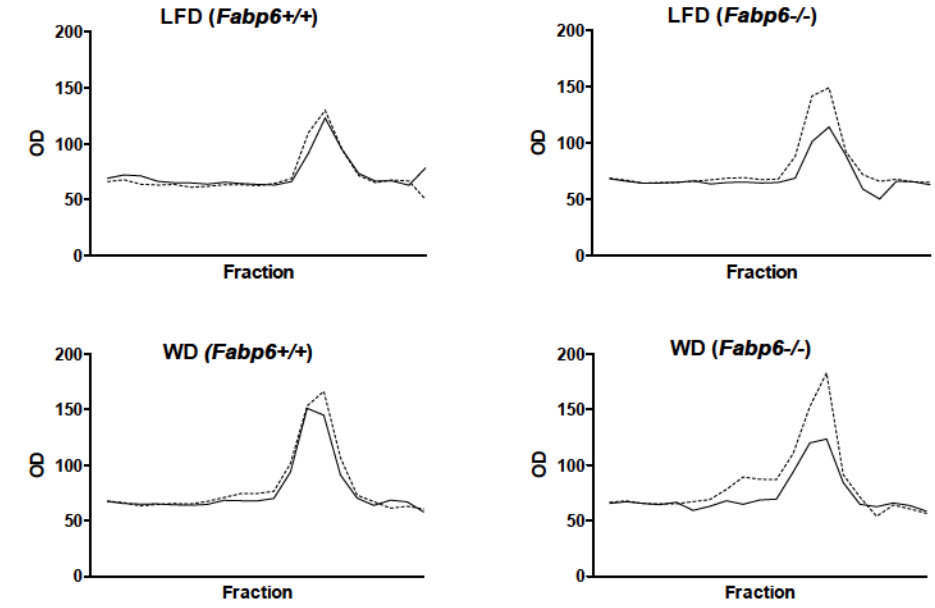
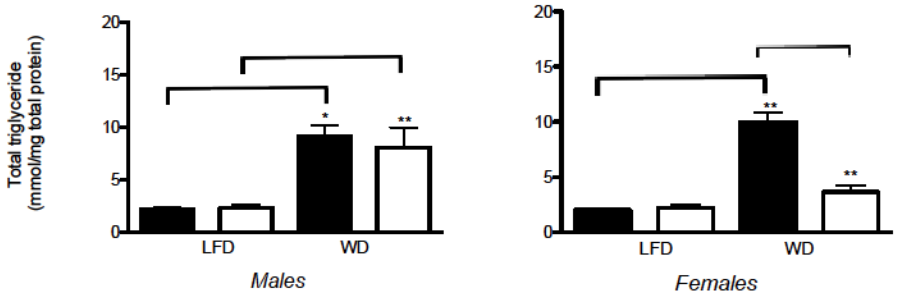
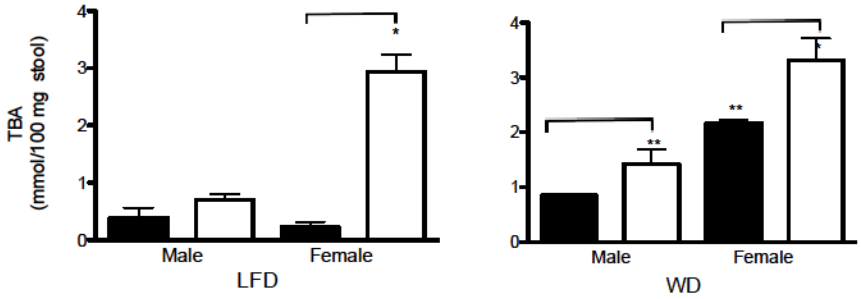


Figure 2.
Lipid profiles of mice on LFD and WD. Male mice are represented by a broken line and female mice are represented by a solid line (n=5-8). (A) Triglyceride lipoprotein profile. (B) Cholesterol lipoprotein profiles.

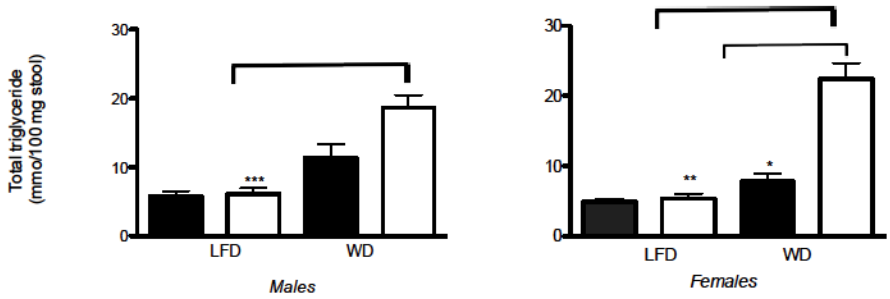
A - Total liver triglyceride



B - Total bile acid excretion



C - Total triglyceride excretion



D - Total cholesterol excretion

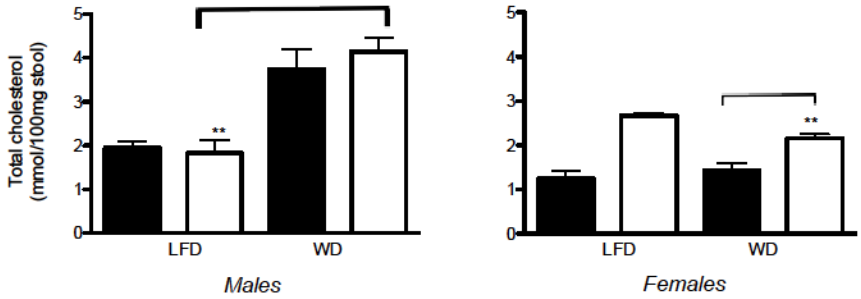


Figure 3.
Impact of WD on lipid metabolism. *Fabp6+/+* mice are represented by black fill bars and *Fabp6-/-* mice are represented by clear fill bars. All values represent means from each group (n=5-8) $\pm$ SEM, *P <0.05, **P <0.01 ***P < 0.001. (A) Total liver lipids of mice on the LFD and WD (mmol) per mg of total protein. (B) Total bile acid excretion (mmol) in 100 mg of stool. (C) Total triglyceride excretion in the stool (mmol) in 100mg of stool. (D) Total cholesterol excretion (mmol) in 100 mg of stool.

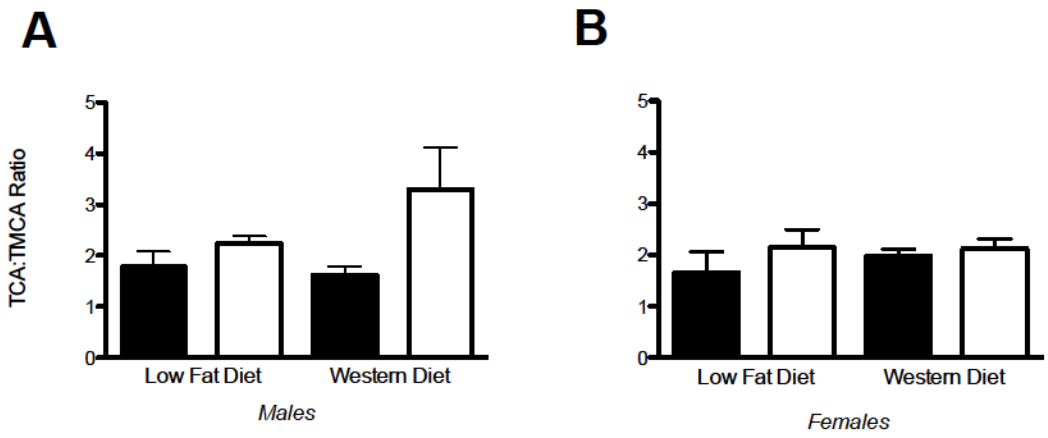
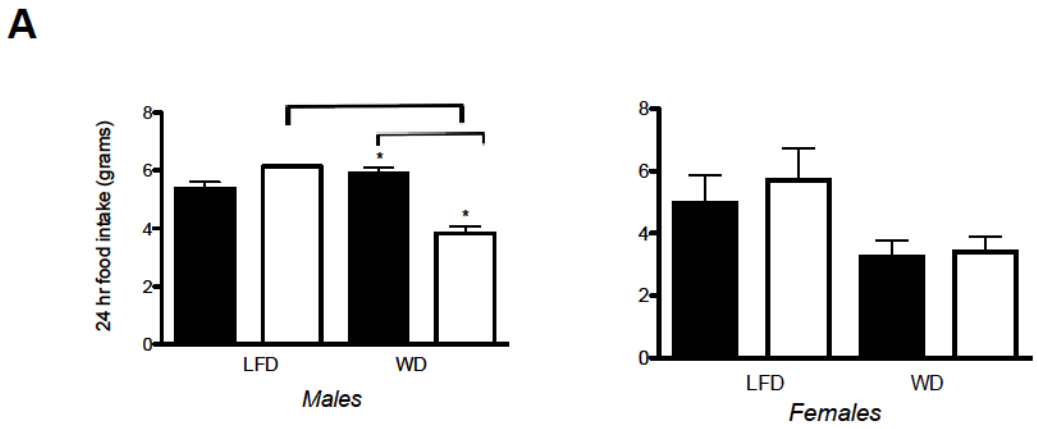
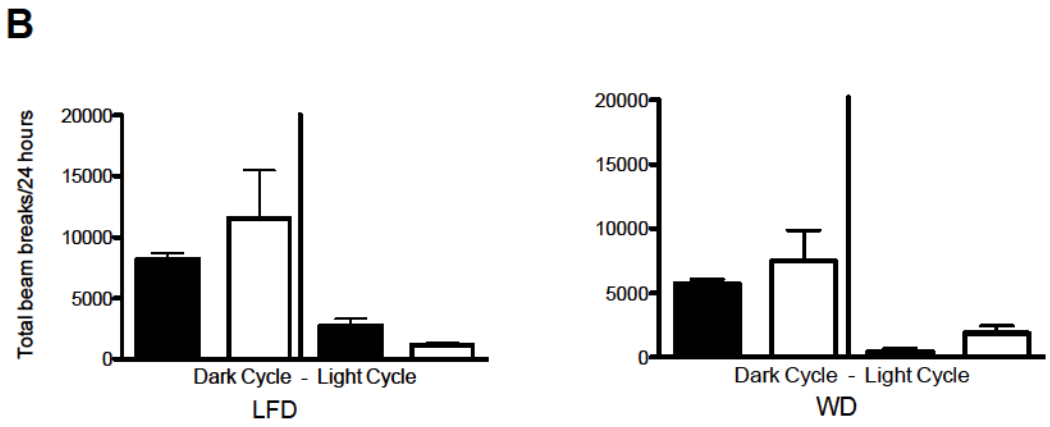

A
B
TCA:TMCA Ratio
0
1
2
3
4
5
Low Fat Diet
Western Diet
Males
Females

Figure 4.
TCA to TMCA ratio. (A) TCA to TMCA in male mice. *Fabp6+/+* males are represented by black filled bars and male *Fabp6-/-* mice are represented by clear fill bars. (B) TCA to TMCA in female mice. *Fabp6+/+* females are represented by black filled bars and female *Fabp6-/-* mice are represented by clear fill bars.


A
24 hr food intake (grams)
8
6
4
2
0
*
*
LFD
WD
Males
8
6
4
2
0
LFD
WD
Females


B
Total beam breaks/24 hours
20000
15000
10000
5000
0
Dark Cycle - Light Cycle
LFD
20000
15000
10000
5000
0
Dark Cycle - Light Cycle
WD

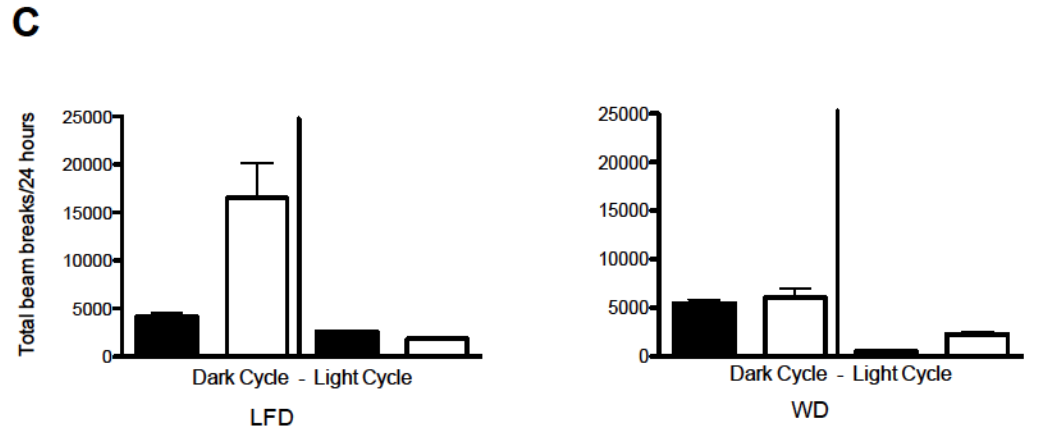

C
Total beam breaks/24 hours
25000
20000
15000
10000
5000
0
Dark Cycle - Light Cycle
LFD
25000
20000
15000
10000
5000
0
Dark Cycle - Light Cycle
WD

Figure 5.
Total vertical and horizontal activity during dark vs. light cycle. Values represent mean ± SEM (n=3). (A) Male *Fabp6+/+* mice are represented by black bars and male *Fabp6-/-* are represented by clear bars, respectively. (B) Male *Fabp6+/+* mice are represented by black bars and female *Fabp6-/-* are represented by clear bars, respectively. (C) Female *Fabp6+/+* mice are represented by black bars and female *Fabp6-/-* are represented by clear bars, respectively.

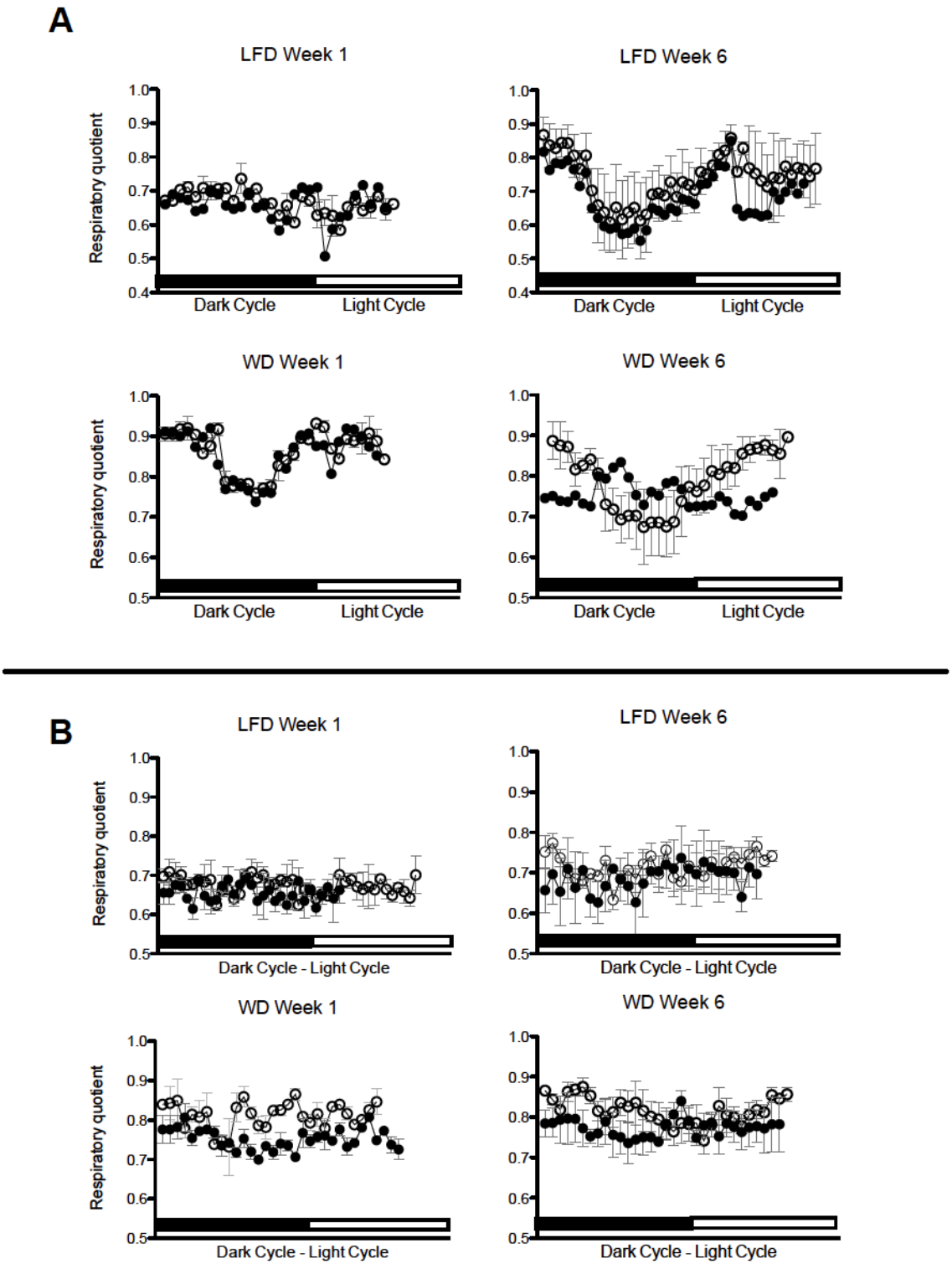
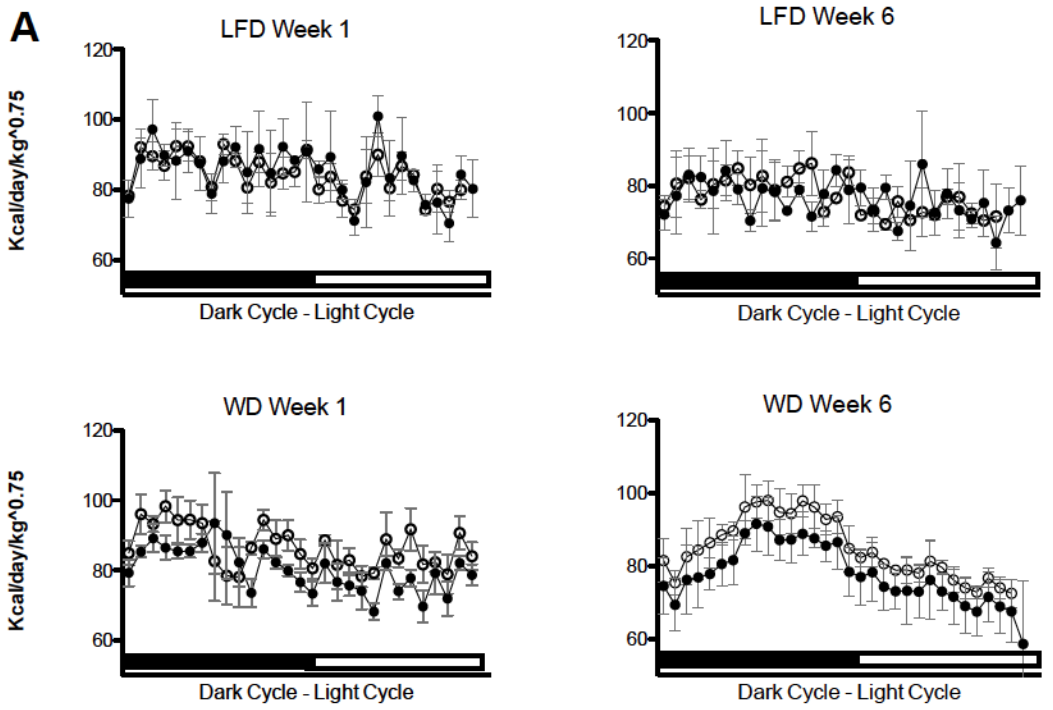

A
LFD Week 1
LFD Week 6
WD Week 1
WD Week 6
Respiratory quotient
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Dark Cycle
Light Cycle
B
LFD Week 1
LFD Week 6
WD Week 1
WD Week 6
Respiratory quotient
Dark Cycle - Light Cycle

Figure 6.
Respiratory quotient is displayed for male and female mice for a LFD versus WD at week one and six. The dark and light cycle each represent 12 h, 24 h total. Data shown is the mean of 72 h $\pm$ SEM (n=3 mice per group). (A) Male *Fabp6+/+* mice are represented by closed circle and *Fabp6-/-* males are represented by open circles. (B) Female *Fabp6+/+* mice are represented by closed circle and *Fabp6-/-* females are represented by open circles.


A
LFD Week 1
LFD Week 6
WD Week 1
WD Week 6
Kcal/day/kg^0.75
120
100
80
60
Dark Cycle - Light Cycle

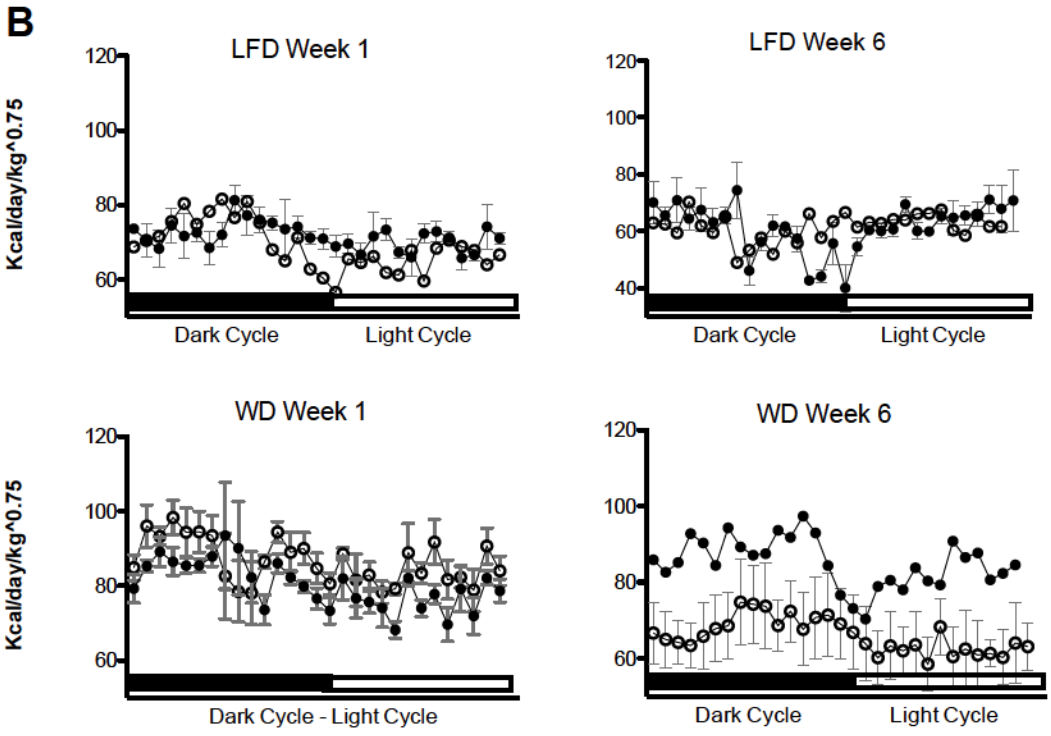

B
LFD Week 1
LFD Week 6
WD Week 1
WD Week 6
Kcal/day/kg^0.75
120
100
80
60
40
Dark Cycle
Light Cycle
Dark Cycle - Light Cycle

Figure 7.
Energy expenditure is displayed for male and female mice for a LFD versus WD at week one and six. The dark and light cycle each represent 12 h, 24 h total. Data shown is the mean of 72 h $\pm$ SEM (n=3 mice per group). (A) Male *Fabp6+/+* mice are represented by closed circle and *Fabp6-/-* males are represented by open circles. (B) Female *Fabp6+/+* mice are represented by closed circle and *Fabp6-/-* females are represented by open circles.

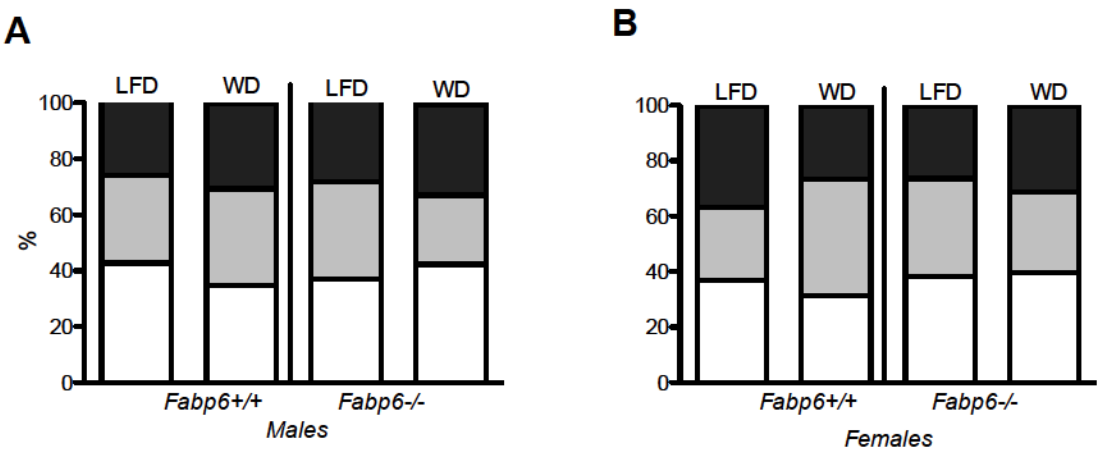

A
LFD
WD
LFD
WD
100
80
60
40
20
0
%
Fabp6+/+
Fabp6-/-
Males
B
LFD
WD
LFD
WD
100
80
60
40
20
0
Fabp6+/+
Fabp6-/-
Females

Figure 8.
Modifications of the gut microbiome. (A) Change in gut microbiome profile of male mice, *Bacteroidetes* (clear fill), *Firmicutes* (grey fill) and *Actinobacteria* (black fill). *Fabp6+/+* and *Fabp6-/-* mice are separated by a black line. (B) Change in gut microbiome profile of female mice, *Bacteroidetes* (clear fill), *Firmicutes* (grey fill) and *Actinobacteria* (black fill). *Fabp6+/+* and *Fabp6-/-* mice are separated by a black line.

Tables.

Table 1.
Effect of the WD on body weight and liver weight. Values represent mean ± SEM (n=5-8). Significant comparisons made between each group on the LFD compared to their parallel group on the WD are denoted by an asterisk (*), where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$. Significant comparisons made between sexes on the same diet are denoted by a number sign (#), where # $P < 0.05$.

	Final Body Weight (grams ± SE)	Liver Weight (grams ± SE)
LFD		
Male *Fabp6+/+*	33.49 ± 3.21	1.22 ± 0.031
Male *Fabp6-/-*	31.09 ± 2.14	0.84 ± 0.045
Female *Fabp6+/+*	25.06 ± 4.03	0.95 ± 0.066
Female *Fabp6-/-*	24.81 ± 3.75	0.90 ± 0.10
WD		
Male *Fabp6+/+*	42.22 ± 6.18*	1.94 ± 0.46
Male *Fabp6-/-*	42.98 ± 5.53***	2.46 ± 0.46*
Female *Fabp6+/+*	33.15 ± 2.16***	1.39 ± 0.11
Female *Fabp6-/-*	34.77 ± 3.00***	1.59 ± 0.11

Table 2.
Effect of the WD on the indicated plasma parameters. For each diet plasma triglyceride, cholesterol and glucose were recorded at week six, values represent mean ± SEM (n=5-8). Significant comparisons made between each group on the LFD compared to their parallel group on the WD are denoted by an asterisk (*), where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$. Significant comparisons made between sexes on the same diet are denoted by a number sign (#), where # $P < 0.05$. Plasma bile acids and ALT/AST were from pooled samples, values represent mean ± SEM (n=5-8) of the replicate assays.

Parameter	*Fabp6+/+* Male	*Fabp6-/-* Male	*Fabp6+/+* Female	*Fabp6-/-* Female
		LFD		
TG (mmol/L)	0.76 ± 0.03	0.78 ± 0.34	0.89±0.45	0.69 ± 0.13
Cholesterol (mmol/L)	1.88 ± 0.22	1.89 ± 0.44	1.87 ± 0.32	1.40 ± 0.16*
Glucose (mmol/L)	10.9 ± 1.71	8.55 ± 0.86	7.81 ± 0.11	8.23 ± 0.16
Bile Acids (μmol/L)	11.09 ± 0.006	33.6 ± 0.019	28.8 ± 0.011	56.5 ± 0.007
ALT (IU/L)	44.9 ± 0.012	52.6 ± 0.011	11.4 ± 0.013	31.62 ± 0.009
AST (IU/L)	51.93 ± 0.017	54.35 ± 0.005	14.59 ± 0.008	43.46 ± 0.023
		WD		
TG (mmol/L)	1.06 ± 0.24	0.54 ± 0.07**,#	0.79 ± 0.31	0.66 ± 0.08
Cholesterol (mmol/L)	4.08 ± 0.84	4.27 ± 0.52	3.34 ± 0.32	3.83 ± 0.76
Glucose (mmol/L)	14.3 ± 1.6	18.1 ± 2.95	7.98 ± 0.35	13.3 ± 0.12 **,#
Bile Acids (μmol/L)	9.34 ± 0.001	47.9 ± 0.005	77.2 ± 0.009	98.9 ± 0.004
ALT (IU/L)	70.46 ± 0.012	94.94 ± 0.001	55.7 ± 0.008	33.76 ± 0.017
AST (IU/L)	48.30 ± 0.023	53.15 ± 0.013	86.06 ± 0.001	94.19 ± 0.005

Table 3.
Impact of ILBP deficiency and WD on BA pool speciation. TCA, TMCA, CA, deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA).

	Fabp6+/+ Male	*Fabp6-/-* Male	*Fabp6+/+* Female	*Fabp6-/-* Female
LFD				
TCA	x	x	x	x
TMCA	x	x	x	x
CA				
DCA			x	
GDCA				x
TDCA				
WD				
TCA	x	x	x	x
TMCA	x	x	x	x
CA	x		x	x
DCA				x
GDCA		x		
TDCA	x		x	

Chapter 4: Overall Summary and Conclusions

Role of bile acids in nutrient harvest

As previously discussed BA malabsorption accompanies fat malabsorption because BA are essential in lipid solubilization and absorption. Previous studies have examined models with BA malabsorption on a LFD with minimal results (142,126), and before this study there was no data examining the effect of a high fat diet in subjects with BA malabsorption. The human WD has been used in many studies to investigate the effects of the WD macronutrient profile on gene expression, metabolism and disease progression in mice (153-160). The studies described in Chapter 3, revealed that the WD, which has high lipid content, caused weight gain in ILBP null mice contrary to expectation. The open source WD contained saturated and unsaturated fatty acids, with the greatest abundance of palmitic acid and oleic acid respectively, from milk fat (163). In fact, ILBP null mice on the WD displayed the greatest degree of adiposity and obesity (mBMI: see Appendix 1). This was surprising as it is reasonable to hypothesize that subjects with fat malabsorption due to primary BA malabsorption would likely be resistant to diet-induced obesity. It appears that primary BA malabsorption is accompanied by a change in the genetic profile of the gut microbiome. BA malabsorption does not provide a protective effect on body weight when consuming a lipid-rich and sucrose-rich diet. BA malabsorption alters the microbiome and it is likely that these changes increase the energy extraction capability from carbohydrates (Chapter 3). As an example in practical application, subjects with Celiac disease may be at an increased risk of becoming overweight or obese when consuming a diet that is high in fat and refined carbohydrates. In the event that a semi-purified high fat diet that does not contain sucrose would have been used these findings would not have been revealed. Therefore, BA malabsorption has a complex effect on the gut microbiome resulting in increased capability of energy extraction from refined carbohydrates.

A major strength of this study was that all mice used in the age and sex-matched groups on both the LFD and WD were siblings. As Turnbaugh et al have demonstrated there is a resemblance between familial core gut microbiomes (143, 148, 168), therefore the conclusions drawn from the gut microbiome profiles in this study are relevant and comparable. Another strength is that the WD utilized in this study was an open source diet patterned after the macronutrient content of the diets humans ingest in western societies. This WD contained sucrose (163) exposing the effect of carbohydrates and the gut microbiome in weight gain despite bile acid malabsorption. On the contrary, a weakness of this study is that human and mouse genomes are different and therefore do not harbor all of the same gut microbiota (175). However, the gut microbiome profile analyzed was focused on broad bacterial phyla (*Bacteriodetes, Firmicutes, Actinobacteria*) that both human and mice have in common (166,168).

Nutrient harvest capability determines metabolic profile

A survey (of gut bacteria) representative of the three major phyla that make up the gut microbiota revealed that the symptoms observed in ILBP null mice on a WD such as obesity, hyperactivity, decreased food intake, hepatomegaly, pale pink livers (likely fatty) and increased hydrophobicity of the gallbladder BA was likely attributable to an alteration of the gut microbiota profile. BA malabsorption affected the composition of the gut microbiota profile, as it is bactericidal and bacteriostatic (41). Before introduction of a WD, ILBP null mice displayed different gut microbiome profiles compared to *Fabp6+/+* mice. In support of the idea that the WD will alter the gut microbiome, the diet further amplified changes in the profile of mice lacking ILBP. This suggests that subjects with BA malabsorption will display a marker for increased susceptibility to obesity; this is due to a different gut bacterial profile that favors saccharolytic fermentation of

refined carbohydrates (such as sucrose). Increased energy harvest from carbohydrates leads to greater adiposity (2) despite lipid malabsorption. The gut microbiome is recognized as a major player in overall energy metabolism because of its ability to extract nutrients based on the speciation within the gut (149). This study provides evidence in a murine model that BA malabsorption may be a disadvantage, because it alters the proportion of phyla in the gut microbiome profile in such a way that increases the potential to extract more energy. This strengthens the idea that nutrient metabolism, BA homeostasis and the gut microbiota profile are interrelated and this provides greater insight into the role these factors play in obesity.

The role of refined carbohydrate in obesity

While excess proportions of any dietary fat in the diet is unhealthy and causes weight gain, the role of excess carbohydrate consumption in obesity is also suspect (178). The purpose of using a western style diet in research is to mimic the average macronutrient distribution intake which is indicative of a diet high in lipids (likely saturated) and refined carbohydrates with high glycemic index values (150). In clarification, this study used a western style diet as an external manipulation that was formulated to meet the nutrient requirements of the murine model as well (167). For example, sugar is one of the lowest cost sources of dietary energy but also has sensory appeal making it a popular choice when the food budget is low. Lower socioeconomic status often correlates with greater rates of obesity attributed to high sucrose and lipid consumption (176). The high refined carbohydrate (high sugar) diet consumed in Westernized nations is a contributing factor to the pathophysiology of obesity and diabetes (177). In vivo studies have demonstrated that a diet rich in sugars increases adiposity (178). Increased adiposity causes the gut microbiome to become enriched with strain specific species capable of increased energy extraction and increases genes involved in carbohydrate metabolism (143) leading to body weight gain.

Public health implications

As we face the obesity epidemic with ineffective tools to date, understanding the role of the gut microbiota in this disease will bring insight to therapeutic treatments. BA malabsorption can occur in humans due to many different disease states such as: cholecystectomy, chronic pancreatitis, diabetes mellitus, ileal resection and chronic diarrhea (17, 41, 126, 177). For example, a patient who has undergone ileal resection and experiences BA malabsorption will have a modified gut microbiome profile due to BA malabsorption (among other factors). If this patient consumes a western style diet with a high refined carbohydrate content it will cause further alterations to the gut microbiome, increasing the ability of gut bacteria to extract energy from refined carbohydrates. Thus, practitioners need to be aware that the gut microbiome regulates obesity on a local (gut) level, and patients with BA malabsorption require a diet prescription that has an appropriately focused nutrient profile. Secondarily, when considering how inexpensive and convenient the westernized diet has become, a global effort needs to be taken to decrease the elitist approach to a healthy diet. Since alteration of the obesogenic gut microbiome is reversible by weight loss, it is important as researchers to highlight the role of nutrient dense food versus refined energy dense foods from not only a scientific perspective but also from an economical and public health standpoint.

Conclusions

Bile acids are important biological molecules not only in fat absorption but also in the regulation of metabolism (see chapter 1). In the case of bile acid malabsorption, exposure of the lower gut to bile acids changes the gut microbiome which can have deleterious effects on the host as previously described. The interrelationship between BA homeostasis, nutrient metabolism and the gut microbiome is complex but these interrelationships can be influenced

by dietary intervention (179). Further investigation is required on the effect of the gut microbiome on intestinal inflammation, insulin resistance and other obesity correlated disease states (180). When formulating a diet in humans with BA malabsorption, the nutrient profile needs to be closely monitored to avoid increased adiposity due to susceptibility markers in the speciation of the gut microbiome (143, 144, 146). Therefore, nutrition support is essential when formulating a diet for a client that exhibits BA dysregulation (181). Future directions include a complete genomic analysis of the gut microbiota of mice exhibiting BA malabsorption and the effects of different nutrients on metabolic profile.

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Appendix

Appendix 1 - Assessment of adiposity

Purpose: To assess adiposity in mice, a new non-invasive, convenient and cheap measure of adiposity was developed: the mouse body mass index (mBMI).

Application: To determine if mice are normal, overweight or obese in a laboratory research setting based on a validated method.

Procedure: After administration of isoflourane, the sedated mouse was scanned using a standard flatbed scanner (CanoScan Lide 20, USA) and the surface area of body was determined using the ImageJ software (Rasband, 1997). The mBMI value was calculated using he formula: mBMI = weight (grams)/(area (pixels) *length (cm). The determined area is placed into the equation and a value is generated that can determine whether a mouse is underweight, regular weight or overweight (161).

Discussion: Mathai et al. (2008), have clearly demonstrated that there is a high correlation (r-squared of 0.79-0.84) between fat mass determined by dual energy x-ray absorbitometry in comparison to measured fat pads. Therefore, measuring total fat pads is a reliable indicator of overall adiposity. To validate the correlation between total fat pads and the mBMI value (generated from the above equation), the values were plotted against each other with the r-squared value of 0.78 which is very comparable to the results by Mathai et al, indicating the mBMI is a reliable indicator of fat mass.

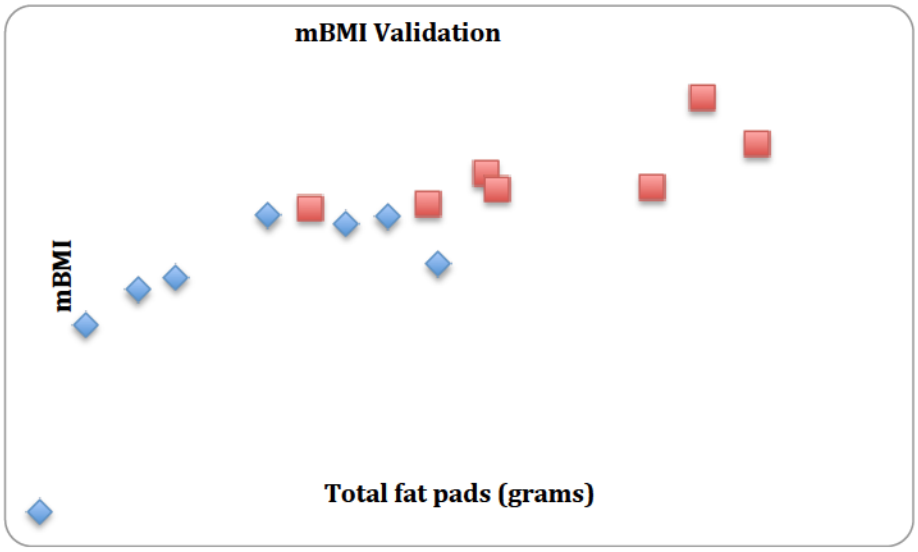

Figure 1 (Appendix 1).
mBMI Validation. On the y-axis the mBMI value was plotted against the total fat pad mass of mesentery, renal and gonadal fat pads (grams) for *Fabp6+/+* on the LFD (diamonds) and the WD (squares).

To generate an index to classify the mouse as underweight, normal or overweight/obese the cut-off weights were determined by using the normal weight range for males and females in Jax physiological data summary of C57/BLJ6 mice (Laboratory J, 2013). The normal Jax weights were identified in the mice, then by looking what their mBMIs are, we were able to translate the weights into indices, where anything below that mBMI is considered underweight and anything above that mBMI is considered overweight/obese.

Cut-offs	Underweight	Normal	Overweight	Obese (I,II,III)
Human BMI	<18.50	18.50-24.99	24.99-29.99	>30.0
MBMI (Wk16+)	<16.99F <24.99M	17.00-26.99F 25.00-34.99M	>27.00F >35.00M	

Table 1 (Appendix 1).
Description of human BMI versus mBMI for mice over 16 weeks of age. M denotes males and F denotes female.

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Appendix 2: Assay for bacteriostatic effect of bile acids

Purpose: To assess the bacteriostatic activity of bile acids, *E. coli* was chosen as a model of gut bacterium.

Application: To determine if bile acid malabsorption has the potential to alter the gut microbiota.

Procedure: Prepare *E. coli* cultures from frozen stock brought to 37° C gently vortexed and inoculated into culture tubes of 2X YT (1.6 % tryptone, 1 % yeast extract, 0.5 % NaCl) and grown for 18 h.

Taurocholic acid (TCA) and cholic acid (CA) were added to 2X YT liquid medium for final concentrations of 10 mM and 50 mM in triplicates, where *E. coli* culture was added at a 1:50 dilution. The control was prepared with 2X YT liquid media, *E. coli* and no bile acids whereas the blank only contained 2X YT liquid media. Samples were cultured at 37° C for 4 h then the optical density was read at 600 nm (DTX-880 Multimode Detector, Beckman Coulter, ON, CA).

Discussion: Increased concentration of TCA and CA (50 mM) decreased bacterial growth indicating that BA have a bacteriostatic effect. However, there was no increase in toxicity for the unconjugated BA, this could be because *E. coli* was used to as a stand alone model when in fact, the gut microbiota is very complex with a diverse population and no anaerobic species were tested.

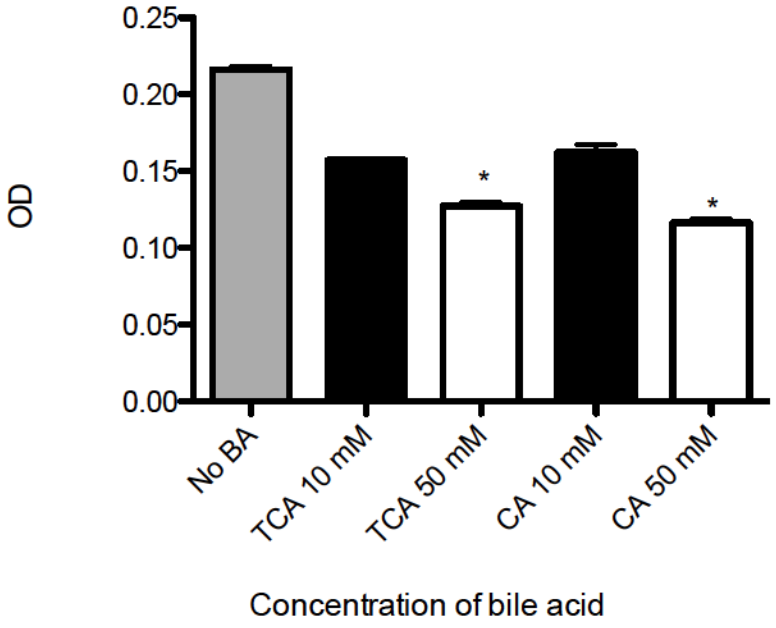

Figure 1 (Appendix 2)
Bacteriostatic effect of BA. The black bars denote the 10 mM concentration of CA and TCA and the white bars denote the 50 mM concentration of TCA and CA.