The effects of microbial metabolites on host physiology

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ABSTRACT

In recent years it has become increasingly clear that the gut microbial community, the microbiota, has a vast impact on obesity, insulin signaling and glucose homeostasis. More specifically, there are microbiota-derived metabolites that are known to possess important functions, both locally in the gut, but also on a systemic level. However, the impact they have on host physiology in terms of contributors to diet-induced obesity (DIO), effects on insulin signaling and obesity-related dysfunctions has been poorly studied. In this thesis the impact on host physiology of the microbial metabolites short-chain fatty acids (SCFA) and bile acids were studied in more detail. As study models conventionally raised mice (CONV-R), mice colonized at birth with the microorganisms present in their environment, and germ free (GF) mice, mice deprived of any microorganism and hence microbiota, were used.

In paper I, we used as study models wild-type mice and a whole-body knockout of the natural bile acid receptor farnesoid X receptor (FXR) on a CONV-R and GF background. These mice were treated with high-fat diet and the results shows that the gut microbiota promotes DIO via FXR signaling, and more importantly, that the altered bile acid profiles and hence FXR signaling affects DIO. Also, our findings suggest that the genotype is also involved in shaping the microbial composition.

In paper II, we observed a prominent difference between GF and CONV-R mice where the former had significantly higher serum levels of the incretin hormone glucagon-like peptide-1 (GLP-1) and increased colonic proglucagon expression; the gene GLP-1 is transcribed from. We demonstrated that the increased GLP-1 levels in GF mice are regulated via energy supply, namely the SCFAs. More importantly, elevated GLP-1 levels slowed intestinal transit.

From **paper I** we conclude that the microbiotas' impact on shaping the bile acid profile has significant impact on DIO and leads to obesity-related dysfunctions. In **paper II** we conclude that GF mice have slower transit time to allow sufficient energy-and nutrient absorption

Keywords: short-chain fatty acids, bile acids, FXR, GLP-1, germ free and conventionally raised.

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SAMMANFATTNING PÅ SVENSKA

Under senare år har det blivit allt tydligare att tarmbakterier, mikrobiotan, har en stor inverkan på fetma, insulinsignalering och glukoshomeostas. Mer specifikt finns det metaboliter producerade av mikrobiotan som är kända för att besitta viktiga funktioner, både lokalt i tarmen, utan även på en systemisk nivå. Däremot, den inverkan de har på värdets fysiologi i form av dietinducerad fetma, effekter på insulinsignalering och fetmarelaterade dysfunktioner, varit sämre undersökta. I denna avhandling har inverkan av de mikrobiella metaboliterna korta fettsyror samt gallsyror på värdets fysiologi studeras i mer detalj. Som studiemodeller har konventionellt uppfödda möss (conventionally raised, CONV-R), dvs möss koloniserade vid födseln med mikroorganismer som finns i deras omgivning, samt bakteriefria möss (germ free, GF) använts.

I artikel I, använde vi oss av vildtyp samt knockout-möss där den naturliga gallsyrereceptorn farnesoid X receptor, FXR, slagits ut i hela organismen. Dessa möss avlades fram på både CONV-R och GF vis. Dessa möss behandlades med fettrik kost och resultaten visar att tarmfloran främjar dietinducerad fetma via FXR signalering, och ännu viktigare, att den förändrade gallsyreprofilen och därmed FXR signaleringen påverkar dietinducerad fetma. Dessutom föreslår våra resultat att genotypen i sig påverkar sammansättningen av mikrobiotan.

I artikel II, noterade vi en framträdande skillnad mellan GF och CONV-R möss, där den förstnämnda hade signifikant högre serumnivåer av inkretinhormonet glukagon-liknande peptid-1 (GLP-1) och ökad utryck av proglukagon (genen som GLP - 1 är transkriberad från) i kolon. I denna studie kunde vi visa att de signifikanta högre nivåerna av GLP-1 i GF möss regleras via energiförsörjning, nämligen korta fettsyror. Ännu viktigare, förhöjda GLP-1-nivåer saktar ner tarmens rörelser och.

Från **artikel I** dras slutsatsen att den påverkan som mikrobiotan har på utformningen av gallsyreprofilen har en betydande inverkan på dietinducerad fetma och leder även till fetmarelaterade dysfunktioner. Från **artikel II** drar vi slutsatsen att GF möss har långsammare transporttid av föda genom tarmsystemet för att möjliggöra tillräcklig energi - och näringsupptag.

LIST OF PAPERS

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

I. Microbiota-induced obesity requires farnesoid X receptor

Ava Parséus*, Nina Sommer*, Robert Caesar, Felix Sommer, Antonio Molinaro, Marcus Ståhlman, Thomas U Greiner, Rosie Perkins and Fredrik Bäckhed.

Manuscript

** Equal contribution*

II. Microbial modulation of energy availability in the colon regulates intestinal transit

Anita Wichmann, Ava Allahyar, Thomas U. Greiner, Hubert Plovier, Gunnel Östergren Lundén, Thomas Larsson, Daniel J. Drucker, Nathalie M. Delzenne, Patrice D. Cani and Fredrik Bäckhed

Cell Host & Microbe 2013; 14, 582–590

CONTENT

ABBREVIATIONS

ASBT	Apical sodium dependent bile acid transporter
BACS	Bile acid CoA synthase
BSEP	Bile salt export pump
BSH	bile salt hydrolase
CA	Cholic acid
CDCA	chenodeoxycholic acid
CLS	crown-like structure
CONV-D	Conventionalized
CONV-R	Conventionally raised
CVD	Cardiovascular disease
DBD	DNA binding-domains
DCA	Deoxycholic acid
DPPIV	Dipeptidyl peptidase-4
FFA	Free fatty acid
Fgf15/19	Fibroblast growth factor 15/19
FXR	Farnesoid X receptor
GF	Germ free
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GPR	G-protein coupled receptor

IAP	Intestinal alkaline phosphatase
I-BABP	Ileal-bile acid binding protein
IL	Interleukin
IR	Insulin resistance
IRS	Insulin-receptor substrates
ITT	Insulin tolerance test
LBD	Ligand binding-domains
LCA	Litocholic acid
LCFA	Long-chain fatty acid
LPL	Lipoprotein lipase
MAP	Mitogen-activated protein
OGTT	Oral glucose tolerance test
PI 3-kinase	Phosphatidyl-inositol 3-kinase
PXR	Pregnane X receptor
RYGB	Roux-en-Y gastric bypass
SCFA	Short-chain fatty acid
SHP	Small heterodimer partner
T2DM	Type 2 diabetes mellitus
TCA	Taurocholic acid
TG	Triglyceride
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha

T-β-MCA	Tauroconjugated-β-muricholic acid
VDR	Vitamin D receptor
VLDL	Very-low-density lipoprotein
WT	Wild-type
α/β/ω-MCA	α/β/ω -muricholic acid

1 INTRODUCTION

1.1 The gut and the microbiota

The human body is home to a complex microbial ecosystem and particularly the gut houses the largest microbial community (the gut microbiota) (1). With a total number of approximately 10^{14} microbes, which is equivalent on 10^{11}-10^{12} cells/g colonic content with a biomass of 1 kg, gut microbiota outnumbers the somatic cells in the human body by a factor of ten (2) and its collective genome (the gut microbiome) recently have been estimated to encode for about 10 million genes (3).Through its mutualistic relationship with us the gut microbiota provide us with a range of metabolic and biochemical functions which we have not been able to evolve by ourselves. Based on its collective metabolic potential the gut microbiota affects host physiology and has been considered as an additional organ that possesses a metabolic potential equal to the liver (4).

'Normal 'gut microbiota is dominated by bacteria, but the gut is also colonized by archaea, eukaryotes and viruses (5). The viriome, consisting of bacteriophages and eukaryotic viruses, have for a long time been thought to be a minority of the microbiota. Recent studies have shown that bacteriophages are ~10 fold more abundant than bacteria and affect the environment to a larger extent than was believed. Thus, the gut microbiota is complex and there are many factors that influence it (6)

Even though the number of bacteria in the gut is so immense there are about 500-1000 species originating mainly from two phyla Firmicutes and the Bacteriodetes. The Firmicutes phyla contain Gram-positive bacteria belonging genera such as the butyrate producers *Eubacterium* and *Roseburia*, but also to *Clostridium* and *Ruminoccous*. Furthermore the Gram-negative Bacteriodetes contain genera such as *Bacteriodes* and *Prevotella*, which are more niched towards degradation of complex carbohydrates (2, 7).

The gut microbiota differs in composition and abundance across the gastrointestinal (GI) tract in 2 dimensions. The first is along the length of the GI tract where microbial density increases from stomach to the distal gut where the former has a microbiota load of 10^{1} cells per gram content, the duodenum (proximal small intestine) 10^{3} microbial cells per gram, the jejunum (mid small intestine) 10^{4} microbial cells per gram, the ileum (distal small intestine) 10^{7} microbial cells per gram and finally the colon containing 10^{12} microbial cells per gram content. The second dimension is across tissue

to lumen-axis, with more diverse and dense microbial population are found in the lumen, and less diverse but quite specific microbiota in the mucus (8).

The colonization of the gut is a complex and dynamic process that begins immediately after birth. At this stage the microbiota pattern depends largely on the mode of delivery, the vaginally delivered infant are first colonized with microbes from the mothers' birth canal, while the C-section delivered babies are colonized with skin bacteria and other bacteria from the hospital environment (9). The colonization process is associated with maturation of the infant's gut and is affected by the nutrition (breast milk or formula food) at early stage of life (9).

The gut is not a constant milieu but a very dynamic organ in terms of fluctuations in available nutrients, diet, hygiene, antibiotics and lifestyle and all these factors shape the microbiota temporarily (10, 11). However, when the faecal microbiota of 39 individuals across the world was sequenced by Sanger technology it was observed that Firmicutes and Bacteriodetes are the two dominant phyla (5). Thus, there is an evolutionary advantage for the adaption to the specific phyla that exists in all individuals regardless of geographical location, environmental effects and diet (12).

1.1.1 Microbiota and diet

Diet is a primary factor that shapes gut microbiota and is determinant in the establishment of gut microbiota composition and function from early life (13). The human milk, primary source of nutrients for the newborn, is rich in oligosaccharides (HMOs) , which promote the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (9, 14), while , formula feeding results in higher abundance of *Bacteroides, Clostridium, Enterobacter* and other facultative anaerobes (9, 15). The cessation of breast feeding has been shown recently to be an important stage in infants' microbiota development, as it leads to a maturation of the microbiome to an adults-like microbiota (9). The introduction of solid food to the infant diet leads to large shifts in the infants' microbiota composition (15, 16). Comparison by 16S rDNA sequencing of the fecal microbiota of children from Burkina Faso consuming a rural African diet with children from Italy consuming a modern Western diet showed no significant differences in the microbiota composition between the two cohorts at breastfeeding period. However, weaning resulted in increased abundance of *Prevotella* and *Xylanibacter* in the microbiota of the Burkina Faso children known to be able to degrade cellulose and xylans; major compounds the African rural diet. In the Italian children, these bacteria were absent (17).

Gut microbiota composition in humans depends largely on diet (18). Long term dietary intake of diet rich in fat and protein increase the levels of *Bacteriodes*, while of diet rich in fibers favors for higher levels of *Prevotella* (19-21). The responsiveness of gut microbiota to short term dietary change in human has been recently reported (22). In particular, short-term animal-based diet lead to increased abundance of bile-tolerant microorganism such as *Bacteriodes* and *Bilophilia*, most probably as respond to an adaption of increased bile acids influx necessary for the emulsification of the fat from the diet (22).

In mice, short term HFD feeding also result in a rapid change in gut microbiota. Mice fed HFD for three days have lower abundance in Bacteriodetes and increase in Firmicutes and Proteobacteria (23). Germ free mice, mice that are sterile and therefore lack gut microbiota, were colonized with human microbiota and fed high sugar/high fat diet (Western diet) showed switch in the functional capacity of the microbiome within 24 hours. Pathways involved in glycosaminoglycan degradation and sphingolipid metabolism that belong to Bacteriodetes, have been strongly enriched (11). Altogether, these studies confirm the immense role of nutrition in shaping gut microbiota composition and its functional maturation, from birth to adulthood.

1.1.2 Bacterial metabolites

There are a wide variety of molecules in the gut that originate from microbial metabolism of food and xenobiotics. There are also metabolites that the microbiota produces directly. The most abundant bacterial metabolite are the short-chain fatty acids (SCFA), produced from fiber fermentation, but many more exists such as secondary bile acids, vitamins, lipids and other metabolites such as ethanol and urea that are found in trace amounts (24, 25).

Dietary fibers are non-starch polysaccharides derived from plant cell-wall polysaccharides such as resistant starch, cellulose, inulin, xylan and oligosaccharides, and are important source of energy for both host- and microbial cells (26). The enzymes produced by the host are limited in amounts and unable to neither digest nor harvest energy from the dietary fibers in a greater extent and the enzymes do not possess specificity to metabolize dietary fibers to a greater extent either. The caecal and colonic microbiome contribute to genes with great capacity to ferment these indigestible polysaccharides (4) into the metabolites SCFAs, which in turn can be utilized by the host as a great energy source (14, 27).

1.1.2.1 Short-chain fatty acids

SCFAs are saturated aliphatic organic acid, such as formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methylbutyric, hexanoic and heptanoic acids; where acetic, propionic and butyric acids are most prevalent (27, 28). As the names imply, these are acids (weak acids of pK of $\leq$4.8), and the internal milieu of the GI tract has a neutral pH. Therefore the SCFAs are present as the anions acetate, propionate and butyrate, rather than free acids (27). The production rate of SCFAs depends on amount and identity of consumed fiber, transit time through the GI tract, and the microbiome (29). Furthermore, due to the variation of bacterial density along the intestine (8) and the diet, the total concentration of SCFAs decrease from 70-140 mM in the proximal colon to 20-70 mM in the distal colon (30) in a ratio of 40:40:20 (27). As the availability of substrates decline towards the end of the colon the SCFAs levels also decrease. Furthermore, the Bacteriodetes produce mainly acetate and propionate whereas Firmicutes are responsible for the vast majority of butyrate production (31). This is a mutualistic relationship where fermentation results in synthesis of gases such as CO_2 and H_2, which then is part of other bacterial pathways, e.g. the synthesis of CH_4 from CO_2 and H_2 by the Archaea (26).

More than 95% of SCFA are reabsorbed by the colonocytes in the cecum and colon, and 5% are lost in the feces. Due to the production site and reabsorption of SCFAs the opportunity to study SCFAs production *in vivo* in humans are strictly limited, since faecal SCFA levels do not represent the correct SCFA concentrations nor the rate of synthesis in the intestine. (32). However, in one study SCFA levels were measured in contents collected from different parts of the intestine from human sudden death victims. They observed that as the pH dropped from ileum to cecum (6.5-5.8) the total SCFA concentration increased to about 140 mmol/kg, where it steadily decreased to reach the rectum. More importantly this study showed that along with increase in pH from cecum to rectum there is a decrease in the SCFA levels (along with SCFA reabsorption). This shows for that fermentation takes place in the cecum and colon (33).

1.1.2.2 Bile acids

Another large groups of microbial metabolites are the bile acids (24). They are a synthesized from cholesterol in the hepatocytes and the neutral form of bile acids, the bile salts, form micelles together with bilirubin, phospholipids and cholesterol which constitute the major part of bile. Cholesterol is an important lipid that possesses further important roles in the body. It also constitutes parts of cellular walls and forms caveloes, just to mention some important functions (34).

500-600 mg of cholesterol is converted to bile acids via enzymatic processes in the liver on a daily basis. This large endogenous production of bile acids is in fact a protective mechanism against pathological accumulation of cholesterol in the liver. The nucleus of the hydrophobic cholesterol molecule consist of four fused carbon rings that are modified via oxidation, hydroxylation and conjugation to amino acids to generate bile salts (35) . Many bile acids have a carboxyl end and a hydroxyl end in a *cis*-configuration to form a hydrophilic face opposed to the other hydrophobic face; making the bile acid amphipathic with detergent properties. The concentrations of bile salts in the gallbladder are 100-1000 times higher compared with hepatocytes (36).

Bile is secreted from the hepatocytes to the gallbladder via bile canaliculi for storage. Postprandially the enteroendocrine I-cells in the duodenum (proximal part of small intestine) secrete CCK that postprandially stimulates gallbladder contraction and release of bile salts into the duodenum (36, 37). Bile acids facilitate intestinal absorption of hydrophobic diet-derived lipids, but also drugs, vitamins and steroids. The amount of bile acids fluctuate during a day throughout the intestinal tract based on the ingestion of meals. After a meal, the concentration of bile acids in liver, intestine and systemic circulation are ~5 μM to ~15 μM (38).

Synthesis. There are two pathways from which the bile acids are produced from. The first *classical pathway* is initiated by the rate limiting enzyme Cholesterol 7α-hydroxylase, denoted CYP7A1 and involves hydroxylation at carbon position 7. The reaction proceeds with further chemical reactions of the sterol ring after which it is modified by either Sterol 12α-hydroxylase (CYP8B1) to generate cholic acid (CA) or Sterol 27α-hydroxylase (CYP27A1) to generate chenodeoxycholic acid (CDCA). The *alternative pathway* is initiated by CYP27A1 and Oxysterol 7α-hydroxylase (CYP7B1) to generate CA, or are further chemically modified to generate CDCA. These two primary bile acids are the predominant bile acids in humans, but in rodents CDCA is effectively converted into β-muricholic acid (β-MCA). The primary bile acids are conjugated to amino acids in a two-step process: first Bile acid CoA synthase (BACS) adds Acetyl-CoA, and thereafter conjugate to predominantly the amino acid glycine in humans or taurine in mice (35). CYP7A1 regulates the rate of bile acid production whereas CYP8B1 regulates the CA/CDCA ratio. It is estimated that ~90% of bile acid synthesis originate from the classical pathway.

The regulation of bile acid synthesis was first discovered when it was seen that the hepatic nuclear hormone receptor Farnesoid X receptor (FXR)

induced the expression of another nuclear receptor called small heterodimer partner (SHP). SHP acts as a corepressor of the transcription of CYP7A1, leading to inhibition of CYP7A1 and hence less bile acid synthesis (39-41). However in further studies performed with *shp* deficient mice it was suggested that another unknown pathway must be involved in the bile acid negative feedback mechanism (34, 41). It was known from previous studies perfomed in rats that intraduodenal infusion of the bile acid taurocholic acid (TCA) lead to hepatic repression of the CYP7A1 gene. As FXR also is expressed in the intestine, it was natural to believe that an intestinal FXR-mediated pathway also has a role. In 2005 it was shown that intestinal FXR activation by bile acids activates intestinal fibroblast growth factor (FGF15 in mice and FGF19 in humans) that via liver FGF4 ultimately repress (until this day incomplete knowledge) CYP7A1 transcription (42). Thus, via the liver derived SHP-pathway and intestinally-derived FGF15/19 pathway homeostasis of bile acid levels are maintained.

Microbiota modulates the bile acids. Once hepatic bile acid synthesis is completed, monovalent bile acids such as tauro- and glyco-conjugated bile acid are secreted into the bile canaliculi with help of bile salt export pump (BSEP) (43). At the same time, divalent bile acids such as sulphated- or glucuronidated bile acids conjugated with glycine or taurine are secreted into the bile canaliculi with help of multidrug resistance-associated protein-2 (MRP2). The bile salt are now stored in the gallbladder, and postprandially will be secreted into the small intestine (36).

The newly synthetized conjugated primary bile acids are further modified by the gut microbiota in the distal part of small intestine and colon. The microbiota produces bile salt hydrolases (BSH) which deconjugates amino acids from the bile acids. Other microbially produced enzymes will chemically modify bile acids by performing dehydrogenation and dehydroxylation and thus generate a wide spectrum of bile acids (44). The secondary bile acids of CA and CDCA are deoxycholic acid (DCA) and litocholic acid (LCA), respectively. The primary bile acids in mice are predominantly CA, UDCA, αMCA and βMCA whereas ωMCA are the secondary bile acids (45). Altogether the effect of the microbiota on bile acid diversity creates 200 different bile acids which ensures a wide capacity for lipid solubility (37). The hydrophobicity of bile acids increase with deconjugation along with increased toxicity. In fact, the hepatocytes protect themselves by conjugating bile acids in order to prevent cell damage.

GF animals lack the BSH and other enzymes which deconjugates the primary bile acids and hence have a different bile acid pool diversity compared with

conventionally raised (CONV-R) animals; animals that are colonized with all the bacteria which are to be found in their environment. Already in 1973 it was shown that GF rats had higher bile acid concentrations in their gallbladders, hypercholesterolemia and increased intestinal cholesterol absorption (46). Researchers in our group and elsewhere have been able to show that various organs of GF mice (such as the liver, gallbladder, intestine and plasma are enriched in tauro-conjugated bile acids, specifically the very potent antagonist T-β-MCA (45, 47).

GF animals have reduced bile acid diversity but larger pool size, whereas CONV-R animals have smaller pool size but more diverse bile acids (48). Due to that the microbiota alters the bile acid composition, the bile acids are considered as microbial metabolites. These differences between the animals have vast effects on host physiology such as glucose metabolism, lipid metabolism and more, both via FXR mediated and non-FXR mediated bile acid signaling (37, 48).

Bile acid reabsorption. Bile acids are effectively reabsorbed at two levels. Firstly, most of the conjugated bile acids are actively absorbed by the ileocyte by the apical sodium dependent bile-salt transporter (ASBT) in the terminal ileum. ASBT is bound to ileal-bile acid binding protein (I-BABP which protects the cells from the deleterious effects of bile acids by transporting them out to the portal system MRP2 and MRP4 (43).Unconjugated bile acids on the other hand are passively reabsorbed in the ileocytes (36). Altogether, the bile acids will be returned back to the liver, secreted into the gallbladder and thereafter to the duodenum, transported throughout the intestinal tract, to once again be reabsorbed (37). These recycling processes is referred to the enterohepatic circulation and stands for 95% of reuptake of bile acids, and only 5% is lost through the feces. The loss of bile acids via feces is replenished by the hepatic *de novo* synthesis of bile acids (49). In this way, bile acids are reused through many cycles during a day and in fact this occurs about 10 times per day where a total of 20 g of bile acids are recycled. It is important to take into account the differences between human and mouse bile acid pool where in humans the pool consists of 40% CA, 40% CDCA and 20% DCA, whereas in mice it consists of 50% CA and 50% α-and β-MCA (49).

Bile acids regulate microbial community. Bile acids have antimicrobial properties where they are able to directly damage bacterial cell membranes (50), and indirectly prevent bacterial overgrowth via FXR and Tgr5 signaling (48). DCA possess an order of magnitude more antimicrobial property than CA, because it is very hydrophobic and act as detergent on bacterial cell

membranes (51). This control mechanism is believed to be important for maintenance of normal microbiota.

Bile acids are in other aspects important regulators of bacterial community, and alterations in bile acid diversity have in several studies shown to lead to pathological states. High fat feeding in mice have shown to lead to increased biliary secretion which reshape the microbiota, leading to a higher Firmicutes: Bacteriodetes ratio (52), and this bacterial profile has previously been seen in obese mice (53). In other studies with Cirrhosis-patients, bile acids levels was compared with bacterial communities and it was observed that in these patients there is a bacterial dysbiosis was present with a significant reduction in gram-positive bacteria linked to low bile acid levels entering the intestine. The altered bile acid pool alters the microbiota composition in these patients (51).

1.2 Obesity and the metabolic syndrome

Overweight and obesity are one of the fastest growing epidemics in our time and are roughly described in the literature as an excess accumulation of adiposity due to intake of high energy rich diet containing fat and sugar, in combination with reduced level of physical activity. The definition of overweight is according to the WHO (World Health Organization) a body mass index (BMI) between 25.0-29.9 kg/m2 and for obesity $\geq$ 30 kg/m2 (54).

Although obesity was once considered only being prevalent in high-income countries where easily accessed high energy rich food goes hand in hand with accessible transportations systems, it is now clear that obesity has also spread to low-and middle income countries. In fact, according to WHOs global statistics from 2014 more than 600 million people suffered from obesity. Thus, obesity arises from a combination of somatic, psychosocial and socioeconomical situation (54) .

Adipose tissue significantly accumulates during obesity. The adipose tissue demands increased energy intake during obesity, and thus it is easy to imagine that lowering the energy intake in these individuals should result in weight loss. However, this is known to be much more complex and far from the solution for many people (55). Obesogenic environments such as in the USA, genetic factors and the previously mentioned factors exacerbate the epidemic, and there are strong indications for that other driver behind the disease exist (56).

Obesity is one of the interconnected factors in the term 'metabolic syndrome' that is known to cause arteriosclerosis and cardiovascular disease (CVD). There are various opinions to which factors should be included in the term metabolic syndrome, however, it is most often agreed that glucose homeostasis disorders such as Type 2 diabetes mellitus (T2DM), but also glucose-and insulin intolerance, dyslipidemia and hypertension are included. Abdominal obesity and IR have gained increasing attention for also being important drivers of the syndrome (57, 58). However obesity is the factor that is growing the fasters and the severity and acute need of finding appropriate treatments against it.

1.2.1 Glucose intolerance and insulin resistance

Glucose intolerance and insulin resistance (IR) are characteristics of obesity and T2DM. Insulin and glucose are two molecules that are tightly related to the epidemic state T2DM, as their levels during fasting, pre, inter -and postprandial reveal significant information of the disease state. A combination of β-cell failure and resistance of main target tissues (skeletal muscles, adipose tissue and liver) to insulin are the main factors driving glucose intolerance and IR.

Prior to the development of T2DM, the main tissues that respond to insulin begin to lose their responsiveness to the insulin molecule. As a consequence of the IR hyperglycemia occurs, and as a compensatory mechanism, β-cells in pancreas elevate the rate of synthesis of insulin. As long as the β-cells are able to produce more insulin to maintain normal blood glucose levels the IR is maintained under controlled state. However if the IR is exacerbated with time, β-cells lose their ability to produce insulin and both hyperinsulinemia and hyperglycemia takes place, and T2DM is now a fact (59).

In the case of IR, insulin does not properly bind, or at all bind to the insulin receptor and the downstream insulin signals are disrupted. Insulin signaling is a complex picture and to properly understand the development of metabolic syndrome one need to understand the downstream pathways of this signaling cascade. Insulin does not only mediate glucose uptake but is in fact involved in synthesis and storage of fat, protein synthesis, cell growth and glucose metabolism (60).

The insulin receptor. The insulin receptor is a tetrameric kinase receptor consisting of two α and two β subunits that upon ligand-binding phosphorylate one or more of the 4 intracellular substrates of the insulin receptor, namely the insulin-receptor substrates, IRS. The IRS differ in tissue

distribution and have different downstream effects (61). However, IRS will activate phosphatidyl-inositol 3-kinase (PI 3-kinase) (62) which in turn activates the serine/threonine kinase Akt through phosphorylation. Akt will in one hand phosphorylate glycogen-synthase kinase 3 leading to increased glycogen synthesis, on the other hand activate FOXO-1, leading to decreased gluconeogenesis (63, 64). Lastly, Akt will activate translocation of glucose transporter Glut4 to the plasma membrane, mediating insulin-stimulated glucose uptake (65). Thus, under normal physiological conditions increased blood glucose levels will ultimately lead to an increased influx of glucose in to the cells, deposition of glycogen in the tissues and lower endogenous glucose production, hence maintaining normal blood glucose levels. A disruption in insulin signaling viewed from this point of the insulin pathway give rise to hyperglycemia and lower glycogen storage.

Another role of PI 3-kinase is involved in lipogenesis and specifically the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c). SREBP-1c is family of three nuclear transcription factors which are mainly expressed in liver and adipocytes and can activate the entire family of fatty acid synthases (66). Insulin has been shown to have a role in the transcription of SREBP-1c in the liver as insulin activates the transcription factor liver X receptor, LXR, which in turn regulates transcription of SREBP-1c (67).

Insulin also activates the so called mitogen-activated protein (MAP) kinase pathway. The downstream activation of insulin leads to MAP kinase activation which in turn activates transcription factors that ultimately leads to cellular proliferation and differentiation (68).

Peripheral insulin resistance. When discussing peripheral IR the common notion tends to be that all tissues become equally resistant to insulin. However this is not the case as it is known that different tissues have different basal levels of susceptibility to glucose and insulin, and in addition have different mechanistic compensatory abilities to decrease hyperinsulinemia in the case of T2DM (63). Studies on tissue specific knockouts mice have enabled us to dissect the mechanistic pathways of the IR in various organs.

Studies in liver-specific knockout mice have shown that liver-derived insulin signaling is important for maintenance of hepatic glucose homeostasis. Under normal conditions hepatic insulin stimulation suppress gluconeogenesis by 50% during a hyperinsulinemic-euglycemic clamp, and in the liver-specific knockout model this effect is lost (63). Even though glucose is not taken up in the liver in an insulin-dependent manner, insulin does block the production

and release of glucose (the latter from glycogen lysis) by the liver (69). Insulin also affects glucose metabolism in the liver indirectly through free fatty acids (FFA) generated by visceral fat. Obesity is known to be an important contributing factor behind IR as the adipocytes have reduced capacity to store FFA. Visceral fat compared with subcutaneous fat, is less sensitive to insulin and postprandially this particular fat depot produce FFAs which travel along the portal vein to reach the liver. In the liver they stimulate gluconeogenesis (70). In the case of hepatic IR, elevated fasting glucose levels and poor glucose tolerance are observed as insulin is unable to suppress gluconeogenesis (71). In case of excessive insulin action in the liver (as in the case of T2DM where plasma insulin levels are elevated), dyslipidemia and liver steatosis are the typical phenotypes.

Skeletal muscles. Skeletal muscles accounts for 70-90% of glucose uptake after an oral glucose load, making them the largest site for glucose oxidation and glycogen storage. Studies on muscle specific insulin knockout mice show that despite being completely insulin resistant they have normal blood glucose and insulin levels (72) and normal glucose tolerance (73). Interestingly, there is a three-fold increase of insulin-mediated glucose uptake in adipocytes isolated from muscle specific insulin knockout mice. In addition, the increased glucose uptake is accompanied by increase in adipose tissue mass, FFA levels and triglycerides (TG) (72). The decreased ability of glucose uptake into muscles suggests at least partly that the adipocytes compensate for this ability to maintain normal blood glucose levels.

Muscles can store up to 400g of glycogen, compared to the 75-100 g stored in the liver, showing the importance of adequate glucose uptake and storage in the myocytes (74). In T2DM patients it was seen that skeletal glycogen storage was 30% lower compared to healthy individuals (75). To continue, elevated FFA levels in the plasma impair insulin-stimulated glucose utilization through inhibition of several enzymes involved in glycolysis (76).

Adipocytes. The adipocytes are the primary site for storage of TG, and during excess intake of energy as in obesity, the excessive energy is stored in the form of TG in adipocytes either through hyperplasia (increased number of cells) or hypertrophy (increased cellular size) (77). obesity in adulthood is associated with hypertrophy and it is the enlarged fat cells that are attributed to metabolic syndrome (78). The original hypothesis that the number of adipocytes are set in childhood and remain constant during life has recently been challenged and hyperplasia occurs (79).

The rapid expansion of the adipocytes during obesity leads to the leakage of end product of lipolysis, namely FFAs, into the portal blood circulation. Once in the liver the FFAs will stimulate for gluconeogenesis, lipid synthesis as well as hepatic IR (80). FFAs also contribute to peripheral IR. However the most prominent effect of FFA is the induction of low-grade inflammation in fat; FFAs bind to toll-like receptor 4 (TLR4) and activate the innate immune system and specifically pro-inflammatory pathways (81). TLR4 activation induces the production of fat-derived cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (81) which in turn attract macrophages to the site where the latter also produces cytokines such as IL-10 and IL-12, exacerbating the pro-inflammation. The cytokines themselves have been shown to induce IR (82) However, the recruited macrophages in turn will phagocyte necrotic cell debris that exists due to the rapidly growing adipocytes (83). As the cellular debris is cleared up, the inflammatory tone goes towards anti-inflammatory and the number of macrophages decreases. In fact, chemical macrophage ablation has been linked to decreased levels of macrophages in adipose tissues in diet-induced obesity and improves insulin sensitivity (84).

β-cells. As all the above scenarios occur during obesity, the elevated glucose levels in the blood need to become decreased, and this is performed by the insulin producing cells, the β-cells, which is one of the cellular components of the islet of Langerhans. (85). The β-cells will at first overcome this by hyperplasia and/or increase in the insulin production. As the demand for insulin is elevated and the peripheral organs are more and more insulin resistant, the β-cells reach a threshold where they cannot cope with the demand and they become dysfunctional, and even terminally apoptosis takes place.

1.2.2 Adipocyte inflammation

Adipocyte inflammation is a common feature for the metabolic syndrome and is known to be one of the early signs prior to the onset of e.g. T2DM (and most prominently during the disease state) (86). Both the innate and adaptive immunity is involved in the development of adipocyte low-grade inflammation, and these will be addressed in this section.

The innate immune system is also called the nonspecific immune system, and is the so called first line of defense against pathogens. It recognizes foreign molecules and pathogens such as cellular debris and bacteria (to mention a few) and provide immediate defense by recruitment of e.g. macrophages (87). The so called anti-inflammatory macrophages, M2, are normally present in

the adipose tissue, even in the case of leanness. As the number and specifically the size of adipocytes increase, a phenotypic shift from M2 to anti-inflammatory macrophages (M1) takes place (88). In 2003 it was clearly demonstrated in two studies that macrophages do infiltrate hypertrophic adipocyte tissue (89, 90).

In attempt to elucidate how this recruitment of M1 macrophages takes place, several suggestions have been made. The FFA-mediated TLR4 activation is one pathway, and was covered in previous section (see 1.2.4). Hypoxia has been shown to also be a contributor, as the researches detected that expanding adipocytes lead to local hypoxia, and that the hypoxia itself lead to upregulation of cytokines, adiponectin and plasminogen activator inhibitor type-1 (91, 92). In another study it was shown that M1 macrophages are most densely localized in necrotic tissue from rapidly expanding adipocytes, and form crown-like structures (CLS) that are visible as round circles when stained (93). To continue the FFA-activated TLR4 pathway is also an important pathway, and was covered in previous section.

The adaptive immune system is the part of the immune system that is viewed as having immunological memory where antibodies are produced against specific pathogens. Unlike the innate immune system that has generic immunity, the adaptive immune system 'remembers' foreign antigens by boosting production of antibodies in re-contact with the same antigen. The distribution and density of cells from the innate and adaptive immune system differ in localization and lean/obese state.

In the visceral fat there are more M1 macrophages present compared with the subcutaneous fat (94). Lymphocytes derived from both innate and adaptive immune system are present in both visceral and subcutaneous fat, however in the visceral fat there are more lymphocytes from the innate immune system compared with the subcutaneous, where the latter has more cells from the adaptive immune system (95). The T_H2 and the T_{REG} cells of the adaptive immune system creates an anti-inflammatory tone in the adipose tissue by secreting the cytokines IL-4, IL-10 and IL-13 so M2 macrophages are recruited to the site (96, 97). T_H1 is a pro-inflammatory lymphocyte and the levels of it increase during obesity. The ratios of T_H1: T_{REG} and T_H1: T_H2 increases and gives a shift towards M1 macrophage recruitment (98).

Recently it was shown that the ileum microbiota after HFD is responsible behind the impairment of the immune system. Specifically, the researchers were able to see that the HFD changes the microbiota composition which in turn regulates differentiation of Th17 cells of the mucosal lining. This

disruption in immune system due to altered microbiota ultimately leads to systemic low-grade inflammation (99).

1.2.3 Liver steatosis

Under normal physiological state TG will become hydrolyzed in the intestinal lumen to render free FFA and 2-monoacylglycerols (MAG), which are absorbed by enterocytes through specific transporters such as CD36. Simultaneously, cholesterol is taken up by the enterocytes where they will be transformed into cholesterol-esters. Once in the enterocytes, FFA and MAG will be reassembled into TG, and together with cholesterol-esters are packed into chylomicrons to enter the blood circulation (100). The chylomicrons travel to the liver where they will together with endogenously produced TG become packed into very-low density lipoproteins (VLDL).

Chylomicrons and VLDL will transport TG to the peripheral organs, in particular organs where lipoprotein lipase (LPL) is strongly expressed (such as heart, skeletal muscles and adipose tissues). LPL will generate FFA, which will be absorbed by the cells and used as energy source. In obesity, LPL action is lower than in lean individuals, which in fact leaves more TG not lypolyzed and a higher deposition of these lipoproteins will ultimately be taken up by the liver (101). Insulin is an important regulator of LPL activity and fuel storage where it has been shown that postprandial rise in insulin will inhibit intracellular lipases in adipocytes, hence lowering the amounts of intracellular lipids. Insulin has also been shown to be able to mobilize FFA from adipose tissue to critical tissues such as heart muscles (102). Therefore, abruptions in insulin levels such as in the case of IR, has obviously negative effects of the maintenance of normal blood lipid levels and lipid deposition into organs.

Liver steatosis takes place in the liver when there is an abnormal load of TG in the hepatocytes, and obesity most often is accompanied with this kind of hepatic TG accumulation. TG accumulates either through increased influx into the hepatocytes or by increased *de novo* synthesis of lipids, or through a combination of the two (103). Prolonged liver steatosis leads to a condition called nonalcoholic steatohepatitis (NASH), which is inflammation and fibrosis of the liver (104). Steatosis and NASH together make up the most common chronic liver disease there is today, namely nonalcoholic fatty liver disease (NAFLD), and its incidence rises with obesity (105).

Hyperinsulinemia caused by IR as a consequence of obesity, increases *de novo* hepatic lipogenesis, decreases hepatic VLDL secretion, decrease fatty

acid oxidation and increase the FFA release from adipocytes due to increased lipolysis (105). The increased FFAs will increase the FFA influx into the liver, further contributing to the steatosis, but more importantly lead to reduced hepatic insulin clearance and ultimately to prolonged elevated circulating insulin levels. Furthermore, the FFA promote endogenous hepatic production of glucose and TG, worsening the already IR state that exists (106).

1.2.4 Pancreas and diabetes

The amount of insulin that is released from β-cells under normal physiological state is carefully regulated to maintain normal blood glucose levels. In a pre-diabetic state, insulin sensitivity might have decreased, but as long as the β-cells are functional this impairment can be compensated. However in prolonged disease state this regulatory mechanism decreases and hyperglycemia occurs. In a non-diabetic patient there are fewer and smaller Islet of Langerhans compared with the T2DM patient, where one would see larger and increased number of islets. This is believed to be a part of the compensatory mechanism for the impaired insulin secretion that takes place in T2DM (107).

Another part of β-cells dysfunction due to T2DM is related to the insulin biosynthesis process. In normal state, the precursor of insulin, namely proinsulin, is cleaved into insulin and C-peptide. In the T2DM individual, there is a significant increased proportion of proinsulin even after release from the β-cells. This means that the decreased ability of enzymatic cleavage of proinsulin leads to more inactive insulin and ultimately another factor contributing to hyperglycemia (108).

1.3 Microbiota and the metabolic syndrome

1.3.1 Microbiota and obesity

GF mice are protected against DIO. The first study that showed for the important role of the gut microbiota in the development of obesity was performed in GF and CONV-R mice, where the different groups were fed the same diet but the GF mice were protected against DIO. Furthermore, it was also shown that the GF animals consumed more diet compared with the CONV-R (109). In a further mechanistic study, unfractionated microbiota from CONV-R mice was transplanted to GF wild-type (WT) (making them conventionalized, CONV-D) and thereafter treated with HFD. The result

showed that GF mice were protected against DIO compared with the CONV-D mice (110).

The gut microbiota encodes for a much larger microbiome than the host, roughly an additional 10 million genes which enables us to possess many vital functions which we otherwise would not have had (3). The microbiome encodes for lipases, hydrolases and other enzymes which enables digestion of complex carbohydrates and hence utilization of energy that would otherwise be indigestible to us (12). Considering the important role of the microbiota in metabolism of macronutrients and energy harvest, much research has tried to establish a link between gut microbiota and obesity. One novel and important way to approach this is the usage of GF animals. The GF animal is housed under such conditions which prevents it from bacterial colonization and makes it an excellent model to study the impact of gut microbiota on host physiology.

The presence of AMP-activated protein kinase, or p-AMPK, in skeletal muscle is an indicator for energy deprived state and acts for supressing ATP consuming activities. GF skeletal muscles have been shown to have approximately 40% higher p-AMPK levels compared to CONV-D counterparts. The p-AMPK shuts down energy consuming activities such as fatty acid deposition into adipocytes and fatty acid oxidation. This may partly contribute to their lean phenotype. Furthermore Angiopoietin-like 4 (Angpl4) is expressed in the intestinal epithelium and is an inhibitor of lipoprotein LPL, the enzyme that hydrolyses TG in lipoproteins. Microbiota supresses Angpl4 expression leading to high LPL activity and TG incorporation in adipocytes contributing to their obese phenotype (110, 111). These primary studies show that the microbiota affect basic phenotype in the host in terms of adiposity.

Human studies on obesity. Twin studies have also been a great tool for studying the effects of microbiota on the development of obesity. Studies on obese and lean monozygotic twin pairs and their mothers show that geographical separation of monozygotic twins does not result in different microbial profiles and the microbiome is shared amongst family members. Thus, host genotype shapes the microbial profile. It was also seen that obesity is linked to reduced bacterial diversity and that the diversity is conserved between twins. High energy input as in the case of obesity reduces the size of microbial niches and the microbiota becomes less diverse (112). Other twin studies have shown that GF mice colonized with microbiota donated from obese and lean twin pairs not only reproduces the obese and lean phenotype

in the recipients, the lean twin pair microbiota in the recipients possess better capacity to ferment non-digestible polysaccharides (113).

Roux-en-Y gastric bypass (RYGB) surgery is an effective treatment against obesity and T2DM and has a success rate of 65-75% weight loss. The proximal small intestine is connected to the esophagus, totally disconnecting the stomach from the GI tract. After RYGB there is a conserved shift in the microbiota which is preserved in humans, rats and mice and independent of diet, showing that the adaption of the microbiota is not randomized. Furthermore transplantation of microbiota from RYGB operated mice and humans into GF recipients protects against obesity, showing that the shift in the microbiota after the surgery protects against obesity (114).

Mouse studies on obesity. The microbiota composition from genetically obese (*ob/ob)* and WT mice on a polysaccharide rich diet nicely showed that obesity does affect the diversity of the microbiota. A 50% increase in the bacterial phylum Firmicutes and a similar reduction in Bacteriodetes were observed in the cecum of *ob/ob* mice compared to their WT counterparts (115), however these particular experiments have not been reproducible to a great extent. In another study the effect of high-fat diet on microbiota composition was studied, and showed that levels of *Eubacterium rectale, Bifidobacteria* and *Bacteriodes* were significantly decreased after the administration of the diet (116) Transplantation studies have shown that transplantation of 'obese' microbiota into GF recipients results in increased body fat compared to 'lean' microbiota transplanted mice, even though the two groups consume equal amount of diet. These studies demonstrated that the gut microbiota can directly transmit the obese phenotype. The obese microbiota recipient lost less energy in their feces compared to the lean microbiota recipients, also indicating for better energy harvesting capacity by the microbiota (111, 117).

In one study GF mice were weaned onto a standard chow diet and colonized with unfractioned microbiota and thereafter given high sugar/high fat Western diet treatment for four weeks. The Western diet treated mice gained significantly more weight compared to the chow fed mice. They also did observe, as in the *ob/ob* study, an overrepresentation of Firmicutes vs Bacteriodetes in the obese mice, however there was an overbloom of the class *Mollicutes* (belonging to Firmicutes) (117). Bacteria belonging to Firmicutes contain genes which are more specialized for lipid- and carbohydrate metabolism compared to Bacteriodetes, whereas *Mollicutes* contain genes which facilitate import and metabolism of simple sugars. This shift in the flora is an adaption to the Western diet in order for the host to utilize

polysaccharide- and fat rich diet, to produce SCFA which in the end will be deposited into adipocytes through the liver (117, 118). Furthermore, to test whether shift in the diet affect the microbiota in obesity CONV-R mice were weaned into Western diet causing obesity and thereafter weaned onto diets with restricted calories. Mice switched onto the diets with restricted calories gained less weight and had less adiposity than those maintained on Western diet. More importantly with weight stabilization followed a significant reduction of the *Mollicutes* and increase in Bacteriodetes (117).

These studies altogether emphasize the importance to consider the gut microbiota as a metabolically active 'organ' which plays a great role in obesity.

1.3.2 Microbiota and diabetes

Diabetes mellitus is one of the most common chronic western-world disorders associated with unhealthy lifestyle such as physical inactivity, poor diet and more (119). The disorder is classified into two categories, namely T1DM and T2DM. In both cases there are impairment in insulin secretion from the insulin producing β-cells or to an impaired peripheral sensitivity to insulin (120) . However that cause of impairment are different.

T1DM is an autoimmune disease where the immune system of genetically susceptible individuals can through external factors such as environmental or infectious agent get triggered to react against the β-cells, hence damaging the insulin production (107). T1DM normally occurs in children and the individuals are not always obese.

The pathophysiology of T2DM is more complex in the way that the onset of the disease can take years and several factors (as mentioned previously) are involved (119). In this disease, the β-cells lose the ability to secrete sufficient amounts of insulin, but the peripheral organs have also lost the sensitivity to insulin. Many obese individuals suffer to some degree of IR, but it is not before pancreas loses its ability to produce enough insulin to overcome the lost insulin sensitivity when T2DM develops. T2DM is also often associated with impaired secretion of incretins as well as presence of low-grade inflammation in tissues such as adipose tissue, liver and muscles (121-123). The individual suffers from fasting glycaemia (124) but also suffers from pre- and postprandial hyperglycemia. IR will lead to elevated FFA levels in the plasma (125) that decrease glucose uptake in skeletal muscles, and as a compensatory mechanism, the liver increases the rate of neogenesis of

glucose. The system will be overloaded with glucose and the IR worsened (126).

The clinical management of obesity-derived metabolic syndrome is today partly focused on addressing dietary- and exercise habits. It has been seen that even under controlled food intake and energy expenditure in subjects with obesity, the weight loss appears not to occur rapidly, indicating for an underlying compensatory mechanism. The gut microbiota has shown to play a significant role in the development of obesity as discussed earlier. The effects of obesity on the development of T2DM, glucose-and insulin intolerance and dyslipidemia will be brought up in the following section.

In a comprehensive human study performed in Chinese and Swedish subjects showed that human T2DM was related to a dysbiotic microbiota (127). Shotgun metagenomics also allows for investigation of functions that are altered in patients with T2DM. Shotgun sequencing of microbial content of T2DM patients showed that these patients had dysbiosis in their microbiota with an increase in opportunistic pathogens and microbes conferring oxidative stress (128). Furthermore, sequencing of faecal material from European woman with diabetes showed for functional changes in the metagenomes (129).

1.3.3 Metabolic endotoxemia

Evolving evidence is demonstrating the important role of gut barrier functions and the development of T2DM. As the microbiota is altered with obesity there is also an altered metabolite profile. The obesogenic metabolites have been shown to downregulate expression of two tight-junction genes the occluding and zonula occludens 1. This altered disruption in host-microbiota symbiotic relationship leads to a leaky intestinal wall (130, 131). One important feature in an intact gut barrier is functional expression of intestinal alkaline phosphatase (IAP). This enzyme will detoxify lipopolysaccharides (LPS), the pro-inflammatory endotoxin found in the outer cell membrane of Gram-negative bacteria (132). The expression of IAP has also been shown to be regulated by the gut microbiota, and an obesogenic microbiota downregulate the expression of IAP (116, 133). The combination of lower IAP expression and leaky intestine eventually leads to a condition referred to metabolic endotoxaemia, i.e. increased plasma LPS levels. (116, 134). The presence of live bacteria has also been detected in tissues in subjects with obesity followed by diabetes (135, 136).

Circulating plasma LPS activates the innate immune system, where they specifically bind to TLR4 present on macrophages. TLR4 activation leads to pro-inflammatory cytokine production such as the IL-6, IL-1 and TNF-α (137). These cytokines induce low-grade inflammation in the body, and it has been shown that the cytokines have negative effect on insulin sensitivity and impair glucose-induced insulin secretion hence the pancreatic β-cells function (138). The inflammation ultimately leads to insulin IR and diabetes (139) (140). Thus, in conclusion, it is important to not neglect the microbiota as an important route for development of diabetes upon obesogenic state.

1.4 The enteroendocrine cells

In experiments performed in the 1960 it was demonstrated that equal amounts of glucose given either orally or intravenously generated higher insulin responses when administrated orally (141). This clear difference in insulin response gave rise to speculations that intestinal-derived factors must exist, and ultimately in 1979 the concept of incretins and the incretin effect was coined (142).

An incretin hormone is a hormone secreted by the gut upon nutrient stimulation, which will lead to insulin secretion from the pancreatic β-cells (143). There are specialized cells in the gut called the enteroendocrine cells, and these cells comprise about 1% of the total cell population (144). The enteroendocrine cells secrete various peptides that regulate appetite but also have a role in the digestive system. There are several types of enteroendocrine cells but two of them, the K and L cells secrete the incretins. Besides the K-and L-cells there is also the I-cells that secrete cholecystokinin (CCK). Postprandially, CCK delays gastric emptying, increases satiety and also release bile juices from the gallbladder (144).

The first incretin hormone that was discovered was the gastric inhibitory peptide (GIP), a hormone secreted by the enteroendocrine K cells, and these cells are predominantly found in duodenum (145, 146). It is secreted upon carbohydrate and lipid stimulation, and it secretes insulin from the pancreatic β-cells, and facilitates fatty acid uptake and lipogenesis in the adipocytes (143, 147).

The next incretin hormone discovered was the glucagon-like peptide-1 (GLP-1) secreted by the L-cells. L cells are located in the ileum, colon and to some extent the rectum (148). As the name implies this hormone is structurally similar to glucagon, and in fact, GLP-1, GLP-2 (another hormone produced by the L cells, known to promote mucosal repair) and glucagon are all

transcribed from the same precursor gene, namely the pre-proglucagon (149). This gene is expressed in the L cells, pancreatic α-cells and in the brain (146). Posttranslational modification of pre-proglucagon yields the *proglucagon* gene which will terminally be translated into GLP-1 and GLP-2 (but also glicentin, oxyntomodulin and glucagon).

Another hormone that is secreted postprandially by the L cells (but not from the *proglucagon* gene) is the polypeptide YY (PYY). This hormone inhibits gastric emptying, slows gastric acid release (150), reduces intestinal motility and acts upon ion channel in the colon so water is retained (144).

Altogether, it is clear that the gut acts as an important endocrine organ that has significant impact on host glucose metabolism, and other digestive function.

1.4.1 Glucagon like peptide-1

GLP-1 does besides stimulate for insulin secretion, also suppress glucagon secretion, reduce GI motility, reduce food intake, inhibit further secretion of digestive enzymes into the intestinal lumen, and gives rise to a feeling of satiety. (151, 152). The release of GLP-1 from L cells are stimulated by simple-and complex carbohydrates, amino acids and long-chain fatty acids (LCFA) (153). It is the intake of carbohydrates that gives rise to earlier elevated levels of plasma GLP-1, and is it believed that this is due to stimulation of sweet taste receptors signaling via neurons to intestinal L cells to ultimately release GLP-1 (154).

As soon as GLP-1 is synthetized, it has approximately a half-life of 30s, this due to the enzyme dipeptidyl peptidase-4 (DPPIV) that removes the 2 first amino acids of GLP-1, hence inactivating it. DPPIV is produced in high amounts by the intestinal epithelial cells, and it is estimated that about 10% of biologically active GLP-1 actually reaches the portal circulation (146). It is suggested that by keeping the half time low, GLP-1 will have a local role. However, it is not known whether the rapid degradation of GLP-1 is to suppress excessive action of GLP-1.

The GLP-1 receptor is a G-protein coupled receptor (GPR) (155) that is expressed in various tissues such as the heart, pancreatic islets, kidney, stomach, brain and GI tract (152, 156). Once GLP-1 is secreted by the L-cells, the small amount of active GLP-1 that is secreted into the bloodstream has both direct and indirect affect. GLP-1 binds the corresponding receptor on the pancreatic α-cells where it decreases glucagon secretion (157) and to the β-cells where it stimulates insulin secretion (158). Furthermore, GLP-1

also binds to GLP-1 receptors on the enteric nervous system, and via the vagus nerve signal to the hypothalamus for further production of GLP-1. This will ultimately lead to several metabolic downstream effects (via the autonomic nervous system), such as reduced food intake, slower gastric emptying, higher blood pressure, increased hepatic glucose-and lipid uptake, and more (158).

1.4.1.1 Short chain fatty acids as potent L cell stimulators

There has been research done to investigate the mechanisms behind GLP-1 release. There are several potent stimulators which are known to be bound to different receptors all expressed on the surface of the L cells, with the downstream effect of GLP-1 release. However, as with all receptors there are different potencies and hence different effects occuring upon specific ligands binding, and some of these ligands will be addressed in this section.

Glucose, amino acids and LCFA have all been best characterized as potent GLP-1 stimulators (159). Several *in vitro* experiments performed on both GLUTag cell line (a commonly used cell line) and explants, have demonstrated the involvement of various uniporter glucose transporters, the GLUT transporters, in sugar-stimulated GLP-1 release (152). To continue, although proteins are considered as weak stimulators of GLP-1 release, it has also been shown *in vitro* that certain amino acids such as alanine and glutamine depolarize the plasma membrane and ultimately lead to GLP-1 release (160). Also LCFA, with different range of saturation, have been shown to lead to GLP-1 release *in vitro* via atypical protein kinase C activation (160). Furthermore, the GPR120 (gene FFAR4) has *in vivo* been shown to be expressed at the largest extent in colon (where the largest density of L cells are to be found) of both mice and man, but is also expressed in ileum, cecum and rectum. GPR120 is activated by LCFA and lead to GLP-1 release (161).

Although these stimulators are per se effective for GLP-1 release from the L cells, in physiological conditions however, these compounds do not reach the colon in large amounts, at least likely not sufficient levels to act as potent stimulators of L cells (162). SCFA on the other hand have been found in large quantities in the colon, as they are derived from bacterial fermentation of fibers. The intraluminal physiological concentrations of SCFAs in cecum is totally 140 mM with 80 mM acetate, 40 mM propionate and 20 mM butyrate (163). Thus SCFA travel along the entire intestinal tract, and they are also known to be potent GLP-1 stimulators (159).

In 2007, one group of researchers were able to show in primary mouse L cell line that upon stimulation with SCFA, they did not only see increased GLP-1 protein levels, but also upregulation of the GPR41 (gene FFAR3) and 43 (gene FFAR2). They were also able to show in *Gpr41-/-* and *Gpr43 -/-* colonic explants that these mice lacked SCFA regulated GLP-1 release (159). Thus, SCFA have significant effects on GLP-1 release.

In 2008, another group was able to show that supplementation of dietary resistant starch, which is a fermentable fiber that is fermented into SCFA, leads to higher SCFA in the distal intestine, higher gene expression of proglucagon and PYY, and as a consequence of the elevated incretins, improved glucose tolerance (164). Recently a group took interest in the investigation of the role of propionate in colonic GLP-1 release. Propionate is the most potent ligand for GPR43, and in contrast to acetate and butyrate it is not cross-metabolized by the microbiota and is thus the end product. By using colonic explants from *Gpr43-/-* mice they were able to show that propionate have a significant impact on GLP-1 (and PYY- release. To continue, they were also able to show with intra-colonic infusion of propionate that GLP-1 and PYY levels were significantly elevated (165)

1.4.1.2 GLP-1 affects gastric emptying and intestinal transit

Gastric emptying is a highly regulated process from which proximal and distal parts of the stomach interplay to release the food and liquid into the small intestine, and it is the nutrient content of the food that regulates the rate of gastric emptying. Food with elevated concentrations of fat slows the rate of emptying to a greater extent than food that contains carbohydrates. Peristaltic and the so called interdigestive motility are the mechanical motilities of the stomach and the small intestine, which help to degrade the food and transport it further distally in the colon.

Enteroendocrine-derived hormones, specifically PYY, has had an established role as an important regulator of satiety as the hormone inhibits gastric emptying, slows gastric acid release and reduces intestinal motility (144, 150). Even though GLP-1 is produced and released much more distally to the stomach, its role in regulation of the gastric emptying rate is has gained more and more attention (166). The conventional view on glucose regulation after a meal is narrowed down to insulin- and glucagon secretion, hepatic glucose production and the rate to which glucose is disposed into the tissues. However, this view has in recent years been shifted towards the effects of GLP-1, as the effects of GLP-1 on gastric motility and rate of emptying and

hence the effects on glucose tolerance has been seen to be more important than believed (167).

In one study subjects were subjected to an oral glucose tolerance test (OGTT) to measure the relationship between the rate of gastric emptying and GLP-1 levels, it was shown that minor alterations increases in gastric emptying rate gave rise to postprandial glycaemia. Furthermore, they were also able to show that increased gastric emptying rate was correlated with higher GLP-1 levels (168). The GLP-1 receptor is known to be expressed in various tissues, and it is also known to be expressed on the parietal cells in the stomach (152). Several human studies has shown that GLP-1 stimulation of the parietal cells inhibits gastrin-induced acid secretion significantly (169).

I one study, the researchers studied the direct effect of GLP-1 on solid gastric emptying (and other physiological effects), and the subjects were injected with GLP-1 intravenously. The results showed that intravenously injected GLP-1 has a significant and profound effect on solid gastric emptying (170). Similar observations have been seen in other studies (171).

Slowing gastric emptying with GLP-1 and its good effects on glycaemia and T2DM is the reason for which GLP-1 receptor agonists exist (172).

1.5 Nuclear hormone receptors and FXR

Nuclear hormone receptors are transcription factors that are modulated through ligands that are of the steroid, thyroid and retinoid kind (173). Nuclear hormone receptors, as the name implies, are receptors present in the cellular nucleus, and there are different kinds of receptors expressed in various tissues (174).

Nuclear hormone receptors possess specific DNA and ligand binding-domains (DBD and LBD, respectively). Bile acids are the natural ligands of a FXR. FXR is expressed in tissues that interact with bile acids in high degree, such as liver and the distal small intestine (174). Upon binding of bile acids to the LBD, a conformational change take place and the recruitment of a second coactivator takes place, in this case retinoid X receptor (RXR) (175). The FXR-RXR heterodimeric complex will now bind the hormone response element on the target gene via DBD. The transcription of the target gene can now take place.

1.5.1 Bile acids are natural ligands of FXR

The potency of FXR activation differs between bile acids, as the hydrophobic/hydrophilic profile affects the ligand affinity (37). Both conjugated and unconjugated bile acids are natural ligands of FXR, and CDCA (mostly occurring in humans) is known to be the most potent natural agonist of FXR (34, 176, 177), followed by LCA, DCA and CA (48). In a study performed in our group recently showed that T-βMCA is a strong antagonist of FXR that is significantly overrepresented in GF mice leading to lower suppression of CYP7A1, ultimately leading to higher rate of bile acid synthesis (45). This does explain the phenotype of increased bile acid concentrations in different organs, specifically the gallbladder (45-47). In parallel, another study showed that the ratio of MCA/CA in bile of GF mice is higher compared with CONV-R mice, and because MCA is a poor FXR agonist and hydrophilic, this leads to reduced FGF15 expression and reduced CYP7A1 expression (178); the same phenotype observed in Islam's paper (45).

Bile acids are known to be strong and potent ligands of 2 other nuclear receptors. These are the pregnane X receptor (PXR) (179) and vitamin D receptor (VDR) (180). LCA is a potent ligand of these receptors (179, 180). PXR is expressed in tissues such as intestine and liver, hence tissues that are more exposed to bile acids (174), whereas VDR is ubiquitously expressed.

There are many target genes that are FXR-regulated and that are involved in not only bile acid synthesis, but also glucose, TG and cholesterol metabolism. Regarding TG metabolism, SREBP-1 is involved in lipogenesis and is repressed as SHP is induced. Also, ApoC-II, ChREBP and PPARα are some examples of genes related to TG metabolism that are FXR regulated (37, 48). To continue, the expressions of BACS and BAT (bile acid-CoA:amino acid N-acyltransferase), two enzymes involved in conjugation of primary bile acids to amino acids are positively regulated with FXR activation. The expression of several bile acid transport proteins, such as I-BABP, MRPs, Bile-salt export pump (BSEP) are also positively expressed by FXR (37, 48). In terms of glucose metabolism PEPCK, G6Pase, and insulin are some examples of genes that are FXR-regulated (partially) and are involved in gluconeogenesis (37, 48).

One important receptor that is bile acid activated is the G-protein-coupled bile acid receptor Tgr5. It is expressed in various tissues and possess various functions. It is expressed in gallbladder, intestine, muscle, brown-adipose tissue and macrophages. Both conjugated and unconjugated bile acids are

potent ligands, and TLCA is known to be the most potent ligand followed by TDCA and TCA. Tgr5 is expressed along the entire intestinal tract with the higher expression in ileum and colon (181).Tgr5 activation is known to induce GLP-1 secretion, and because GLP-1 improves glucose metabolism, Tgr5 agonist are a potential drug against impaired glucose metabolism (182).

1.5.2 FXR and metabolism

The role of FXR in the development of obesity and glucose metabolism has been for some time under investigation. Even though data has to some extent been contrary, recent publications have presented robust and reliable data regarding FXR and metabolic parameters which will be addressed in this section.

In a study performed in 2011, whole-body FXR knockout mice and control counterparts were phenotyped for diet-induced obesity (DIO) and were given HFD for 20 weeks. The results showed that FXR deficiency protects against DIO followed by lower glucose- and insulin levels and improved IPGTT (183). As FXR is expressed in intestine, kidneys and liver (174) the investigation for which tissue/tissues that are responsible for FXR-derived impairment of DIO and glucose metabolism proceeded.

Tempol is an antioxidant that was reported to prevent obesity in mice (184), and later it was shown through metabolomics that the microbiota-derived metabolites was altered in tempol-administered mice and also the composition of the gut microbiota (185, 186). Thus, tempol-treatment alters the bacterial composition and hence the metabolite-profile in the gut which through different mechanisms helps in prevention of DIO. To continue, HFD itself is also known to modulate the microbiota where higher abundance of Firmicutes to Bacteriodetes develops (111, 117). In a recent study intestinal specific FXR knockout mice were given HFD and tempol in order to study wheter or not the shift in microbiota during HFD affects DIO, but also to detect if it is the intestinally derived FXR that is important. The results from bile acid analysis showed that T-β-MCA was significantly higher (approximately 9 times higher) in the intestine of Tempol-treated mice vs. control counterparts. Also, they could see that tempol-and HFD treatment of intestinal specific FXR knockout and WT mice protected against DIO, improved OGT and insulin tolerance test (ITT) in the latter. The conclusion is that the increased levels of T-β-MCA antagonize FXR in the WT and not in the intestinal knockouts, giving rise to the different phenotypes (186).

In a similar experiment as the Li study 2013 researchers wanted to investigate if intestinally-derived FXR is important for the development of NAFLD and went on in s similar manner by administrating tempol and HFD to intestinal specific FXR knockout and WT mice. In the first part of their study they showed that WT mice administered tempol vs control vehicle had less TG accumulation in their livers. To elucidate if intestinally expressed FXR is important for the development of NAFLD intestinal FXR knockout and WT mice were treated with HFD or chow diet. The results showed significantly fewer lipid droplets in the livers of the knockout vs the WT mice. This study shows that inhibition of intestinal FXR, e.g. via T-β-MCA mediates NAFLD via the microbiota (187).

Other studies have shown the importance of inhibiting FXR to prevent HFD-induced NAFLD. Experiments performed on CYPA1-tg mice, mice that overexpress CYP7A1 and hence overproduce bile acids and in particular T-β-MCA, show that they are more protected against developing NAFLD and have improved IR (188). This study can be compared to the studies where FXR is deleted or higher levels of T-β-MCA antagonize FXR.

Chronic administration of the FXR agonist GW4046 has shown to cause more weight gain than mice administered HFD (189), and other studies using FXR deficient mice on HFD have shown that FXR attenuates weight gain, OGT and IT (183). The molecular mechanisms to why this occurs are poorly understood but the phenotype is clear. Regarding glucose metabolism, FXR mediates GLP-1 release via TGR5 signaling (182) which is contrary to data showing that FXR in fact attenuates glucose tolerance and insulin sensitivity. However, as FXR has many target genes, one can not neglect the fact that there may be other mechanism which take over and hence give rise to the phenotype.

2 AIM

The aim of this thesis was to elucidate how the two most common microbial derived metabolites short-chain fatty acids and bile acids affect host physiology.

I. Microbiota-induced obesity requires farnesoid X receptor

In this paper the specific aim was

- ✓ to investigate if the microbiota affects FXR signaling and if it affects the development of obesity.
- ✓ to investigate how the obesity-associated phenotypes manifest.

II. Microbial modulation of energy availability in the colon regulates intestinal transit

- ✓ to test the hypothesis that the gut microbiota inhibits proliferation and/or differentiation of intestinal L cells.
- ✓ to determine the mechanism by which the gut microbiota regulates expression of GLP-1.
- ✓ to test the hypothesis that germ free mice exhibit increased insulin sensitivity due to increased GLP-1 signaling.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Animal models

In paper I and II two different mouse strains were used, namely Swiss Webster's and C57Bl/6J. These two strains of mice were used in GF and CONV-R state and also with different knockout models.

3.1.1 Genotype and phenotype differences between Swiss Webster's and C57Bl/6J

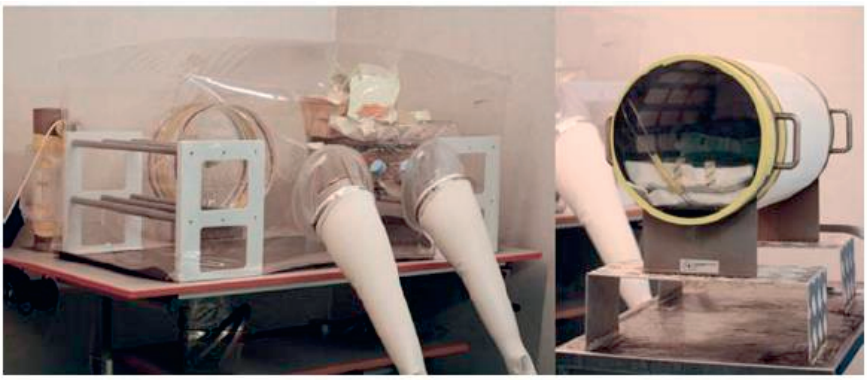
Experiments in paper I were conducted with the C57Bl/6J mouse model. We chose this model based on the type of questions that were asked and the experimental design. The C57Bl/6J is an inbred mouse model that is first and foremost well-characterized but also genetically uniform as the inbreeding causes smaller genetic variations. The genetic homogeneity allows for reproducible experiments but does also mimic the genetic variability as in a district group of humans (190). More importantly the C57Bl/6J possesses desirable phenotype well suited for our experiments. The model is often used for induction of obesity, have fairly low weights (average 26 grams) around 10 weeks of age (the time point which we start treating with HFD) and are more prone to develop T2D (191, 192). These characteristics make it well suited for our type of experiments.

Experiments in paper II were conducted with the Swiss Webster mouse model and it is an outbred strain. In comparison to inbred strains, outbred strains are genetically randomized and the goal is to maintain maximum heterozygosity and to keep the coefficient of inbreeding (probability that two alleles at a locus are identical as previous generation) less than 1% per generation (190, 193). A cohort of Swiss Webster's mimic similar genetic variation as would be found in humans (190). Also, one physiological trait that differentiates outbred strains to inbred due to the genetic heterozygosity is the body weight. Outbred strains tend to weigh more at a given time point than the inbred (about 40 grams at week 10). The genetic trait of Swiss Webster's minimize the risks of developing specific diseases as well as lacking specific tendency for developing e.g. obesity upon diet (194, 195). Therefore it is a model used as an all-purpose stock for research.

3.1.2 Breeding and colonization of germ free animals

The generation of GF animals has introduced a great tool for mechanistic studies for exploration of links between the gut microbiota and pathological conditions such as e.g. obesity, diabetes and IBD. The first GF animal was generated already in in the 1920s and 1930s by James Reyniers, resulting in the first GF guinea pig in 1936. The GF guinea pig was delivered after hysterectomy of a conventional female and the animals had to be hand fed one by one (196). Today the technique has evolved to such extent enabling rederivation of several generations of GF mice with specific desired genotype. Furthermore, the invention of GF animals has opened up a pathway to the generation of gnobiotic animals (from Greek roots *gnotos* "known" and *bios* "life"). This field is another very important research field that enables studies on the contribution of specific stain/strains on host physiology.

Breeding and handling GF animals require extreme cautions and sterility, as any mistake can result in contamination. The GF mice in our facility are maintained in custom designed flexible film isolators as viewed below.



Left: Isolator for housing germ free mice. Right: Sterilizing cylinder.

Arvidsson, C., Hallén, A., and Bäckhed, F. (2011). Generating and Analyzing Germ free Mice. In Current Protocols in Mouse Biology (John Wiley & Sons, Inc.)

Food pellets and bedding for the cages are placed into sealing bags whereas water is placed into a container. They are thereafter placed into the sterilizing cylinder (see figure to the right above) together with Steris DART for confirmation of sterility. All other equipment that tolerates autoclavation are

also sealed and transferred into the cylinder. After autoclavation the cylinder is connected to the isolator and the equipment are brought into the isolator. Some diets and chemicals do not tolerate the heat in the autoclave and can be irradiated instead. In some cases some liquids no not tolerate irradiation either and these have to be sterile filtered and sterilized with Clidox® (197).

Gnotobiotic mice are generated colonizing GF mice with one or several strains of bacteria, Regardless of the number of strains the bacteria are colonized with, the mice will be housed in separate isolators to avoid contamination of other unwanted bacteria.

There are some technical limitations with handling and performing experiments on mice in isolators. One of the issues is the number of mice in each isolator. Due to limitation of space in the isolators and number of mice per cage (maximum 5) it is not always possible to metabolically phenotype the mice as one wish. For an experiment running for a longer time e.g., it is not always possible to perform all the experiments as one wish on all the mice and still maintain sufficient number of mice for each experiment. Therefore, many long-term experiments will have to be repeated to collect all data needed, or set up several isolators simultaneously, but most often it is a matter of cost that would be a limiting factor.

Food monitoring requires sophisticated cages such as the metabolic cages. Today we have the possibility at our animal facility to maintain mice, both colonized and GF, in metabolic cages in which we can measure food consumption, energy expenditure and locomotor activity. Unfortunately this system was not up and running at the time of the experiments and left us only with the possibility to weigh the diet before and at specific time points (normally once per week). However this method makes it hard to account for spillage especially on mice fed high-fat diet in the bedding and since small changes in daily food intake can have large cumulative effects we did not manage to collect robust data on food intake in this experiment.

Our studies could have been completed with metabolic chambers to properly monitor rates of carbon dioxide production and oxygen consumption. This kind of data would have simplified data regarding weight gain during HFD treatments. This system is up-to-date not available in a sterile milieu.

3.1.3 Antibiotic treatment

In paper II CONV-R Swiss Webster mice were treated with an antibiotic cocktail of bacitracin, neomycin, and streptomycin. The purpose of the

particular experiment was to partially deplete the microbiota in order to decrease levels of SCFA and hence see the effect on colonic *Gcg* expression. These antibiotics were chosen based on several criteria which will be brought up in this section.

Bacitracin is a mixture of secondary polypeptide-metabolites and is produced by microbes belonging to *Bacillus subtilis*. They have a wide diversity of antimicrobial properties where they disrupt the cell walls of both gram negative and gram positive bacteria. Bacitracin is clinically used in topical treatments but is in preclinical studies also used orally. More specifically it is used in microbiota-related studies due to its good capacity to target a wide diversity of the microbiota and hence reduce the risk of over-and underrepresenting species (198).

Neomycin is an aminoglycoside antibiotic produced by *Streptomyces fradiae* (199) and is mostly used in topical treatments, but is also administered orally. It is not absorbed from the GI tract and is traditionally used in treatments such as small intestinal bacterial overgrowth. The bactericidal spectrum of neomycin is specific against gram negative bacteria and has partial activity against gram positive bacteria. Because of this niched spectrum, Neomycin it is often used in combination with other antibiotics. Due to the highly nephrotoxic ability of Neomycin it is never given intravenously.

Streptomycin is an antimycobacterial drug that as the name implies targets mycobacteria and does so very efficiently. It is synthetized by *Streptomyces* griseus (200). This antibiotic is nephrotoxic in addition to several other toxic features, which is why this is never given intravenously. However, in microbiota-related studies Streptomycin is a good supplement in a cocktail of other antibiotics as it target the mycobacteria.

As mentioned, these antibiotics were chosen in our study as they altogether cover a wide bacterial spectrum. In this manner one lowers the risk of over- and underrepresenting species. They are also preferred to use as the spread of the antibiotics are limited to the intestine where we only want to study the bactericidal effect, and thus eliminate possible systemic effects.

3.2 **Diet**

Prior to diet-treatment with specific composition the mice were kept on a standard aautoclaved chow diet from LabDiet (Diet-5021-3-BG) and had access to diet and water *ad libitum.* The calories are provided by ~24% proteins, ~fat 22% (the majority being saturated fatty acids, but minor parts

monosaturated fatty acids, linoleic acid, cholesterol, omega-3 and fatty acids) and 55% carbohydrates (the majority is starch with minors parts glucose, sucrose, lactose and galactose) (201). This diet is produced according to the Constant Nutrition® manufacturing assuring minimal inherent biological variations in long-term studies. More importantly, this production method delivers a constant level of nutrients that are on a daily basis assayed to ensure minimal nutrient variations, withstands changes in the levels of nutrient during autoclavation and lastly assayed so that environmental contaminants have minimal impact on the diet. The standard diet hence provides a predictably and maintenance of baseline data (202).

3.2.1 High fat diet treatment

Obesity was induced in the mice by using an established high fat diet from Harlan Laboratories (Diet TD.96132). This diet contains 4.5 Kcal/g diet where ~19% Kcal are from proteins, ~ 41% from carbohydrates and ~41% from fat. The type of carbohydrates added in the diet are mainly sucrose, which is common carbohydrate source used for induction of metabolic syndrome. Sucrose is known to increase hepatic TG and glucose production, ultimately leading to IR and liver steatosis. The fatty acids in this diet are derived from beef tallow and are ~41% saturated, 17% trans, 35% monosaturated and 7% polyunsaturated, hence the majority of fat are saturated and solid, which have a much greater contribution for development of DIO (203).

3.2.2 Tributiryn diet treatment

The energy availability in the colon was measured by a diet from LabDiet (204) supplemented with 10% tributyrin oil. The calories in this diet is not much different from the standard laboratory diet (201) and are derived from ~23% proteins, 21% ~fat and 55% carbohydrates. The diet contains 9% fat, making it a high energy diet suitable for various experiments for mice under stress. The diet is more importantly suitable for complementation with other substances such as tributyrin as it is stable.

3.3 Metabolic phenotyping of glucose metabolism

For good understanding of the pathogenesis of T2DM and other metabolic syndromes proper diagnostic tools are of most importance. In our studies robust and well-established tools were used to measure glucose-and insulin tolerance; however we did encounter some technical limitations due to the

GF state. These limitations and further aspects will be discussed in this section.

When metabolically phenotyping, circulating glucose is one of the important biochemical measures. One way of measuring glucose tolerance is to perform an OGTT. In an OGTT a fix and standardized glucose load is administered to the body and its clearance is measured. The procedure is performed by gavaging a bolus of total weight-adapted glucose solution via the mouth of the fasted (4 h) mice followed by time-wise monitoring of blood glucose levels during a 2 hour time period. In contrast to intra-peritoneal glucose tolerance test (IPGTT), where glucose in injected intraperitoneal (IP) into the abdomen, glucose takes the natural route in the OGTT via the mouth and is thereafter subjected to intestinal absorption. When glucose is administered orally instead of IP, incretin effect also plays a role in the glucose. IPGTT resembles a more 'artificial' test compared to the OGTT since it does not take the natural route of administration in consideration.

The D-glucose dosage in our OGTT studies was 3 g/kg body weight for both Swiss Webster's and C57bl/6. The D-glucose concentration for lean mice (<35 g) were 20% and obese mice (>35 g) 30%. Concentration of the solution was increased with increased body weight as we noticed that volumes of the glucose solution exceeded the maximum volume of the stomachs of the mice (500 µl). If more than the approved volume is gavaged, the risk of overfeeding and hence stressing the mice increase and could potentially perturb the results. Furthermore, glucose uptake takes place to about 85% in the skeletal muscles, whereas the rest is distributed amongst tissues such as white adipose tissue. We, and most people performing OGTT in both humans and mice, adjust the glucose concentration to total body weight. This is not optimal and would ideally be based on lean weight to ensure the most corrected glucose concentrations for muscle mass. To obtain lean mass we would have needed to run Magnetic resonance imaging (MRI), which once again leads to the problematics with the GF mice. As soon the GF mice are taken out from the isolators they are contaminated. We prioritized minimizing the stress impact from the MRI and risk of contaminations, after fasting and the following OGTT (or ITT) test.

The time of fasting has significant effects on the results since longer fasting time leads to much lower fasting glucose-and insulin levels but also eliminates effects from glycogen storage. It would have been preferred to fast our mice for a longer time to eliminate possible contributions derived from 'background' glucose. Our animal permits only allow 4 h of fasting.

Peripheral tissue insulin tolerance is measured with an ITT. In an ITT a fix and standardized insulin load is administered to the body and the subsequent glucose levels are measured. The procedure is performed by IP injection of total weight-adapted insulin solution in the abdomen of the fasted (4 h) mice followed by time-wise monitoring of blood glucose levels during a 120 minutes time period.

3.3.1 Gastric emptying and intestinal transit

Transit studies in humans are performed to detect the time required for a specific substance/food to travel along the entire GI tract, thus from the mouth to feces. Such study requires substances that are not absorbed along the GI tract and can easily be detected, most often via coloring of the substance. One parameter that affects the intestinal transit time is the rate of gastric emptying. Intestinal transit- and gastric emptying rate are examined differently in humans and mice and will be brought up in this section.

Regarding intestinal transit experiments in humans, food coloring is added to a specific food or substance as an indicator, and this method is noninvasive and harmless. However, as mentioned previously, in these studies one detects the traveling along the entire GI tract. In our study we wanted to elucidate the *intestinal* transit and for this purpose methylcellulose, nondegradable by the microbiota, was gavaged to the mice. Since it is known that solid materia takes longer time to travel through the GI tract compared to liquids we performed pilot studies and concluded that a concentration of 1.5% methylcellulose was optimal for both Swiss Webster's and C57Bl/6. However, the treatment time differed between the two strains. Evan's blue was added to the methylcellulose as the color-indicator and it is a strong dye (and harmless) that enables good visibility through the intestinal wall. The data was presented as the traveled distance in % relative to the total length of the small intestine.

Gastric emptying can be assessed by an acetaminophen absorption test and it is a test for measuring the rate of gastric emptying. It is a substance that is not absorbed in the stomach but in fact occurs later in the duodenum, making it suitable for this kind of test. The test is harmless, easy to conduct and provides robust data. In addition it is also the most commonly used test in mice. The rate at which the acetaminophen starts to appear in the blood is correlated to the rate of gastric emptying; the sooner it appears the faster emptying rate (205).

When performing intestinal transit- and gastric emptying rate experiments it is important to take into account the contribution of fasting times. It is important to make sure the stomach is completely empty (exception water) as remaining food itself will prolong the gastric emptying time and hence the transit time. For our mice we evaluated this and concluded that 4 hours are enough. Furthermore different strains of mice have different GI motilities which will give differences in migration distance. We concluded that for the Swiss Webster mice 45 min treatment after gavage was optimal, for the C57Bl/6 and *Glp-1r−/−* mice 30 min (intestinal transit).

3.4 Statistical analysis

Paper I. Data are represented as mean ± SEM. The effects of colonization and genotype were studied using two-way ANOVA followed by Tukey's multiple comparison tests. Specific individual bile acid levels were analyzed using one-way ANOVA. Multiple groups were analyzed by two-way ANOVA followed by *post hoc* Bonferroni post-tests. The effect of *Fxr* knockout on weight gain over time was analyzed by using a mixed two-way ANOVA test in R software. In this test the genotype was considered as a between-sample variable and the time as a within-sample variable.

Paper II. Data are presented as mean ± SEM. Statistical differences between groups of two were analyzed with a Student's t test, comparisons of three or more groups with one independent variable (e.g., colonization status) were analyzed by one-way ANOVA with ad hoc Bonferroni posttests, and comparisons of groups with two or more independent variables (e.g., colonization status and diet) were analyzed by two-way ANOVA with ad hoc Bonferroni posttests with the use of GraphPad Prism 5.

4 RESULTS

4.1 Paper I

The gut microbiota promotes diet-induced obesity and glucose metabolism through FXR.

To investigate whether microbiota induces diet-induced obesity through FXR we transferred GF and CONV-R WT and *Fxr*-/- mice on 10 weeks of HFD. In this manner we were able to investigate the role of gut microbiota on diet-induced weight gain. CONV-R WT mice gained significantly more weight than GF counterparts, but this difference was not observed in mice deficient in *Fxr*. We observed that WT mice had increased fasting glucose levels compared with *Fxr*-/- counterparts, but that CONV-R mice hade increased fasting glucose levels compared with GF counterparts on both genotypes. Similar result was obtained from an OGTT and suggests that the genotype is more important than the colonization state for these phenotypes.

In contrast we observed FXR-dependency for insulin related phenotypes. CONV-R WT mice had elevated fasting insulin levels, increased beta-cell mass, and impaired insulin tolerance compared with GF counterparts. No differences were observed between GF and CONV-R mice on *Fxr*-deficient genetic background. Taken together our results suggests that the gut microbiota modulates IR in an FXR-dependent fashion, whereas oral glucose tolerance is more dependent on the genotype (Figure 1, paper I).

Obese CONV-R WT displayed increased adiposity and larger adipocytes compared with GF counterparts, a phenotype that was abolished in *Fxr*-/- mice. The number of the CLS, accumulation of macrophages surrounding apoptotic adipocytes, and expression of macrophage markers *Emr1* and the pro-inflammatory genes *Saa3* and *Tnf-α* were markedly increased in adipose tissue from CONV-R versus GF WT mice but not in adipose tissue from *Fxr*-/- mice with or without a microbiota. These data suggest that microbiota-induced obesity is associated with increased macrophage infiltration and that this process requires functional FXR signaling (Figure 2, paper I).

Altered gut microbiota from Fxr-/- mice modulates glucose metabolism upon transfer to GF mice

To investigate whether FXR modulate the microbiota and whether such modulation contributed to the phenotype we first analyzed the fecal microbiota by performing 16S rRNA sequencing on samples harvested at the

end of. Data showed a clear separation between the groups (Figure 4, paper I). We also observed decreased levels of Firmicutes and increased levels of Bacteriodetes in *Fxr-/-* compared with WT mice after HFD.

Next, we wanted to investigate whether the altered gut microbiota of *Fxr-/-* mice could contribute to the metabolic differences between WT and *Fxr-/-* mice. GF WT mice were colonized with caecal microbiota from HFD-fed *Fxr-/-* mice *(*CONV-D(*Fxr*-/-) *or* WT mice (CONV-D(WT) and treated with HFD for 10 weeks. CONV-D(*Fxr*-/-) gained less weight compared with CONV-D(206) accompanied by lower adiposity. In addition glucose tolerance was improved in CONV-D(*Fxr*-/-) mice compared with CONV-R mice (Figure 4, paper I). These data suggest that the altered gut microbiota in HFD-fed *Fxr*-deficient mice may contribute to some of the beneficial metabolic effects observed in these mice.

FXR alters gut microbiota and bile acid composition

The gut microbiota and FXR have profound effects on bile acid metabolism. We found that GF mice had a similar bile acid composition independent of Fxr genotype. In contrast, bile acid profiles differed between CONV-R WT and *Fxr-/-* mice. CONV-R *Fxr-/-* versus WT mice had a shift towards higher proportion of tauro-conjugated CA and βMCA and reduced proportion of the secondary bile acids ωMCA as well as DCA. The altered bile acid composition between CONV-R WT and *Fxr-/-*mice may thus be the result of altered microbiota composition (Figure 4, paper I).

4.2 **Paper II**

Basal GLP-1 levels are elevated in GF mice

To investigate how the microbiota affects the production of GLP-1 GF and CONV-R mice were used as model. A primary screening of plasma GLP-1 levels in fasting GF and CONV-R mice revealed that microbiota suppress GLP-1 production. This difference could not be explained by decreased DPPIV activity from plasma in the GF group. *Gcg* expression was significantly higher in the sites with the most bacterial density, namely the cecum and proximal colon, in GF mice compared with CONV-R (Figure I, Paper II). This observation explains to some part the observed elevated GLP-1 levels. In addition, GF mice had approximately 4-fold more GLP-1-positive L cells in the cecum and 2-fold more GLP-1-positive cells in the proximal

and distal colon in comparison to CONV-R mice, also contributing to elevated GLP-1 levels. Thus, elevated plasma GLP-1 levels in GF originate from the cecum and colon, and the gut microbiota affects both *Gcg* expression and L cell number (Figure I, Paper II).

SCFAs increase and proglucagon expression decreases upon colonization

Butyrate is a SCFA and the primary energy source for colonocytes and colonocytes in GF mice has previously been shown to be energy deprived. We colonized GF mice with an unfractionated microbiota from a CONV-R donor and analyzed SCFA levels in cecum to detect if one can rescue the energy deprived state and alter GLP-1 levels. In the GF mice SCFA levels were as expected low, with small amount of acetate whereas propionate and butyrate were barely detectable (Figure S1, Paper II). SCFA concentration started to increase rapidly already after 24 hours (7-fold) and resembled the levels of CONV-R at that timepoint. Consequently the increased SCFA levels were associated with significant decreases in colonic *Gcg* expression after 24 and 72 hr. The number of GLP-1-positive cells in the proximal colon did not change significantly after 24 hours but decreased to a level similar to CONV-R 72 hours after colonization. Although not statistically different the average plasma GLP-1 levels decreased gradually after colonization (Figure 2, Paper II).

SCFAs suppress proglucagon expression in GF colon

Next we wanted to investigate how the microbiota effects *Gcg* expression and GLP-1 levels. We suggested that SCFAs is involved in this, as it is known that one SCFA in particular, butyrate, act as sole energy source for colonocytes; the same site in the intestine as the L-cells are situated. To continue, as colonocytes in GF mice has previously been shown to be energy deprived, we hypothesized that the energy deprived state of the GF colon could be a driving factor behind the observed high GLP-1 levels. In order to address this question we colonized GF mice with unfractionated caecal microbiota to detect if one can rescue the energy deprived state and alter GLP-1 levels.

In the GF mice SCFA levels were as expected low, with small amount of acetate whereas propionate and butyrate were barely detectable (Figure S1, Paper II). SCFA concentration started to increase rapidly already after 24 hours (7-fold) and resembled the levels of CONV-R at that timepoint.

Consequently the increased SCFA levels were associated with significant decrease in colonic *Gcg* expression after 24 and 72 hr. The number of GLP-1-positive cells in the proximal colon did not change significantly after 24 hours but decreased to a level similar to CONV-R 72 hours after colonization. Although not statistically different the average plasma GLP-1 levels decreased gradually after colonization (Figure 2, Paper II).

Thus, now we were able to detect a clear link between low Gcg expression and high levels of SCFAs, and vice versa.

SCFAs suppress proglucagon expression in GF colon

In order to more specifically investigate the role of microbial production of SCFA on *Gcg* expression GF mice were individually colonized with two single bacterial strains that possess different fermentation capabilities. *Escherichia coli* (gram-negative bacterium that ferments simple sugars) and *Bacteroides thetaiotaomicron* (gram-negative bacterium that ferments a wide range of complex polysaccharides) were used. We confirmed this hypothesis and observed a small increase in acetate but no significant alteration in total SCFA levels, *Gcg* expression, or GLP-1-levels was observed in *E. col*i colonized mice. In contrast, colonization with *B. thetaiotaomicron* produced significant increases in acetate and propionate resulting in a 4-fold increase in total SCFA levels, a 2.5-fold decrease in colonic *Gcg* expression, and a 1.7-fold decrease in GLP-1-positive cells (Figure S1 and Figure 2, Paper II).

In another approach to confirm our observation we treated CONV-R mice with antibiotics for 3 days, which resulted in a 13-fold decrease in SCFA concentration and a 3-fold increase in *Gcg* expression (Figure 2, Paper II). Thus, we observed an association between high levels of SCFA and low levels of GLP-1..

In order to show the direct link between SCFA levels and *Gcg* expression proximal colonic tissue was incubated *ex vivo* with a physiological concentration of SCFAs. SCFA treatment resulted in significantly lower *Gcg* expression in GF colon but did not have a significant effect on *Gcg* expression in CONV-R colon. Furthermore, we treated fed GF mice a diet supplemented with tributyrin, a TG that is hydrolyzed to butyrate in the colon. This treatment increased caecal butyrate levels 2.8-fold and resulted in a 1.3-fold decrease in colonic *Gcg* expression compared with GF controls. Altogether, these data show that SCFAs suppress *Gcg* expression in GF colon and that this effect does not appear to be specific for a particular SCFA, given

that increasing levels of acetate and propionate (*B. thetaiotaomicron* colonization) or butyrate (tributyrin diet) suppress *Gcg* expression (Figure 2, Paper II).

Given that diet and gut microbiota change considerably at the suckling-to-weaning transition, we analyzed colonic *Gcg* expression in GF and CONV-R mice from birth through young adulthood, to see if diet composition affects *Gcg* expression. Given that energy availability in neonatal colonocytes are restricted to milk lactose, amino acids and lipids we hypothesized that there would be little difference in dietary energy availability and hence *Gcg* expression, between GF and CONV-R mice during the suckling period. Colonic *Gcg* expression did not differ between GF and CONV-R during the first 3 weeks of life but increased substantially in GF mice in comparison to CONV-R mice at 4 weeks of age as they were weaned into standard chow diet and was even greater at 8 weeks of age (5-fold) (Figure 3, Paper II).

SCFAs are signaling molecules that can activate G-coupled receptors such as Gp431 and 43 (159). In addition, butyrate can signal through Gpr109a and also act as an histone deacetylase inhibitor (207). Experiments using Gpr41 and 43 deficient mice excluded the possibility that microbial effects observed in this model was mediated through Gpr41 and 43. Since acetate and propionate also had similar effects as butyrate, we reasoned that it was unlikely that the signaling was mediated through Gpr109a and modulation of histone acetylation. Since butyrate is an important energy source and in the gut epithelium and that acetate and propionate may have similar effects we weaned mice onto a HFD (40% calories from fat) to test whether increasing energy supply from the diet could suppress the increase in *Gcg* expression in GF mice. Caecal levels of the LCFA palmitate were analyzed due to its abundance in diet and the fact that is can rescue the defect in mitochondrial respiration in GF colonocytes. HFD-fed mice had higher levels of palmitate and reduced levels of *Gcg* expression compared with chow fed controls. In addition we found that colonization status had a smaller effect on SCFA levels for HFD-fed mice than for chow-fed mice (Figure 3, Paper II) suggesting that the gut microbiota suppress GLP-1 by providing energy and that GLP-1 may act as an important energy sensor in the colon.

As GLP-1 is known to have important physiological roles we next examined whether increased GLP-1 levels in GF mice contribute to improved oral glucose tolerance. GF mice were found to have significantly better oral glucose tolerance compared with CONV-R mice, and by blocking the GLP-1 receptor glucose tolerance curves shifted to a similar extent for GF and CONV-R mice (Figure 4, Paper II). Furthermore, the fold increase in insulin

after glucose gavage was similar in GF and CONV-R mice indicating for different glucose metabolisms but not due to GLP-1. We conclude that enhancing glucose metabolism in GF mice is not the primary function of increased basal GLP-1 levels (Figure S2, Paper II).

Increased GLP-1 in GF mice results in slower intestinal transit

Another physiological roles of GLP-1 is modulation of gastric emptying and intestinal GI transit. We next investigated whether increased GLP-1 levels in GF mice play a affected these phenotypes. GF mice exhibited similar rate of gastric emptying but significantly slower small intestinal transit in comparison to CONV-R controls (Figure 2S and Figure 4, Paper II). By blocking the GLP-1 receptor we were able to elucidate if the differences in transit were GLP-1 derived, and we could see that blocking the receptor completely rescued the transit phenotype in GF mice (Figure 4, Paper II).

As additional support for the role of elevated GLP-1 levels in slowing intestinal transit, we investigated transit in *Glp-1r-/-* mice and C57Bl/6 controls after treatment with antibiotics. Antibiotic treatment reduced caecal SCFA levels, 3-fold higher colonic *Gcg* expression, and approximately 3-fold higher GLP-1 levels in both C57Bl/6 and *Glp-1r-/-* mice (Figure S2, Paper II). Although intestinal transit was significantly slower after antibiotic treatment in C57Bl/6 mice, there was no significant difference in transit between control- and antibiotic-treated *Glp-1r-/-* mice. Altogether these result show that intact GLP-1 signaling is required to slow intestinal transit after antibiotic treatment (Figure 4, Paper II).

Upon conventionalization of GF mice and monocolonization with *B. thetaiotaomicron* and *E. coli* we could see an increased transit only 72 hours after conventionalization and *B. thetaiotaomicron* colonization, but not *E. coli*. We also found that intestinal transit was normalized to the CONV-R rate in GF mice that were fed an HFD (Figure 4, Paper II).

5 DISCUSSION

Many different metabolites in the gut are microbially-derived e.g. bacterial fermentation of dietary fibers, and xenobiotics. Although there is ongoing research and data published regarding effects of microbial metabolites on host physiology and health, we are just scratching the surface.

SCFAs are the most abundant bacterial metabolite produced from fermentation of fibers (otherwise indigestible by the host) and are in this form a useful form of energy for the host (24, 25) . In fact, 6-10% of the total energy consumption by the host is from SCFAs (27). The SCFA butyrate is the preferred energy source of colonocytes, and because GF mice lack the ability to produce butyrate, their colonic epithelium is energy deprived (208). Bile acids are another relatively well-studied group of microbial metabolites. The microbiota chemically alters the bile acids and thus generate a wide spectrum of bile acids (44). Bile acids are known primarily for their detergent properties of ingested dietary lipids and activation of FXR, however, bile acids do activate other target receptors such as TGR5, PXR and VDR (48). Thus, the microbial metabolites SCFAs and bile acids have significant effects on host physiology. We have in our 2 papers demonstrated how these metabolites affects host physiology. Although different approaches was used in the two papers, we were able to show the important role of the gut microbiota in the profiles of these two groups of metabolites, and more importantly, their effects on host physiology.

5.1 Paper I

In the first paper we investigated whether the gut microbiota induced DIO through FXR by feeding GF and CONV-R WT and *Fxr-/-* mice HFD for 10 weeks. After the HFD feeding the primary and most prominent phenotype was that DIO required intact FXR signaling via the gut microbiota, as mice lacking the microbiota and/or FXR were protected against DIO. A previous paper has demonstrated that the FXR contributes to DIO (183) and other investigators have demonstrated that GF mice are protected against DIO (REFS Bäckhed 2007). However, we were the first to demonstrate that the combined effects. Furthermore, we found that the microbiota promoted adipocyte inflammation and liver steatosis in a strict FXR-dependent fashion.

Since obesity and metabolic inflammation is associated with impaired glucose metabolism we continued to analyze insulin tolerance. As expected,

obese CONV-R WT mice had impaired IT compared with GF WT controls, whereas GF *Fxr-/-* and CONV-R *Fxr-/-* exhibited similar fasting insulin levels, similar beta cell mass and IT. This highlights the importance of microbial signaling through FXR. However, during an OGTT we noticed that GF *Fxr-/-* mice had improved fasting glucose levels and OGT compared with CONV-R *Fxr-/-* mice. It has previously been shown that FXR activation in various tissues occur at different degrees, and in fact only 10% overlap between liver and intestine (209). Our data suggests in concordance with the Thomas' publication that FXR may have tissue-specific effects and hence the observed OGT and IT phenotype.

In an attempt to target possible explanations for the prominent phenotype that DIO and other metabolic disruptions occur via intact microbiota-mediated FXR signaling, we next addressed the FXR ligands: bile acids. Bile acid have for some time been known to be modulated by the gut microbiota (210) and FXR activation by bile acids has in several studies shown to be important in various metabolic features (183, 187, 188). Bile acid analysis in serum after HFD administration confirmed the lack of secondary bile acids in GF mice. However the CONV-R *Fxr-/-* sera had significantly higher proportions of T-βMCA and an increased trend towards higher TCA levels compared with the WT counterpart. This suggests for that in the CONV-R *Fxr-/-* mice there is a reduced capacity for deconjugation of bile acids. More importantly, the differences observed in bile acid profiles between CONV-R *Fxr-/-* and CONV-R WT suggested that there is an altered microbial community, and/or increased bile acid production. Studies have shown that bile acids through their acidic properties can directly modulate bacterial growth in the gut (53, 211) or indirectly via production of antimicrobial species (212).

As expected we found clear differences in the microbiota of CONV-R *Fxr-/-* and CONV-R WT, which is in line with the altered bile acid profiles. We found that the lean *Fxr-/-* mice had reduced Firmicutes: Bacteriodetes ratio in the WT counterparts; associated with leanness (115, 117). Also, we were able to reproduce the obesogenic phenotype of when lean GF mice were transplanted with either CONV-R WT or *Fxr-/-* faecal material after 10 weeks of HFD feeding demonstrating that the altered gut microbiota directly contributes to the metabolic phenotype..

Altogether, the results from paper I shows that the gut microbiota promotes DIO via FXR signaling, and more importantly, that the altered bile acid profiles and hence FXR signaling affects DIO. Also, our findings suggest that the genotype is also involved in shaping the microbial composition.

5.2 **Paper II**

In initial experiments, we were able to see that GF mice have significantly higher levels of serum GLP-1 levels (3-fold difference) compared with CONV-R mice. We excluded possible contributions of DPPIV activity, which degrades GLP-1, to the observed GLP-1 levels. However we did instead detect significantly higher levels of *Gcg* expression in caecum and colon in GF mice as well as higher number of L-cells in the same parts of the intestine. Due to that colonocytes use the SCFA butyrate as the preferred energy source, and that GF colonocytes are energy deprived (Donohoe 2011) we hypothesized that the energy deficiency could be a regulating factor behind increasing GLP-1 levels. To test the hypothesis we performed short-term colonization experiments, showing that already 24 h colonization is sufficient for normalizing the levels of SCFAs, but more importantly, suppressing *Gcg* expression and decreasing L-cell number. This shows for a link between energy availability and *Gcg* expression. Follow-up experiments modulating energy availability with monocolonizations with good and poor fermenters, or treating the mice with HFD, all reduced *Gcg* expression, once again showing that L cells sense available nutrients in their surrounding environment and thereafter regulate *Gcg* expression in another attempt to show the link between available SCFAs and *Gcg* expression, mice were treated with antibiotics, showing once again that reduction in microbiota leads to lower SCFA levels and hence higher *Gcg* expression.

As GLP-1 is an incretin hormone (148) we next examined the physiological consequences of increased GLP-1 levels and found that GF mice exhibited improved OGT versus CONV-R mice. Treatment with GLP-1 antagonist showed impairment in OGT but at a similar degree, between GF and CONV-R mice.

Considering that GLP-1 is known to increase the number of β-cells (213) and induce insulin secretion, we also examined these phenotypes, but did not observe any differences between GF and CONV-R mice, highlighting that there is a fundamental difference in glucose metabolism but through another GLP-1 independent mechanism.

GLP-1 is also involved in slowing intestinal transit (166) and we were able to show that GF mice have slower transit time versus CONV-R mice, and treatment with GLP-1 antagonist accelerated transit time in the GF group but not in the CONV-R mice. Also, the antibiotic-induced effect in transit time is abolished in mice lacking GLP-1 receptor. The experiment thus demonstrated that functional GLP-1 receptor is required in absence of microbiota.

Altogether, in paper II, we demonstrated that the increased colonic GLP-1 levels in GF mice are regulated through available energy (SCFAs). More importantly, we see that the physiological consequence of the elevated GLP-1 levels slows intestinal transit, and we propose that GF mice have slower transit time to allow sufficient energy-and nutrient absorption. Given that intestinal transit time also regulates and prevent bacterial overgrowth (Stephen 1987) we also propose that during colonization, when the levels of SCFA increase, intestinal transit time decreases to prevent bacterial overgrowth.

6 CONCLUSION

Altogether the conclusions to be drawn from paper I and paper II are the following:

- Gut microbiota mediates host physiology via the microbial metabolites.
- Microbial-mediated DIO requires intact FXR signaling.
- Bile acids and FXR are not only modulated by the gut microbiota but they also modulate the microbiota composition.
- The FXR genotype shapes the microbiota composition.
- The gut microbiota increases colonic energy availability by fermenting dietary fibers into SCFAs.
- The low SCFA levels in GF colon lead to increased *Gcg* expression and GLP-1 levels, leading to decreased intestinal transit time.
- The decreased intestinal transit time in GF mice is a compensatory physiological mechanism to allow for more time to absorb nutrient and energy.

7 CLINICAL IMPLICATIONS

The results from paper I and II show potential clinical implications. In paper I we see that the gut microbiota promotes diet-induced obesity and associated phenotypes through effects of the bile acid profile and altered FXR signaling. Furthermore, we were also able to see that the FXR genotype affects the microbiota composition. Thus, one novel approach for clinical application is manipulation of the microbiota in order to achieve similar bile acid profile and hence altered FXR signaling that terminally could protect against DIO and associated phenotypes.

In paper II we conclude that increased GLP-1 levels in the GF mice have significant impact on glucose metabolism, as the GF mice have improved glucose tolerance compared with the CONV-R. Our study confirms the usage of GLP-1 agonistic drugs which are already used as a treatment method against diabetes. Another clinical implication can be the usage of probiotics which alter the microbiota composition so that less SCFA are produced, which ultimately increase colonic proglucagon expression.

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