On the role of gut microbiota in intestinal physiology and hepatic metabolism

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Gothenburg 2017

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ISBN 978-91-629-0121-9 (TRYCK)
ISBN 978-91-629-0122-6 (PDF)

Printed in Gothenburg, Sweden 2017
Ineko AB, Gothenburg

To my Mother

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ABSTRACT

The gut microbiota, a complex and dynamic community of microbes in the mammalian gut, has coevolved with us for ample time providing mutual benefits. However, mechanistic knowledge of these has been limited, but are now becoming increasingly clear. We used germ-free mice to study three aspects of host physiology; the effects of the microbiota on small intestinal postnatal vascularization (I), small intestinal permeability (II), as well as the interplay between the gut microbiota and a steatogenic diet and the subsequent effects on hepatic metabolism (III).

In **Paper I** we found a new mechanism underlying microbiota-induced vascular remodeling in the small intestinal villi. This mechanism involves activation of protease activated receptor-1(PAR1) induced by microbial regulation of tissue factor activity. As a consequence of PAR1 signaling we observe increased angiopoietin expression in the intestinal epithelium and subsequent expansion of blood vasculature.

In **Paper II** we applied Ussing chambers to determine small intestinal permeability and observed increased permeability in conventionally raised (CONV-R) mice, compared with germ-free (GF) mice. This was accompanied by reduced mRNA expression of tight junction proteins and ultrastructure analyses revealed wider tight junctions and reduced numbers of desmosomes. The alterations between GF and CONV-R mice were abolished in the absence of farnesoid X receptor.

In **Paper III** we investigated if the gut microbiota interacted with dietary sucrose to induce hepatic steatosis. GF and CONV-R mice were fed a zero-fat, high-sucrose diet (ZFD) or control diet and we observed a synergistic

effect of diet and microbiota on hepatic steatosis by induction of *de novo* lipogenesis. Furthermore, we could establish a central role for the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) in this process.

In conclusion, these studies show that the microbiota induces expansion of intestinal vasculature and increased permeability, which may both contribute to metabolic effects. Further, the microbiota is required for a zero-fat, high sucrose diet to be steatogenic. This could give rise to novel treatment options for non-alcoholic fatty liver disease.

Keywords: Gut microbiota, Intestinal permeability, Non-alcoholic fatty liver disease

ISBN: 978-91-629-0121-9 (TRYCK)

ISBN: 978-91-628-0122-6 (PDF)

SAMMANFATTNING PÅ SVENSKA

Tarmfloran, den samling bakterier som lever i våra tarmar, har länge ansetts ha positiva effekter för sin värd, genom vitaminsyntes och ökat energiupptag från födan. I en värld med allt större tillgång till mycket energirik mat, och avsaknad av komplexa kolhydrater, har man kunnat visa att tarmflorans en gång viktiga roll numer bidrar till utveckling av stora folksjukdomar som fetma och typ-två diabetes med kardiovaskulära följdsjukdomar. Tarmfloran är mycket komplex det finns lika många bakterieceller som det finns mänskliga celler i kroppen. Det är fortfarande inte klarlagt exakt vilka mekanismer som ligger bakom tarmflorans effekter, och eftersom tarmfloran är dynamisk i sin sammansättning kan de variera mellan olika scenarier. I denna avhandling har bakteriefria möss använts för att studera några av de effekter som tarmfloran har, och vi kan visa att tarmen, som är det organ som är närmast i kontakten med bakterierna, uppvisar bredare tarmvilli när den jämförs med tarmen från bakteriefria möss. För att detta ska ske måste kärlsystemet i dessa strukturer expandera och vi fann att detta sker via en mekanism där tarmfloran modifierar tissue factor i tarmpeitelceller, vilket leder till att proteaset trombin bildas och verkar på proteasaktiverade receptorer. Detta i sin tur signallerar till cellkärnan där angiopoietin sedan uttrycks och inducerar blodkärlsexpansion. Vi såg också att barriärfunktionen är högre i det bakteriefria epitelet och att de sammanbindande tight junctions är mer slutna. Dessa skillnader mellan bakteriefria och konventionella möss finns dock inte i möss som saknar gallsyrareceptorn FXR, vilket indikerar att den kan vara inblandad i mikrobiell reglering av tarmpermeabilitet, som är en viktig faktor i både hälsa och sjukdom. Till sist undersökte vi hur en diet bestående av sukros och protein (och helt utan fett; zero-fat diet, ZFD) samverkar med tarmfloran för att inducera fettlever. Bakteriefria möss som fick denna diet uppvisade betydligt lägre fettinnehåll i levern vilket tyder på att tarmfloran är nödvändig för att levern ska bilda nytt fett. Vi identifierade en transkriptionsfaktor, SREBP-1c, som en central faktor i induktionen av fettbildning. För att förstå vad som i sin tur reglerar denna undersökte vi sammansättningen av tarmfloran och fann att den ändras drastiskt när möss gavs ZFD. Dessa förändringar kan, tillsammans med bakteriernas ämnesomsättning, förklara de stora skillnader i olika metaboliter som återfanns i mössens portalven. Förhoppningen är att identifiera en specifik metabolit eller en (grupp av) mikrob(-er) som inducerar den SREBP-1c-beroende fettsyntesen, men mer arbete krävs för att göra detta.

LIST OF PAPERS

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

I. Tissue factor and PAR1 promote microbiota-induced vascular remodeling

Christoph Reinhardt, Mattias Bergentall, Thomas U. Greiner, Florence Schaffner, Gunnel Östergren-Lundén, Lars C. Petersen, Wolfram Ruf, Fredrik Bäckhed

Nature 2012 Mar 11;483(7391):627-31. doi: 10.1038/nature10893.

II. Microbial Regulation of Tight Junction Ultrastructure and Intestinal Permeability – Potential involvement of FXR?

Mattias Bergentall, Jenny K. Gustafsson, Bengt R. Johansson, Fredrik Bäckhed

In Manuscript

III. The gut microbiota is required for sucrose-induced steatosis through SREBP-1c

Mattias Bergentall, Rozita Akrami, Valentina Tremaroli, Marcus Ståhlman, Antonio Molinaro, Louise Mannerås Holm, Geesje M. Dallinga, Adil Mardinoglu, Max Nieuwdorp, Fredrik Bäckhed

In Manuscript

CONTENT

ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ANGPTL4	Angiopoietin-like protein 4
ASO	Anti-sense oligonucleotide
BA	Bile acids
BSH	Bile salt hydrolase
CD	Crohn's disease
CDCA	Chenodeoxycholic acid
ChREBP	Carbohydrate response element binding protein
CNS	Central nervous system
CoA	Coenzyme-A
CONV-R	Conventionally raised
DIO	Diet-induced obesity
DKO	Double knock-out
Dll4	Delta-like ligand 4
DSS	Dextran sulfate sodium
ELOVL6	Elongation of very long chain fatty acids protein 6
ENS	Enteric nervous system
FAS	Fatty acid synthase
FGF	Fibroblast growth factor
FXR	Nuclear receptor farnesoid X receptor

GF	Germ-free
GLP-1	Glucagon-like peptide 1
GLUT5	Glucose transporter 5
GPCR	G-protein-coupled receptor
HFD	High-fat diet
IBD	Inflammatory bowel diseases
IEC	Intestinal epithelial cells
IF	Immunofluorescence
LPS	Lipopolysaccharides
LXRα	Liver X receptor α
M-BAR	Membrane-type receptor for bile acids
MCA	Muricholic acid
MLCK	Myosin light chain kinase
MyD88	Myeloid differentiation primary response gene 88
NAFLD	Non-alcoholic fatty liver disease
OST	Organic solute transporter
PAR	Protease activated receptors
PECAM1	Platelet endothelial cell adhesion molecule 1
PEPCK	Carboxykinase
PNPLA3	Patatin-like phospholipase domain-containing protein 3
PUFA	Polyunsaturated fatty acids

RXR	Nuclear receptor retinoid X receptor
SCD1	Stearoyl-CoA desaturase 1
SCFA	Short-chain fatty acids
SHP	Small heterodimer partner
SPF	Specific pathogen-free
SREBP-1c	Sterol regulatory element-binding protein-1c
TEM	Transmission electron microscopy
TER	Transepithelial resistance
TF	Tissue factor
Tie2	Angiopoietin tyrosine kinase receptor
TJ	Tight junction
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
VEGFR	VEGF-receptor
WT	Wilde-type
ZFD	Zero-fat, high sucrose diet
ZO-1	Zonula occludens-1

1 INTRODUCTION

This thesis will investigate three areas of how the gut microbiota affects mammalian physiology. Two projects concern fundamental biology of the intestine, angiogenesis as well as intestinal permeability and epithelial ultrastructure. The third project is focused on how the gut microbiota affects hepatic sugar and lipid metabolism, and steatosis.

1.1 Historical introduction to gut microbiota

The gut microbiota has been considered an important factor to health at least since Hippocrates who stated "All disease begin in the gut"[1]. Because of the small size of bacteria, and later the observed complexity of microbiota, research has moved in distinct steps dictated by technological advances. Antonie van Leeuwenhoek studied several different aspects of biology with his newly inveted microscope. He made his most celebrated observation by microscopy in 1683, in that bacteria reside in both oral cavity and other areas of the body[2]. Thereafter, many years would pass until the area was further investigated. Towards the mid 19th century researchers again turned to investigate intestinal bacteria. However, there was a widespread notion that the presence of intestinal bacteria had little effect on host physiology, and that they were mere passengers that could propagate due to the presence of suitable substrate upon feeding by the host[3].

This notion was subject to change by Louis Pasteur's pioneering work on identifying a bacterium as the cause of anthrax[3]. Later work, for instance by Ilya Metchnikoff, proposed that bacteria in the gut could be implicated in regulation of host physiology. He concluded that presence of intestinal bacteria may in fact reduce longevity of the host, but, in contrast, also that by ingesting bacteria found in yoghurt they may confer health[4].

Already in 1879 it was realized that bacterial shape and amount of bacteria were different between normal and pathological stool, but also that morphological diagnosis was impossible because many bacteria were indistinguishable based solely on their morphology[3]. The great number of bacteria, many of which are similar to one another, revealed a high complexity and showed the demand for the next technical breakthrough to study the impact of intestinal microbes on their host: animals completely

devoid of bacteria. This idea was conceived in 1885 by Pasteur, who postulated that germ-free (GF) life would be impossible[5].

Rearing GF animals is technically challenging, and historical documentation reveals several attempts that did not succeed in creating viable GF animals. The omnipresence of the very small bacteria poses a major problem, especially in regards to feed and water that need to be entirely free of bacteria. Moreover, it was also required to produce a barrier that prevents bacterial contamination yet allowing intervention to the animals. Based on the fact that mammals are germ-free *in utero*, guinea pigs were delivered by Caesarean section into a germ-free environment by Nuttal and Thierfelder in 1895[6]. They were subsequently fed sterilized cow's milk and it was observed that the guinea pigs died within two weeks, in line with Pasteur's initial idea that GF life would be impossible. Other attempts included chickens (1898, Shottelius)[7,8] , which are somewhat easier to maintain given the fact that they reside in an egg and are more independent after hatching. Küster concluded that the GF goats developed similarly to conventionally raised (CONV-R) counterparts, at the longest experimental duration of 35 days under GF conditions[8] (1912, by Küster). Thus, in contrast to guinea pigs, chickens and goats survived under GF conditions. Twenty years later, Glimstedt sought to gain understanding of the importance of nutrition in GF animals and turned to the guinea pig as an experimental model[9]. In addition to sterilized milk, vitamins and solid food was added to the feed, rendering an increased life span of up to two months, suggesting that the microbiota produce essential nutrients[9]. No differences in survival between GF and CONV-R guinea pigs were observed when both groups were fed the same diet. Glimstedt next continued the characterization and observed increased cecal size and a more watery texture of the feces in the GF animals, as well as an increased food intake in the latter group[9].

Still it was not possible to rear GF animals over generations and subsequent attempts were made to achieve this. Reyniers, active at the University of Notre Dame, performed experiments using various animals and developed laborious protocols, contributing greatly to the development of functional GF breeding during the 1940's[10-12]. Deriving animals into the GF steel isolator was complicated by the fact that the pups lacked a mother to feed them. Instead, technicians had to work in shifts to feed the sucklings manually every couple of hours. Finally, Reyniers and coworkers succeeded in maintaining guinea pigs GF for two generations, thereby facilitating the experimental conditions as the pups no longer needed manual feeding during the suckling period[13]. However, success was limited as the animals succumbed to starvation prematurely.

In Sweden progress was made at the University of Lund where Gustafsson and coworkers resumed GF research, and in 1946 rats were successfully kept GF over generations[14].

These technical achievements were crucial for the initial understanding of gut microbiota-host interactions. For instance, it was clear that mammals could survive without bacteria, but survival depended on the supplementation of vitamins.

1.2 Gut microbiota and host physiology

As illustrated by GF animal experiments, the gut microbiota is dispensable for mammalian life, but some factors contributed by the gut microbiota provide an essential aid for the host. Because most mammals lack the capacity to synthesize several vitamins, they must rely on external supplies. Importantly, it has since been shown that several groups of bacteria contribute to the production of B vitamins, implicated in several aspects of cellular metabolism, as well as vitamin K, important for blood coagulation[15]. As such, the genes present among the bacteria residing in the gut constitute an important complement to the mammalian genome which lacks molecular tools to metabolize many complex carbohydrates, including plant polysaccharides[16]. In line with this notion, the mammalian genome also lacks coding material for certain enzymes needed to degrade potentially harmful xenobiotic substances, which are instead broken down by members of the gut microbiota[17].

The gut microbiota is implicated in energy uptake

From an evolutionary perspective, mammalian life has been characterized by fluctuation and limitation in food accessibility. Fluctuations make the capability to store energy necessary, and limitation demands efficiency in energy extraction from the ingested food. On a macronutrient level, food consists of carbohydrates, fat and protein which can all be converted to adenosine triphosphate (ATP) for energy production; however, ATP cannot be stored. Carbohydrates may be converted to sugars, mainly glucose, and proteins are broken down to amino acids – both of which are unsuitable for long term storage, mainly due to the osmotic pressure created by solutes within a cell. Instead, most storage occurs in the form of glycogen in the liver and fat in adipose tissue.

The early observation that GF mice have increased cecal size is related to bacterial fermentation of dietary fiber. Lack of bacteria leads to the

accumulation of fiber which exhibit an osmotic pressure that retains water in the cecal lumen. In normal CONV-R mice, bacteria degrade these complex carbohydrates and produce short-chain fatty acids (SCFA). SCFA serve as the preferred energy source for colonocytes[18], but are also involved in intricate signaling both locally in the intestine, and systemically[19].

In the intestine, SCFA act as signaling molecules that bind to G-protein-coupled receptors such as free fatty acid receptor 2 and 3 (FFAR2/GPR43 and FFAR3/GPR41) as well as GPR109A[19]. Activation of GPR43 by acetate or propionate in the colon leads to repression of glucagon-like peptide 1 (GLP-1) production, in turn reducing intestinal transit[18]. Activation of GPR41 by propionate or butyrate leads to inhibition of histone deacetylases, which regulate gene expression by removing acetyl groups in the histone structures[19]. This is implicated in both protection against cancer development and inflammation. Butyrate can also signal through GPR109A, with reduced inflammation and carcinogenesis as consequence. Furthermore, these receptors are expressed by lamina propria cells such as immune cells and the neurons of the enteric nervous system (ENS)[19,20]. The overall effects of signaling through these receptors in immune cells is reduced tumorgenesis and inflammation, for instance by the production of interleukin-10, an antiniflammatory cytokine. The ENS responds by increasing secretory activity and gut motility[19].

SCFA are metabolized in both the large intestine and the first pass through the liver, leading to low systemic concentration. However, acetate can reach concentrations of physiological relevance and signal to the brain to increase satiety and neurogenesis, and to white adipose tissue to reduce lipolysis[19]. Systemic responses to SCFA may also be indirect, such as the incretin effect where intestinal GLP-1 leads to pancreatic insulin secretion, or intestinal gluconeogenesis which has metabolically beneficial effects[21].

Ample evidence prove that GF animals have less body fat and higher energy content in feces and urine compared with CONV-R counterparts[22,23]. This phenomenon is partly counteracted by higher food intake in GF animals and it shows that the microbiota is required for efficient energy extraction and utilization from diet. SCFA used for energy is an important branch of this process, but it has been shown that also other molecular pathways are involved. For instance, angiopoietin-like 4 (ANGPTL4), is expressed in the small intestine and is a potent inhibitor of lipoprotein lipase which is an enzyme that mediate uptake of triglycerides from the blood into cells in muscle and adipose tissue. Expression levels of ANGPTL4 are reduced in CONV-R mice, leading to increased uptake of triglycerides in both adipose

tissue and muscle[23]. Furthermore, it has been shown that obese mice have more efficient energy uptake from the diet, compared with lean counterparts, as a direct consequence of altered microbial ecology[24].

Microbial regulation of intestinal epithelial renewal and immune system

The gut microbiota has been shown to modulate several physiological systems within the host. These effects range from fundamental differences in organ morphogenesis, and cellular turnover to more specific effects. The intestinal epithelium in CONV-R animals renews itself in 3-5 days, replacing the epithelial cells along the length of a villus. However, GF animals have reduced cellular renewal[25].

The host immune system has developed to provide a protective response to pathogens[26]. The maturation and functionality of the immune system requires interaction with non-self material and a major site for this interaction is the intestine. This process is strongly affected by the absence of intestinal bacteria. GF animals display several morphological and functional anomalies compared with CONV-R or specific pathogen-free (SPF; animals reared with limited microbiota to exclude pathogenic bacteria). Notably, secretion of antimicrobial peptides by specialized intestinal epithelial cells called Paneth cells is reduced in GF animals[26]. Furthermore, Peyers patches and lymph nodes located in the mesentery are smaller and contain less plasma cells, which are responsible for secretion of large amounts of immunoglobulin A (IgA)[26]. IgA opsonizes certain intestinal bacteria, thereby functioning as a barrier to bacterial invasion and a selective regulator of microbial composition[27].

1.2.1 Composition of the gut microbiota

The gut microbiota is composed of bacteria, viruses, archaea and fungi, of which the bacteria are the most well studied entity[28]. For the remainder of this text, the term microbiota should be interpreted as the bacterial fraction of the ecosystem. The gut microbiota consists of trillions of cells and it is estimated that the colonic content is 10^{11} microbial cells per gram of content, totaling approximately 0.2 kg of dry weight[29]. The colonic microbial niche has the highest density of all known microbial ecosystems. It has been estimated that within the host-microbial mutualism, the ratio between bacterial cells to nucleated host cells is ten to one. Including also anucleated red blood cells the ratio is 1:1[29]. At the genomic level, it is believed that the collective of genes found in the microbiota (the microbiome) is a 100-fold larger than the

host genome. To date, over 1000 different bacterial species have been identified in the human gut microbiota[30]. Within each host at least 160 bacterial species are typically found, and reduction of this individual diversity has been linked to metabolic aberrations[31].

Establishment of the gut microbiota is characterized by large initial fluctuations. Major differences are induced by the mode of birth (vaginal or Caesarean section), gestational age and use of antibiotics[28,32]. When following full-term babies over the first 24 weeks the microbiota is dominated by the genus *Bifidobacterium*. *Escherichia/Shigella* is found at higher levels one week after birth, and levels normalize at four weeks to remain stable up to 24 weeks after birth[33]. The adult microbial community is remarkably stable, and, without dramatic perturbations as e.g. during antibiotic treatment, a core group of approximately 40 bacterial species (representing 75% of the microbiota as measured by abundance) remain unchanged during one year[34]. Although characterized by high diversity at species level, it is strongly dominated by the two phyla Bacteroidetes and Firmicutes to which 90% of the bacteria belong[16]. Other phyla represented are Proteobacteria, Actinobacteria, Verrucomicrobioa, Fusobacteria and Tenericutes[16].

The gut is highly compartmentalized, with gradients of oxygen, pH, bile acid concentration and nutrient availability. This leads to difference in microbial composition along the intestine, but sequencing of bacterial 16S from stool and cecal samples provides a valuable proxy for the overall microbial composition. The relative sparse microbiota (10^3 cells/mL) of the proximal small intestine is dominated by *Lactobacillus* and *Streptococcus*[28]. In comparison the distal small intestine harbors higher numbers of microbes (10^6–10^8 cells/mL), which are more diverse and dominated by *Bacteroides, Clostridium, Enterobacteria, Enterococcus, Lactobacillus and Veilonella*.[28] The large intestine has the highest microbial density and is sometimes referred to as a bioreactor due to its multitude of metabolic processes. This microbial ecosystem, most densely populated of all presently known, consists of several genera, notably *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Escherichia*, *Lactobacillus*, *Peptostreptococcus*, *Propiobacterium*, *Ruminococcus* and *Streptococcus*[28,35].

Alterations of gut microbial composition have been observed in both experimental diet-induced and genetic obesity as well as in patients with obesity and inflammatory bowel diseases[24,36,37]. Recently, a remarkable recovery from *Clostridrium difficile* infection was observed after fecal microbiota transmission using material from healthy donors[38]. This highlights

the importance of a normal composition, in this case probably by restoring a more healthy competition among microbes.

1.2.2 Metabolite production by the gut microbiota

The metabolic activity of the gut microbiota is complex, and the produced metabolites reflect the complexity. It has been observed by mass-spectrometry-based metabolomics analyses that the mouse plasma metabolite profile is strongly affected by the presence of microbes[39]. The profiles revealed an overlap of nearly 4000 metabolites, and 152 unique metabolites in CONV-R mice compared with 52 unique for GF mice. In these samples 318 metabolites were significantly elevated and 148 were significantly reduced in CONV-R mice compared with GF mice. These changes may be the result of direct microbial metabolism, such as the conversion of tryptophan to indole by microbial tryptophanase, or a host response to altered compounds from the microbiota. For instance, some sulfate conjugates and glycine conjugates originating from hepatic detoxification are enriched in, or even unique to, CONV-R mice[39].

Microbial SCFA production is abundant. Primarily butyrate, propionate, acetate and lactate are produced. Acetate production is possible in many bacteria. Propionate is produced by *Bacteroides* spp., *Veillonella* spp., and others. Some notable butyrate producers are *Eubacterium rectale, Eubacterium hallii, Faecalibacterium prausnitzii* and *Roseburia* spp.. Among these, acetate is the most commonly utilized substrate, but *E. hallii* can also use lactate[19]. It is evident that cross-feeding mechanisms are common, which in the case of lactate has the important function to stabilize the luminal milieu by preventing lactate build-up[19].

Due to bile salt hydrolase (BSH) expressed by many bacterial species in the gut microbiota, the amino acids are deconjugated from bile salts in the intestinal lumen. Importantly, aerotolerant bacterial species such as *Lactobacilli* represent an important bile salt modulator in the small intestine. The deconjugation may have several biological roles. For instance, deconjugated bile acids (BA) have been shown *in vitro* to have lower antimicrobial effects, but *in vivo* the deconjugated BA confer antimicrobial protection by activation of the immune system. This indicates the crucial, and recently discovered, concept that BA signaling has different effects depending on their composition. It has also been hypothesized that the free amino acids may serve as nutrients for the microbes. Further effects by microbial modulation include 7α-dehydroxylation which is required for production of the secondary BA litocholic acid and deoxycholic acid.

1.3 The intestines

1.3.1 Intestinal functions – an overview

The intestine is responsible for nutrient uptake from the ingested food. The intestinal wall is made up of a circular and a longitudinal muscle layer which enable peristalsis.

In the mouth food is digested to smaller pieces and amylase acts to break down starch to smaller sugars. Food is then transported through the esophagus to the stomach, where it is further processed. The luminal pH of the stomach is very low which aids in protein digestion and acts as a microbial barrier, thus representing the first selection point for microbial presence in the intestine.

Gross morphology and functions of the small intestine

The small intestine is divided into duodenum, jejunum and ileum in humans, but has less clear borders in mice. Because of the acidic discharge from the stomach, the duodenum secretes large amounts of bicarbonate to neutralize luminal pH[40]. The duodenum is also the site for bile release into the intestine. In brief, bile consists of enzymes released from the pancreas (mainly to break down proteins to amino acids) and BA produced by the liver. BA are primarily involved in fat uptake by emulsifying lipids. Due to its detergent and proteolytic activity, bile acts as a bactericidal or bacteriostatic agent, thus representing the second point of protection from and selection of microbes. However, recent research has provided ample evidence that BA are important signaling molecules regulating several aspects of immunology and metabolism.

Distally to the duodenum, in the jejunum and ileum, the bulk absorption of nutrients takes place. The surface area of the intestine is debated and some estimates[41] claims it is up to 300 m^2, whereas others suggest it to be around 30 m^2, the length being around 3.5 m[42]. This surface enhancement is due to several morphological features of the intestine. Macroscopically this includes the circular folds of the intestine and the villus structures[42]. Villi are dynamic, vascularized and innervated structures which are covered by the intestinal epithelium.

Cellular composition of the epithelium

The epithelium is made up of several different cell types carrying out different functions. The majority of intestinal epithelial cells (IEC) are columnar absorptive enterocytes, which possess apical microvilli that further dramatically increase the luminal surface area of every enterocyte, and thus the intestine. Other cell types are summarized in Table 1[43,44].

Table 1 Overview of epithelial cells along the intestine. Abbreviations used: 5-HT, serotonin; AMP, antimicrobial peptides; CaSR, Calcium sensing receptor; FFAR free fatty acid receptor; LPAR5, lysophatidic acid receptor 5; GB, gall bladder; GIP, glucose-dependent insulinotropic peptide; GLP, glucagon-like peptide; GPR, G-protein coupled receptor; IL, interleukin; Li, Large intestine; PP, Payers patches PYY, peptide YY; Si, small intestine; TLR, toll-like receptor.

Cell type	**Function**	**Receptor**	**Location**
Goblet cell	Mucus secretion		Si, Li, Along the villi
Paneth cell	AMP secretion		Si, crypts
Tuft cell	Protection against parasite infection, IL-25 secretion		Si
Microfold cell	Antigen uptake		Si, Li, in PP
Enteroendocrine cells	-	-	-
Enterochromaffin cell	5-HT, gut motility, nausea	FFARs 2-3, toxin receptors, TLRs	Si, Li
I cell	GB contraction, pancreas secretion, CCK, (5-HT)	GPCRs, FFA1, LPAR5, CaSR, TLRs	Proximal Si
K cell	GIP secretion (stim. of insulin secretion)	GPR119, GPR120, FFAR1	Proximal Si
L cell	GLP-1, GLP-2, PYY secretion	T2Rs, FFARs, GPR119, GPR120, LPAR5, CaSR	Distal Si, Li
M cell	Motilin, peristalsis	Bile receptors	Si
N cell	Neurotensin, inhibition of intestinal contractions	FFARs	Si, Li
S cell	Secretin secretion (stim. of bicarbonate)	Acid receptor	Proximal Si

The microvilli harbor transmembrane channels and pumps that are required for uptake of nutrients and osmotic regulation. These serve to provide optimal ratios of specific ions intracellularly and extracellularly. Much of the absorption uses the gradient of Na^+ as driving force; organic solutes such as amino acids and glucose are co-absorbed with Na^+ against their own osmotic gradient, but following the gradient of Na^+[45]. During physiological

conditions, secretion of ions is a prerequisite for the absorptive function and to control cell swelling due to osmotic pressure following absorption of ions. During diarrheal responses to pathogens, ion secretion is strongly enhanced, leading to increased water flux to the intestinal lumen to clear the infective agents[45].

Nutrient sensing, gut hormones and nervous signaling

The small intestine is also an important player in nutrient sensing and signal transduction to distant organs. In fact, the gastrointestinal tract can be viewed as the largest endocrine organ; it produces more than 20 peptide hormones[46]. These are involved in regulation of several systemic responses to food intake. Some, for instance gastric inhibitory peptide and GL-1, stimulate insulin secretion when secreted, a response referred to as the incretin effect[47,48]. Enteroendocrine cells are responsible for the production of gut hormones, and the different subsets of cells express receptors that enable appropriate sensing. For instance, SCFA and BA have been shown to stimulate GLP-1 secretion through GPR41 and TGR5, respectively, thereby promoting insulin release[49].

Innervation of the intestine is extensive and the number of neurons, 400-600 million, is equal to that of the spinal cord. Important functions of the enteric nervous system (ENS) include regulation of contractile activity of the muscle layer, transmucosal fluid fluxes and local blood flow. The ENS has capacity to function even when separated from the central nervous system (CNS), but under normal conditions it is a complex network integrating the ENS with the CNS[50].

1.3.2 Intestinal barrier function

The intestine contains all domains of life, and a dynamic and functional border to separate the host from the intestinal contents is crucial to mammalian physiology. While allowing uptake of nutrients and controlled secretion, the epithelium must also serve as a fence to avoid harmful pathogens to invade their host[51].

Intestinal mucus – more than lubrication

In addition to secretion of antimicrobial peptides and antibodies, the epithelium also produces large quantities of mucus. Goblet cells produce, store and secrete mucins upon stimulation. Mucins make up the protein backbone of mucus, and are heavily glycosylated to bind water. In the small intestine mucus lubricates the epithelium and offer protection against

bacterial adhesion[52]. In contrast, the mucus layer of the large intestine, which has slower transit time and higher bacterial load, is compartmentalized. The colonic epithelium is coated by a firmly adherent mucus layer that is believed to be largely sterile[52]. This layer devolves to a loose outer layer of mucus which contains bacteria. In addition to a physical barrier, the mucus also delays the diffusion of antimicrobial peptides and antibodies to create a gradient with high concentration close to the epithelium. However, it should also be considered that the heavy glycosylation of mucins (notably Mucin) serves as energy source for certain bacteria[53].

Epithelial permeability

In this context, permeability is the facility by which molecules are subjected to non-mediated passage across the epithelium. The barrier function of the epithelium can be divided into transcellular and paracellular pathways. The transcellular path is mainly governed by the impermeable plasma membrane of the IEC, whereas the paracellular route is controlled by cell-cell junctions. From apical to basal localization these are: tight junction (TJ), adherens junctions, desmosomes and gap junctions[51].

The rate limiting structures of paracellular permeability are the TJ. These are multi-protein complexes consisting of up to 30 different proteins. These proteins can be divided into two categories; transmembrane and anchor proteins[51]. Anchor proteins serve to link the transmembrane proteins to the cytoskeleton and provide physical integrity to the structure. The connection to the cytoskeleton also provides dynamics because the actomyosin ring, encircling the cells apically, is contractible and can thereby widen or tighten the paracellular space at the TJ[51]. This contractive regulation is in part controlled by myosin light chain kinase (MLCK)-dependent phosphorylation. Upon phosphorylation of myosin II regulatory light chain, the ring contracts and increases paracellular permeability. Pharmacological inhibition of MLCK prevents contraction and restores barrier function[54]. Many endogenous factors regulate MLCK activity, such as the proinflammatory cytokine tumor necrosis factor (TNF)[55]. By using anti-TNF antibodies the deleterious effect can be reversed, a strategy applied in inflammatory bowel diseases, where levels of TNF and other cytokines are elevated[56]. In addition TJ are also responsible for formation of apical and basolateral membrane domains, i.e. cell polarization, such that a separation of membrane transporters and channels is possible[51].

Permeability across the epithelium is determined based on ion flux and passage of larger molecules. Both reflect paracellular leakage through TJ, but

since the epithelium has an inherent ion transport this needs to be accounted for during measurements of ions[57]. Because ions are charged, they are readily detected as current and the barrier function is expressed as transepithelial resistance (TER). Permeability to larger molecules is measured by estimating the passage of labeled substances of known size, such as radioactive ^{51}Cr or fluorescently labelled sugars[57].

Notably, the first characterized TJ protein is zonula occludens-1 (ZO-1), which is a key anchor molecule. Genetic deletion of the ZO-1 gene (*Tjp1*) in mice leads to embryonic lethality due to hemorrhage, indicating its important role also in endothelial integrity.[58] The ZO-family of proteins (ZO-1/2/3) are structurally similar, but have separate functions as knockout of *Tjp1* cannot be compensated for by any of the other proteins[58].

Many transmembrane proteins are involved in shaping the cell-cell bindings in the TJ and thereby regulating the permeability. Occludin was the first protein to be identified as a transmembrane member of the TJ[59]. It interacts with ZO-1 intracellularly and occludin on the adjacent cell extracellularly[60]. Unlike ZO-1, complete genetic knockout of occludin did not cause lethality, and TJ were formed seemingly intact[61]. However, the mice suffered various inflammatory responses and histological abnormalities. By means of small interfering RNA silencing of the occludin gene it was shown that occludin regulates flux of macromolecules *in vitro* and *in vivo*[62]. This finding is in line with the characterization of the occludin deficient mouse which did not show an altered electrophysiology[61]. Instead, members of the large claudin family of proteins largely determine the electrophysiological properties of the TJ. There are 26 claudins in human and 27 in rodents[63]. The extracellular domains have different amino acid composition which leads to pores that are charge selective. For instance, claudin-2 is typical for leaky epithelia and known to form a cation specific channel[64], whereas claudin-1 and -8 act to seal the epithelium and reduce the passage of cations[65].

Consequences of abnormal intestinal permeability

Increased permeability, the consequence of impaired barrier function, leads to uncontrolled influx of harmful substances from the intestinal lumen into the interior milieu. This is implicated in several diseases, notably diseases with inflammatory etiology. Locally in the intestine, increased permeability is seen in patients with Crohn's disease (CD), one of the major inflammatory bowel diseases (IBD), and their first degree relatives[66]. This is true also for ulcerative colitis (UC)[66]. While UC is confined to the colon, CD can be manifested throughout the intestine. Both are characterized by serious

inflammation of the affected areas and initial treatment includes immunomodulation, which also improves the intestinal permeability by reducing levels of pro inflammatory cytokines.

A drastic increase in intestinal permeability is seen also in celiac disease where wheat protein induces a potent immune response with damage to the epithelium as a consequence[67]. Furthermore, in patients suffering from burn injuries, a common complication is sepsis with intestinal origin[68]. This is thought to be due to high levels of circulating cytokines, which affect the intestinal epithelium and increase the permeability. In turn, this increases the influx of bacterial toxins, notably lipopolysaccharides (LPS), creating a cycle of events that enhance each other, with dramatic consequences.

In more chronic states, a low-grade systemic inflammation has been suggested to be part of the metabolic syndrome, which is defined as an array of comorbidities including obesity, elevated blood sugar, insulin resistance and high blood pressure. In animal models of genetically-induced obesity (obese or diabetic mice) increased permeability appears to be a hallmark[69]. Indeed, increased intestinal permeability is associated also with human obesity and[70,71], albeit at low magnitude, this elevation takes place over extended time periods and impacts distal organs such as the liver and adipose tissue. A decade ago it was shown that metabolic endotoxemia may act as an initiator of obesity and insulin resistance in mice[72]. Mice that were fed a high-fat diet (HFD) displayed elevated plasma LPS levels, and upon experimental endotoxemia (LPS infusion by subcutaneous osmotic pumps) several features of HFD-fed mice were recapitulated; body weight gain, visceral adipose tissue mass and expression of proinflammatory cytokines. Notable is also the increased visceral adiposity often seen in patients suffering from IBD, pointing to a role for intestinal permeability at the crossroads of inflammation and metabolic dysregulation[73].

The low-grade inflammation in obesity manifests also as macrophage infiltration into adipose tissue. Accumulation of macrophages, and other leukocytes, makes up the primary source of secreted inflammatory cytokines from adipose tissue. It has been shown that gut-derived LPS induce the accumulation of adipose tissue macrophages[74].

1.3.3 Angiogenesis

Overview of angiogenesis

All eukaryotic cells require oxygen for efficient energy production. On single cell level, oxygen diffusion from the surroundings covers the need, but more complex organisms require a vascular system for appropriate oxygen delivery[75]. In addition, the vascular system also transports waste, enables hormonal signaling and regulation of cellular responses, and serves as transportation of immune cells[75]. During development, when organisms are expanding and requirements for oxygen and nutrient transport increase, a vascular plexus is formed *de novo* in a process referred to as vasculogenesis. In contrast, angiogenesis is defined as the creation of new blood vessels from preexisting ones. This process occurs in two different ways; angiogenic sprouting and intussusceptive vessel splitting[75].

A well balanced regulation of vascular function is of crucial importance. In case of injury, coagulation prevents excess blood loss and the subsequent obvious risks. However, coagulation must not be uncontrolled or exaggerated due to the risk of thrombus formation, leading to blood stasis and ultimately risk of necrosis or death. Instead, following strictly regulated coagulation, angiogenesis follows to reestablish adequate blood supply to the affected tissue. In an adult animal, blood vessels are kept in a quiescent state, with a lining of long-lived endothelial cells supported by podocytes[75]. A common marker for endothelial cells is platelet endothelial cell adhesion molecule 1 (PECAM1, also known as CD31) which is expressed mainly on endothelial cells and mobile cells such as platelets. The endothelium however remains responsive to angiogenic cues. On a molecular level, angiogenesis and coagulation coincide. It is interesting to note that platelets, which aggregate during coagulation, contain and secrete several factors that have positive or negative impact on angiogenesis.

In brief, the angiogenic cascade is a series of events leading to expansion of the vasculature[76]. Initially vascular endothelial growth factor (VEGF, formerly known as vascular permeability factor[77]) cause an increased permeability leading to leakage of plasma proteins which form an extravascular temporary matrix that serves as a migratory scaffold. Endothelial cells migrate with a tip cell leading the migration. Tip cells express delta-like ligand 4 (Dll4) and VEGF-receptor (VEGFR), both of which are crucial for the tip cell phenotype. VEGFR is required to direct the new vessel toward the VEGF gradient. Dll4 is a ligand for Notch signaling, and serves to balance the formation of tip and stalk cells to avoid

uncontrolled branching[78]. General pharmacological or genetic inhibition of Notch signaling leads to multiple tip cells and disorganized vascular structure. In contrast, when Dll4-Noch signaling is intact, the stalk cells divide to "follow" the migrating tip cell and form the vascular lumen[78].

The angiogenic cascade is also orchestrated by angiopoietin 1 (Ang1), Ang2 and their tyrosine kinase receptor Tie2. Mice deficient in Tie2 die around embryonic day 10 due to hemorrhage and display reduced vascular complexity[76]. A similar phenotype is seen in Ang1-deficient mice. In contrast, Ang2-deficient mice on C57Bl/6J background are born normally but display vascular abnormalities in adulthood. This shows that Ang1-Tie2 interaction is indispensable during embryogenesis, however in adult angiogenesis, Ang-Tie2 signaling is thought to occur mainly to assist in regulating the stalk cells during sprouting[76]. As the perivascular cells are an important source of Ang1, this signaling is thought to be crucial for vascular stability following angiogenesis[76].

Coagulation and protease activated receptors (PAR)

Coagulation may occur via the intrinsic or the extrinsic pathway. The intrinsic pathway is initiated by activated platelets or collagen and requires several steps to form a stable fibrin clot; the extrinsic pathway is initiated by tissue damage as detected by tissue factor (TF) activity, and converges onto the intrinsic pathway by activating coagulation factors IX and X to form active forms (IXa and Xa, respectively). However, the role of TF has expanded from a simple initiator of coagulation in the vessel wall, to a ubiquitously expressed transmembrane protease coreceptor with a range of functions. TF is the cellular receptor of coagulation factor VII (FVII), rendering FVII active, which in turn can activate factor X (FX). This factor has proteolytic activity on prothrombin, thereby generating thrombin that has strong affinity for several protease activated receptors.

A main signaling path for TF is through PARs, which are expressed in several tissues including many epithelia[79]. These are transmembrane surface receptors that belong to the G-protein-coupled receptor (GPCR) family. However, unlike other GPCR's, PAR's are not activated by binding of a soluble ligand, but by proteolytic cleavage of an extracellular loop[80]. This prototypically occurs by thrombin mediated cleavage however, a vast array of proteases have PAR activating capacities. As a consequence, activation is irreversible. However, there are also proteases that cleave the extracellular domain in ways that render the PAR irreversibly inactive.

There are four PAR's, PAR1-PAR4, which each have their distinct repertoire of activators and inhibitors and even if they have overlapping tissue expression pattern, the PAR's have various physiological outcomes upon activation. These include inflammation, cell migration, cell growth and receptor transactivation. The final response elicited also depends on the interaction with different G-protein subunits, although the exact mechanism for this biased response remains unclear. Furthermore, to the complexity of PAR activity regulation it can also be added that post-translational modifications and subcellular localization contribute strongly. For instance phosphorylation of the cytoplasmic tail leads to rapid dissociation from the GPCR and inhibited signaling. TF together with FVIIa has been shown to strongly induce PAR1 and PAR2 activation[81].

Angiogenesis in health and disease

Perhaps most prominent is the link between angiogenesis and disease progression in cancer. Because cancer is an expanding tissue with extraordinarily high demands in oxygen and nutrition, it relies heavily on sprouting angiogenesis from the surrounding tissue. This process is required for each new tumor to grow during metastasis, making anti-angiogenic treatment a compelling option in this setting. Tumors frequently produce angiogenic factors to promote this process, and recent treatment strategies in a range of different cancers also include agents that block angiogenesis. For instance blockade of VEGF-induced angiogenesis by tyrosine kinase inhibitors to block VEGFR signaling, or antibodies directed to VEGF has showed promise in clinical studies. Treatment options that include binding to several members of the VEGF family (VEGF-A, VEGF-B and placental growth factor) showed even stronger effects. Interestingly, it was hypothesized that targeting angiogenesis, being a relatively rare occurrence in adults, would have few side effects. However, blocking angiogenesis led to several side effects, notably gastrointestinal perforation.

The hallmark feature of obesity is an expansion of adipose tissue, which relies on increased vascularization through angiogenesis[82]. Adipose tissue is one of the most highly vascularized tissues in the body and a capillary network surrounds each adipocyte. Indeed, adipose tissue modulates its angiogenic environment through several mechanisms. Primarily adipose tissue expresses several proangiogenic substances from the VEGF family[82]. Furthermore it has been shown that hormones produced by adipocytes, such as leptin and resistin as well as neuropeptide Y produced by macrophages in the adipose tissue, contribute to angiogenesis. It has also been hypothesized that adipose tissue-derived stem cells have the capability to differentiate into

vascular cells based on surgical procedures where adipose tissue is transplanted to infarcted hearts[82].

Intestinal angiogenesis

The intestine is subjected to dramatic postnatal vascular remodeling. Shortly after birth, the vascular system of the villus is very simple, consisting of an unbranched vessel at the villus core. This system evolves to a more complex network, presumably in order to optimize transport of nutrients from, and distribution of circulating immune cells to, the intestine. Earlier studies on this matter showed that the gut microbiota contributes strongly to this process. The GF mouse had approximately 50% less vascularization as assessed by microscopical quantification, but already 10 days following colonization with a complete microbiota, the vascularization appeared to stabilize at the normal level[83]. Interestingly, it was also established that colonization with a single known microbial species (*Bacteroides thetaiotaomicron*) was sufficient to recapitulate the development[83]. It was hypothesized that Paneth cells were involved in the establishment of a microbially modulated mature vascularization. However, no specific angiogenic factor produced directly by Paneth cells was identified and GF Paneth-cell deficient mice had a similar reduction in vascularization, when compared with *B. thetaiotaomicron* colonized mice[83], indicating that much is still unknown.

1.4 The liver

1.4.1 Liver functions

Overview

The liver plays a central role in diverse physiological systems such as energy metabolism, detoxification of xenobiotics and immunology. Receiving venous blood directly from the intestine, the liver is exposed to all compounds taken up by the blood. The detoxification system includes a two-phase process; however, not all compounds are processed through both steps. During phase I the compound is most commonly oxidized by the activity of members of the cytochrome P450 (CYP450) superfamily of proteins, and sometimes generates products more harmful than the substrate[84]. During phase II the intermediary metabolite is conjugated to any of a number of conjugates (e.g. sulfate, glucuronidate, amino acids) before excretion to bile or serum, leading to fecal or urinal excretion, respectively[84].

The liver in energy metabolism

The liver is placed at the center of mammalian energy metabolism. Due to its capability to store and redistribute energy systemically, it is involved in regulation of many branches of metabolism. Glucose is taken up (and, when needed, excreted) through the bidirectional glucose transporter 2 (GLUT2). Absorbed glucose can then be used to produce energy via glycolysis and the mitochondrial citric acid cycle. Unlike glucose, fructose is taken up by GLUT5 but can also be converted to pyruvate and provide substrate for the citric acid cycle. Thus it serves as substrate for both glycogen synthesis and lipogenesis[85]. In the fed state, glycogen is produced by the liver to store glucose with less effects osmotic effects. Glycogen is rapidly depleted by glycogen phsophorylase during the initial phase of fasting[86]. Accordingly, the liver relies on other mechanisms to provide energy during extended periods of fasting.

One way to maintain blood glucose at a steady level during prolonged fasting is gluconeogenesis. In essence, this pathway relies on reversing the outcome of glycolysis and the citric acid cycle. Most steps in these processes are either reversible by the same enzyme, or catalyzed by different enzymes with opposing effects[86]. In order to perform gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), the rate determining enzyme, catalyzes the conversion of oxaloacetate derived from the citric acid cycle to phosphoenolpuryvate. Substrates for gluconeogenesis include amino acids, glycerol and SCFA[86].

Lipid production, storage and systemic delivery are performed by the liver. Dietary lipids can be stored but the liver also synthesizes lipids *de novo* using non-lipid substrates. Central to this pathway is fatty acid synthase (FAS) which is the rate limiting enzyme converting malonyl coenzyme-A (CoA) to palmitate[86]. Production of malonyl-CoA relies on release of citrate from the citric acid cycle, which is transformed to acetyl CoA by ATP citrate lyase. Acetyl CoA is then converted to malonyl CoA by acetyl CoA carboxylase[86]. The produced palmitate is a fatty acid of 16 carbons length, but can be elongated by elongation enzymes (notably elongation of long chan fatty acids (ELOVL) enzymes) that add two carbons transferred from malonyl-CoA. Stearoyl-CoA desaturase 1 (SCD1) can desaturate stearic acid to produce oleic acid. The fatty acids produced *de novo* may either be stored locally in lipid droplets, or transported out from the liver in very low density lipoprotein particles secreted into the blood where they serve as energy source for distant organs or are taken up by adipose tissue[86]. Alternatively, lipids can also be used for hepatic energy production by β-oxidation in

mitochondria, which can also lead to formation of ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) which are exported to the systemic circulation and provide energy mainly for the brain during fasting[86]. Mitochondrial β-oxidation is intimately regulated by lipogenesis in that the malonyl-CoA, the substrate for FAS, is a potent allosteric inhibitor of carnitoyl palmitate transferase which imports long-chain fatty acids into the mitochondria[86].

High caloric diets are known to contribute to obesity and *de* novo lipogenesis in the liver. In particular, recent research has pointed to the role of diets rich in (simple) carbohydrates. Regulation of *de novo* lipogenesis is complex and determined by fluctuations between fasted and fed state, primarily as determined by levels of insulin and glucagon[86]. Also high intracellular levels of cyclic adenosine monophosphate (cAMP) serve as an indicator of the fasted state. Acute regulation of the enzymes involved in *de novo* lipogenesis may be allosteric or due to post-translational modifications such as phosphorylation[86].

However, long-term control of *de novo* lipogenesis largely depends on transcriptional regulation by different transcription factors. Two central regulators are carbohydrate response element binding protein (ChREBP) and sterol response element binding protein-1c (SREBP-1c). As indicated by the name, ChREBP is induced by intracellular levels of carbohydrates. SREBP-1c, despite its name, mainly regulates enzymes in *de novo* lipogenesis in response to insulin and upon stimulation by liver X receptor α (LXRα)[87]. Lipogenic enzymes regulated by these transcription factors include FAS, ACC, SCD1.

Bile acid metabolism

A major role for the liver is to produce bile, store it in the gall bladder and release it to the duodenum upon food intake. Bile is composed of BA, cholesterol and bilirubin. Furthermore, bile is also the main excretion route of harmful substances that have been modified in the liver during detoxification. BA are produced by hepatocytes, which make up approximately 80% of the cell population in the liver, and are released to the gall bladder via the bile canaliculi. BA are amphipathic molecules, which assures their function as detergent for dietary fat in the intestinal lumen[88]. The hydrophilic-lipophilic balance is determined by the number and position of hydroxyl groups on the nucleus cholesterol. By creating micelles, BA are essential for absorption of lipids (and other hydrophobic substances) from the intestine.

Cholesterol serves substrate for BA synthesis, and it is estimated that approximately 0.5 grams of cholesterol is used daily for BA production. Cholesterol is modified in a series of enzymatically catalyzed steps where the molecules undergo modifications to the cholesterol ring and side chains (notably elongation and oxidation)[89]. The rate limiting enzyme is cholesterol 7α-hydroxylase[90]. Primary BA produced by this pathway are cholic acid and chenodeoxycholic acid (CDCA) in human. In mice CDCA is converted to α-muricholic acid (αMCA) or βMCA, which together with ursodeoxycholic acid make up the primary mouse BA, presumably through a Cyp2c70 dependent pathway[91]. In high concentration, the BA are cytotoxic and are therefore rapidly conjugated to amino acids, which are less reactive. These conjugated BA are then exported to the gall bladder for release to the intestine.

Bile acids are efficiently reabsorbed from the distal small intestine via the enterohepatic recirculation, and 95% are taken up from the intestine to be reutilized[89]. The ileal bile acid transporter is expressed apically in the ileal epithelial cells, and organic solute transporter-α and –β (OST-α, OST-β) are expressed basally to transport BA's across the epithelium to the portal vein[92]. The bile acids are then taken up by hepatocytes via several transporters and can be reprocessed and transported to the gallbladder.

Signaling through bile acid receptors

Regulation of bile acid synthesis is tightly controlled[89]. This requires a system for detection of BA throughout the enterohepatic system. The two primary receptors for BA are expressed in both the liver and in the intestine. The nuclear receptor farnesoid X receptor (FXR) was identified in 1999 and membrane-type receptor for bile acids (M-BAR, or more commonly TGR5) was identified in 2002[88].

FXR is expressed widely in the body, with highest levels in the liver and intestine. It is also expressed in kidney, adrenal glands, immune cells and white adipose tissue. Intestinal FXR expression increases dramatically along the length of the intestine with maximal expression in the terminal ileum[88]. Activation of FXR in the epithelium stimulates expression of fibroblast growth factor-15 (FGF15) in mice and the ortholog FGF19 in humans. FGF15/19 is transported via the portal vein to the liver where it binds to its receptor complex, consisting of FGF receptor-4 (FGFR4) and βKlotho[88]. Activation of the receptor complex leads to a signaling cascade that via JNK and ERK pathways, as well as the cytoplasmic tyrosine kinase Shp2, inhibit the expression of Cyp7a1. Furthermore, reabsorbed BA can also bind directly

to FXR in the liver, which leads to induction of small heterodimer partner (SHP), a potent inhibitor of Cyp7a1 expression. Thus, FXR is crucial in a negative feedback loop that regulates bile acid synthesis.

FXR activation is highly complex, involving several levels of regulation. FXR response is tissue specific and involves both co-activators and co-repressors[93]. For instance, FXR forms a heterodimer with the nuclear receptor retinoid X receptor (RXR), which responds to retinoic acids[88]. Much effort has been put into deciphering FXR signaling, which has unraveled an extensive network of FXR-regulated pathways.

By generating whole body FXR knock-out (*Fxr*$^{-/-}$) mice it has been shown that lipid metabolism is under regulation of FXR. Levels of triglycerides and cholesterol are elevated in both serum and liver in the *Fxr*$^{-/-}$ mice indicating a negative regulation[94]. The exact underlying mechanisms are still under investigation, but it has been shown that the gut microbiota metabolizes a very potent FXR-antagonist, tauro-β-muricholic acid, in mice and thus alleviating strong inhibition seen in GF mice[95]. Enrichment of tauro-β-muricholic acid has also been observed in mice treated with tempol, an antioxidant with capacity to modulate the gut microbial composition to favor Bacteroidetes, reduce Firmicutes and, notably, act with strong selectivity against lactobacilli[96]. The loss of lactobacilli was shown to reduce the overall bile salt hydrolase activity in the microbial community and thus inhibit deconjugation, explaining the elevated levels of tauro-β-muricholic acid. The link between microbiota and FXR appears partly reciprocal because *Fxr*$^{-/-}$ mice display alterations in their microbial ecology compared with wild-type mice fed the same high fat diet, indicating that genetic loss of FXR is a modulator of microbial composition. At phylum level, *Fxr*$^{-/-}$ mice, like tempol treated mice, display decreased Firmicutes and increased Bacteroidetes, and upon colonization of GF wilde-type (WT) mice with the different microbiota, the obese phenotype of WT mice could be partly recapitulated[96].

Recent studies have highlighted the requirement of tissue specific knockouts as FXR stimulation in different parts of the enterohepatic circuit may have different effects. Therefore, mice with intestine-specific *Fxr* deficiency were generated and fed a high fat diet (HFD). Interestingly, these mice showed reduced hepatic lipid content, whereas liver-specific *Fxr* knock-out mice fed the same diet showed no reduction[97]. The specific role of FXR stimulation was to, when intact, induce intestinal ceramide synthesis. Ceramides are then transported to the liver where SREBP-1c is activated, leading to induction of *de* novo lipogenesis. This is indicative of the important role of intestinal FXR

signaling in the regulation of liver metabolism. However, simultaneously to this study, another study was published where a synthetic BA analogue that is not absorbable from the intestine (and thus acts to only stimulate intestinal FXR) was shown to mediate the opposite effects with reduced steatosis and expression of lipogenic genes including *Srebf* (SREBP-1c)[98], underpinning the complexity of FXR-mediated metabolic signaling.

FXR has also been shown to be involved in immunomodulation, and thus in inflammatory diseases. From studies concerning IBD, it has been shown that overall microbial diversity is reduced, and that bacterial groups with BSH activity may have lower relative abundance, leading to BA dysregulation[99]. Indeed, in patients with Crohn's disease reduced intestinal FXR activation has been shown[100]. This could lead to a loss of the otherwise anti-inflammatory responses elicited by FXR stimulation. DSS-induced IBD in rodents has been shown to be ameliorated by FXR stimulation, correlating with an improved epithelial barrier and less transepithelial flux of pro-inflammatory substances[101].

Bile acids can also signal through other receptors such as transmembrane G protein-coupled receptor 5 (TGR5) which is ubiquitously expressed in both mice and humans. Interestingly, it is not expressed in hepatocytes, indicating distinct differences between TGR5 and FXR[88]. Importantly, for both receptors different bile acids show different affinities and the bile acid composition, largely modulated by microbial activities, is therefore of importance for the downstream effects of bile acid receptor signaling. While chenodeoxycholic acid is the most potent FXR agonist, TGR is more strongly activated by litocholic acid and deoxycholic acid. TGR5 is involved in overall metabolism, in part by regulating energy expenditure. Similar to FXR, TGR5 activation also reduces expression of inflammatory cytokines[88].

Other BA receptors include pregame X receptor (PXR), vitamin D receptor (VDR) and constitutive androstane receptor (CAR)[88]. These are responsive to litocholic acid and the resulting activation leads to reduced BA synthesis (PXR), reduced bile salt synthesis (VDR) and increased detoxification (all)[88]. Notably VDR is expressed in the intestine but not in the liver[88].

1.4.2 Non-alcoholic fatty liver disease

Overview

Non-alcoholic fatty liver disease (NAFLD) is associated with obesity and considered part of the metabolic syndrome. NAFLD is mainly characterized as hepatic lipid content over 5-10% of the liver weight without abnormal alcohol consumption (or consumption of certain medications)[102]. It is also the most common liver disorder worldwide. NAFLD does usually not cause symptoms, but and is present in a significant part of the population. Typically around one-third is cited for developed societies and around 20% is the world median[103]. In essence, hepatic steatosis is the outcome an imbalance between lipid input from diet or *de novo* lipogenesis and the export of lipids.

It is believed that hepatic steatosis represents the first "hit" in a pathological system involving a number of hits. The initial phase of NAFLD is reversible and relatively benign but it is associated with dyslipidemia, high blood pressure and systemic insulin resistance. However, in around 10% of patients, NAFLD progresses to non-alcoholic steatohepatitis. This progression may be induced by a "second hit" such as; lipotoxicity due to the elevated hepatic lipid levels, increased levels of reactive oxygen species formed during oxidation of fatty acids, or endotoxemia of intestinal origin leading to increased inflammation. The progress to NASH increases the risk of metabolic complications, and is also a prerequisite for progression to hepatocellular carcinoma and end stage liver disease with liver failure as consequence.

Risk factors and mechanisms

The underlying etiology to NAFLD has not been mapped to completeness but several risk factors have been linked to development of NAFLD. Broadly these can be characterized to environmental factors such as diet, host genetics and gut microbiota, which in turn are interconnected[104]. Environmental factors are dominated by diet and the role of high fat and/or high carbohydrate has been studied intensely[105].

In the case of lipid rich diets, fat is taken up and may contribute directly to increased storage of lipids. However, the lipid quality has been shown to be important. Diets rich in saturated and monounsaturated FA's, from e.g. lard, are a well-established inducer of hepatic steatosis in rodents. The role of polyunsaturated fatty acids (PUFA) is more complex and it has been shown that n-3 PUFA appear protective, whereas n-6 PUFA contribute to

steatosis[106]. This is partly because the n-6 PUFA serve as precursors for pro inflammatory eicosanoids.

Intake of excess carbohydrates has been shown to be inducers of *de novo* lipogenesis, converting the carbohydrates to lipids within the liver. A key inducer to this process is insulin. However, fructose has been found to be a highly lipogenic sugar. Interestingly, the intake of simple carbohydrates (especially sucrose and fructose) has risen dramatically in most societies and is paralleled by increased prevalence of NAFLD. Hepatic uptake of fructose occurs mainly by glucose transporter 5 (GLUT5) and is insulin independent[107].

The prevalence of NAFLD is not uniform over populations. Male sex is a risk factor, and it seems that Hispanic-americans are overrepresented, whereas African-americans are protected. This ethnic discrepancy has been attributed to polymorphisms in the gene patatin-like phospholipase domain-containing protein 3 (PNPLA3), which has a role in hydrolysis of fatty acids[108,109]. The polymorphism leads to structural alterations near the catalytic site and decreased enzymatic activity. Reduced fatty acid hydrolysis promotes steatosis. Furthermore, polymorphisms in this gene have been associated with progression of NAFLD to NASH and subsequent hepatocellular carcinoma, the end stage of a NAFLD.

2 AIM

Paper I

To elucidate the underlying mechanisms of microbiota induced angiogenesis in the intestine.

Paper II

To test the hypothesis that the microbiota regulates small intestinal permeability at steady state, as well as to investigate potential underlying causes.

Paper III

To investigate the role of the gut microbiota on the development of NAFLD in mice fed a high-carbohydrate diet (without fat).

3 METHODOLOGICAL CONSIDERATIONS

This section covers conceptual aspects of the methods applied in the papers that form the basis for this thesis, and that are not feasible to comment in the manuscripts. For detailed methodological descriptions, please refer to the individual papers.

3.1 Using mouse models to study human disease

The majority of biomedical research is conducted to increase understanding of human disease in order to establish or improve treatments. The vast majority of data in this thesis is derived from work using mice. Performing *in vivo* experiments in animals is, and has for long time, been a cornerstone for knowledge expansion in biomedical research. Relevant for this thesis, there are three reasons for this; (1) a non-animal based model corresponding to GF animals is not present, and in a foreseeable future mice are unlikely to be replaced in this branch of research, partly due to their small size which make them feasible to keep in GF isolators (2) the possibility to use specific knock-out mice (or mice where specific genes are knocked down) helps in deciphering inter-organ signaling events, especially when relevant inhibitors are not applicable (due to availability or specificity), and (3) the possibility to modulate the experimental set-up with e.g. strict dietary alterations *in vivo* (at "whole body") level.

The main reasons to use animals in research is to elucidate mechanisms, their similar genetics (especially in inbred mice), and their compliance to interventions. Furthermore, most *in vitro* systems fail to represent the complex *in vivo* situation. However, *in vitro* studies facilitate dissection of organ specific cellular pathways, but this mostly requires previous work *in vivo* to direct the *in vitro* experiments. Using animals has been debated from an ethical view over the past fifty years. Initially viewed as scientific necessity, today's view on animal use is characterized by awareness and in most countries regulations to assure animal welfare. However, in the growing biomedical research field animal use parallels the expansion. A major concern is the translatability to human relevance. In a spectrum of diseases where animal studies are the foundation, experimental treatments that, despite showing promise in animals, has an effect in patients are relatively sparse[110]. This however must be weighed against the potential benefit of the identification of a treatment that works. It also points to the importance of

selecting the right model and carefully designing experiments. In conclusion, using animals is valuable and indispensable, but should be done with care to experimental design and interpretation of (the expected) results.

Two different types of mice have been used in these studies; the inbred C57Bl/6J and the outbred Swiss Webster. Inbred mouse strains have minimal genetic diversity which serves to enhance reproducibility and reduce noise in the experimental outcomes. Outbred mouse stocks have the benefit of better representing the diversity of the normal human population, potentially at the cost of decreased signal-to-noise ratio. This has two possible effects, namely the risk of "missing" effects, but also increased use of animals to render the observed putative difference statistically significant [111].

C57Bl/6J mice are well characterized and represent the most widely used mouse strain; it was also the first strain to be fully sequenced. Phenotypically, it is responsive to diet-induced obesity (DIO) and related insulin resistance. The C57Bl/6J is a relatively small strain with adult weight around 25 grams when fed normal diet. It is also a common background strain used to generate transgenic mice.

Swiss Webster mice have been extensively used as an all-purpose stock and in pharmacological research. It is also responsive to DIO and has been used in metabolic research. Being larger (body weight approximately 40 g) than C57Bl/6J these mice produce larger litters, and the mothers are known to care well for the pups.

The microbial composition in mice and humans is comparable at phylum level, with Firmicutes, Bacteroides and Proteobacteria being dominant and representing over 70% of the total microbiota[112]. Two independent studies have identified 33 genera in humans[30] and 60 genera in mice[112] that were present in all samples, and 13 of the top 20 most abundant genera are overlapping. At microbiome level, there are remarkable differences and only 4% of the genes overlap between mouse and human microbiota. However, based on functionality, i.e. KEGG orthology, the overlap between human and mouse microbiome is 80%[112]. It should also be noted that microbial composition in mice differs between laboratory sites[112] with detectable effects on host physiology[113].

3.2 Microscopy analysis in morphological and physiological studies

Microscopy is an essential part of experimental and clinical medicine. In these studies, light microscopy, fluorescence microscopy and electron microscopy have been applied to answer both qualitative and quantitative questions. The perhaps strongest and most straight forward analysis using microscopy is qualitative analyses; visualizing expression of target proteins in cell subtypes or defining the subcellular location.

Quantitative microscopy is capable of combining spatial information with information about the amount of a given molecule. However, this requires many technical replicates and is thus laborious. In Project I immunofluorescence (IF) was crucial to compare the villus vasculature between GF and CONV-R mice, but in Project II, when observing tight junctions, the limitation of IF was noted; observing small subcellular structures can be impossible due to relatively low resolution. In theory, the resolution is limited by the diffraction of visual light and the microscope set-up (notably the numerical aperture). In practice however, this resolution is further decreased by the relatively large antibody complexes which add substantial size to the observed structure. Therefore, staining for tight junction components is valuable when large effects are expected, such as in IBD, but smaller differences may not be seen. Instead transmission electron microscopy (TEM) was applied. This method is label free and enables visualization of intact tissue at much higher resolution. By applying this method it was possible to detect nanometer differences in paracellular space at tight junctions. These measurements were performed as previously described[114] by measuring the width of the tight junction at three points to provide an average width. This was done at 100,000 fold magnification. These examinations also made possible the observation that the number of desmosomes was different between GF and CONV-R mice, which would not have been possible using other methods.

Microscopy is however limited by technological boundaries. The overall aim is to visualize tissue in a life-like state, while still maintaining suitable processing procedures as well as possibility to detect specific structures. Therefore the fixation protocols needs to be tightly controlled.

3.3 Analyzing intestinal permeability

Assessing the intestinal permeability is important and can be done using various techniques. In these studies the Ussing chamber was applied. It was established by Hans Ussing in 1946 to study the ion transport across frog skin, but is now routinely used for other tissues and cultured cells. It is a sensitive method, but in practice this sensitivity is partly blunted by other factors and care must be taken when dissecting the biopsies. For instance, when excising the intestine, the mesenteric attachment points should be cut carefully with little tension to the attachment point (as opposed to crude dissection used for other types of samples where hastiness may be more important). This is in order to prevent holes that induce artificial leakiness and thereby making results impossible to interpret. Also during mounting into the chamber there is a risk of stretching the tissue and damage the biopsy. This can partly be overcome by using more technical replicates per biological sample, making the methodology relatively time consuming.

The intestinal muscle layers were left intact during Ussing chamber analysis, which could be a confounding factor. It will influence the passage of labelled dextran, but need to be weighed against the fragility of the mouse intestinal epithelium, and the risk of decreasing tissue viability due to increased handling time.

The Ussing chamber is an *ex vivo* system, and as such may have less context than *in vivo* measurements, such as tracer-based methods e.g. lactulose-mannitol intake followed by detection in urine to estimate passage across the epithelium in humans. In mice, oral gavage with labelled substances followed by detection in blood is also possible. These methods however, are also affected by other factors such as intestinal transit time, which has been shown to differ between GF and CONV-R mice. Furthermore, any structural changes that affect the surface area are likely to have an even greater effect if the entire intestine is involved. Another advantage of Ussing chamber analysis is the possibility to identify spatial differences, e.g. specific segments of the intestine that show differentially regulated permeability. For instance, the expression of FXR is highly compartmentalized and, since the expression is highest in the distal small intestine, it is useful to be able to pinpoint such locations. Such detailed analysis has the potential to provide data that could otherwise be masked.

3.4 Anti-sense oligonucleotide-mediated knockdown

A subset of mice used in paper III were subjected to treatment with anti-sense oligonucleotide (ASO) in order to specifically knock down SREBP-1c. The advantage of this method, compared with transgenic mice, is that the effect is limited in time. Many knock-out models show embryonic lethality, and can therefore not be used for experiments in adult mice. Furthermore, in this setting where GF mice are used, transgenic mice would be required to undergo rederivation to the GF state, which is time consuming and expensive. In the present experiment it was also advantageous to be able to combine the knock-down with the diet that was expected to induce expression, and thereby potentially minimize the risk of development of compensatory responses.

4 RESULTS AND DISCUSSION

This section serves to emphasize key findings in paper I-III.

4.1 Paper I

Figure references below refer to the figure numbers in paper I.

4.1.1 A new mechanism for postnatal intestinal vascularization induced by the microbiota

The gut microbiota induces reorganization of villus architecture upon colonization, leading to shorter and wider villi[25]. This increase in volume requires increased vascularization to provide the cells with oxygen and nutrients. Increased vascular density in the intestine of CONV-R mice has been observed earlier[83]. Here we show that the gut microbiota contribute to vascularization by a novel mechanism involving tissue factor (TF) and angiopoietin-1 (Ang1).

TF glycosylation and surface localization

TF is localized throughout the body, including the intestinal epithelium, but it's procoagulant and proangiogenic properties rely on cell surface localization. Here we showed that this is dependent on glycosylation, a process induced by the gut microbiota because primary enterocytes from CONV-R mice have higher levels of fully glycosylated TF (Fig.2d) whereas GF mice have more underglycosylated TF in the cytosolic fraction (Fig.2e). We identified that TF is N-glycosylated (Fig. S9a, b) to higher extent in CONV-R and conventionalized mice than in GF mice. Furthermore, mannose residues were observed on the mouse TF (Fig. S9a). Also TF in humans is glycosylated, mainly by fucosylation[115]. It should be noted that there are large interspecies differences between glycosylation patterns in rodents and humans, such that human TF is void of mannose residues. However we did not analyze the presence of fucose. Epithelial fucosylation has been shown to be induced by the gut microbiota via a mechanism involving innate lymphoid cells[116]. Furthermore, it has also been shown that immunological sensing of the microbiota by the immune system is required for microbiota-induced vascular remodeling. By generating mice deficient in two adapter molecules for toll-like receptor signaling (myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), referred to as double knock-out, DKO) it was shown that postnatal

angiogenesis was strongly reduced when compared with WT counterparts[117]. Taken together, these data suggest that immune sensing and subsequent increased glycosylation may act upstream of TF-dependent microbial regulation of vascular remodeling.

Unexpected PAR1 activation after TF-induced thrombin formation

Tissue factor is well known to activate PAR2. However by using PAR2-deficient mice, we could establish that this receptor is dispensable during vascular remodeling as vascularization, and mRNA levels of PECAM-1 and Ang1 were unaffected by the knock-out (Fig. 4b,c,d,e). This could however be explained by the involvement of thrombin in our model, which is not capable of cleaving and thereby activating PAR2[81]. Instead, PAR1 was found to be central for increased vascularization. Not only is PAR1 mRNA specifically upregulated in CONV-R mice compared with GF counterparts (Fig.4a), but knockout of PAR1 is linked to reduced vascularization, as well as PECAM-1 and Ang1 mRNA expression levels (Fig.4b,c,d,e).

PAR1 activation leads to downstream phosphorylation of TF because phospho-TF was reduced in the PAR1 deficient mice. Hirudin is a potent anticoagulant found naturally in leeches, and functions by strongly binding to thrombin. Experimentally inhibiting thrombin-dependent PAR1 activation with hirudin during a 6 hour colonization revealed impaired TF phosphorylation (Fig.g,h).

Ang1 as the functional outcome of microbiota-stimulated angiogenesis

Angiogenesis has been extensively studied during development and in cancer biology; however the mechanisms for physiological postnatal angiogenesis are less well studied. Here we observed that the ultimate result of the microbiota-TF-PAR1 cascade was increased Ang1 production by the intestinal epithelium (Fig.1f). Ang1 signaling is extraordinarily complex and may differ between different tissues and situations. Ang1 is at the center of angiogenesis regulation and plays a bifurcated role; it may promote endothelial stability and suppress plasma leakage at steady state, but is also involved in promoting angiogenesis[118]. It has for instance been shown that Ang1 promotes endothelial cell migration *in vitro*[119]. Furthermore, by inhibiting Ang1 during colonization, and observing subsequent reduced vascularization, we provide strong evidence for the proangiogenic role of Ang1 in this pathway. Ang1 is a secreted protein with capability to interact with the extracellular matrix differently than Ang2[120]. This could enable

Ang1 to form a gradient, with the epithelium as high-concentration origin, and thereby contribute to directing angiogenesis.

4.1.2 Microbiota-induced vascular remodeling is required for villus maturation

That small intestinal villi in CONV-R mice are wider compared with their GF counterparts was observed by Abrams[25] and was confirmed in these studies (Fig.1a). However, we have linked this finding to the expansion of villus blood vasculature and observe that vascular expansion is a prerequisite for villus reorganization. As widening of the villus leads to greater distance between epithelial cells and the villus core, increased vascularization appears to be a logical requirement that needs to be met in order to supply oxygen to the epithelium as well as to assure uptake of nutrients from the intestine. Indeed, we saw that in conventionalized mice treated with anti-TF antibodies, and thus had reduced vessel density (Fig.1p), the villus width was reduced (Fig.1n). This point to that vascular density is a prerequisite for villus widening. To date it can only be speculated whether this is a reflection of a reduced surface area that minimizes potentially harmful bacterial contact in CONV-R mice, or an increased surface area in GF mice to optimize nutrient uptake due to their energy deprived state. Presumably villus vasculature is modulated for an optimal balance between uptake and minimization of infection risk.

4.2 Paper II

Figure references below refer to the figure numbers in paper II.

4.2.1 The gut microbiota increases intestinal permeability

Gut microbiota induces increased permeability in the small intestine

In the literature there is much evidence that the gut microbiota[22,121,122] and intestinal permeability[123,124] affects metabolic and inflammatory pathologies. Studies linking the microbiota to alterations of permeability are limited and are in part based on probiotics effects of selected microbial species. Because probiotics, by definition[125], should confer health benefits to the host and would therefore be likely to positively affect barrier function. Here we addressed how the gut microbiota affected small intestinal permeability by performing Ussing chamber analyses. To date, small intestinal permeability at steady state has not been investigated in GF and CONV-R mice. Our

results show that transepithelial flux of FITC labeled dextran of molecular weight 4000 Da (FD4) is increased in CONV-R mice (Fig.1A). This method is frequently used experimentally and clinically and represent a "gold standard" in epithelial investigations[57]. Since mouse intestine have relatively thin muscle layers it is common that these are left intact during Ussing chamber analysis[57], as was done also in these studies. No effects were observed on TER indicating similar diffusion of ions through the paracellular route.

mRNA levels of TJ components are reduced in CONV-R mice

Since the gut microbiota exerts much of its effects by, directly or indirectly regulating gene expression[126,127] we selected a number of genes to analyze by qRT-PCR and found that two genes, *Tjp1* and *Ocln* (encoding ZO-1 and occludin, respectively) were significantly reduced in CONV-R mice (Fig.1B). ZO-1 is a crucial adapter molecule that links transmembrane TJ proteins to the cytoskeleton[128], whereas occludin is an important transmembrane protein that binds to occludin found in the adjacent cell. Both of these proteins are implicated in barrier function. Others have shown that by siRNA-mediated knock-down of occludin (residual mRNA expression approximately 30%) in mouse small intestine, permeability towards larger molecules was drastically increased, but TER remained unaltered[62], largely resembling the phenotype at hand. Furthermore, claudin-family proteins are known regulators of ion flux through TJ, and we did not observe changes mRNA expression for any of the selected claudins, also in line with unchanged TER. However, even if mRNA expression levels of ZO-1 and occludin have been shown to correlate with intestinal permeability, gene expression provides only partial information regarding TJ regulation. Localization of TJ proteins to the TJ is one of the established regulatory mechanisms[129], and staining for TJ markers is commonly used to assess barrier integrity[130-132]. When staining for ZO-1, no differences were seen (Fig.1C). However, it could be argued that much of previous studies using this method represent acute inflammatory responses, pathological or experimental, rather than the physiological normal state as has been done here. For instance, in IBD epithelial erosion is present[133], which leads to drastic alterations in stainings for TJ markers[134].

Transmission electron microscopy reveals ultrastructural TJ alterations

To study the TJ in greater detail, electron microscopy was applied. To test the hypothesis that the gut microbiota affected the ultra structure of TJ's we performed TEM and observed that CONV-R mice had increased TJ width without any significant effects on the length of this structure (Fig.1D,E). A

widening of the TJ opening could explain the increased flux of FD4 across the epithelium, but might also suggest regulation beyond expression levels, such as contraction of the actomyosin ring via myosin light chain phosphorylation.

Furthermore, we unexpectedly observed fewer desmosomes in CONV-R mice (Fig.1F,G). These structures are less prominent in the field of intestinal permeability because TJ are the rate limiting structures. Nevertheless it has been shown that inhibition of desmosome formation leads to opening of the apical TJ in the colon carcinoma cell line T84[135]. This could therefore represent a parallel path in TJ and permeability regulation. However, much more work is needed to establish this branch.

4.2.2 The gut microbiota does not alter permeability in FXR-deficient mice

We next sought to determine microbially regulated upstream factors that could influence permeability. The nuclear receptor is emerging as a key regulator in both inflammation and metabolism, and Gadaleta[101] has previously shown that FXR is implicated in regulation of colonic permeability. We therefore repeated the experiments in GF and CONV-R mice lacking the FXR gene (*Nr1h4*, mice referred to as *Fxr*$^{-/-}$ for simplicity). Differences in permeability and occludin mRNA expression between GF and CONV-R mice were abolished in these mice (Fig.2A,B) and expression of ZO-1 was elevated in CONV-R mice (Fig.2B).

CONV-R *Fxr*$^{-/-}$ mice revealed similar permeability levels to CONV-R WT mice. This result differs from those obtained by Gadaleta[101], but might reflect different mechanisms in different parts of the intestine. For instance, FXR expression levels are lower in colon compared with ileum in WT mice, and could suggest that the effects observed in the colon is mediated through other cell types, such as immune cells.

Interestingly, while FXR signaling is repressed in GF mice[95], the GF *Fxr*$^{-/-}$ mice more closely resembled the CONV-R mice. This is surprising but could be explained by activation of a FXR-dependent repressor. Notably, microRNAs (miR) are implied in regulation of intestinal homeostasis, and may be microbially regulated[136]. More specifically, it has been shown that intestinal TJ are under negative regulation by miR-122[137]. Furthermore, it has been shown that miR-122 is an induced target of by FXR signaling in hepatocytes[138], but the link between intestinal FXR and miR-122 remains to be studied.

4.3 Paper III

Figure references below refer to the figure numbers in paper III. When specified, additional data that has not been integrated to the manuscript is presented in Appendix IV, in which case the reference is "(Appendix IV, xy)" where xy are the individual figures.

4.3.1 The gut microbiota is required for sucrose-induced steatosis

Western style diet is a well-known inducer of obesity and steatosis, however more investigation is needed to understand the distinct roles of fat and sucrose[139,140]. Fleissner *et al*[139]. have previously observed that the propensity for CONV-R mice, as compared with GF mice, to develop steatosis depends on sucrose content of the diet. To this end we fed a zero-fat, high sucrose diet (ZFD) to GF and CONV-R mice. Analyses of liver lipid content, histologically and by lipidomics, we showed here that the gut microbiota contributes strongly to sucrose-induced steatosis in CONV-R mice receiving the ZFD (CR-Z) (FIG. 1D, E) because GF mice receiving the same diet (GF-Z) have significantly lower levels of hepatic fat content. However, GF mice receiving chow diet (GF-C) do in fact have the lowest levels of all groups, while CONV-R mice fed chow diet (CR-C) display slightly elevated fat content, indicating synergism between diet and microbiota in steatosis induction. In addition, we observed a microbiota-independent shift in saturation of the fatty acids, where both GF and CONV-R ZFD-fed mice displayed higher levels of monounsaturated fatty acids, and lower levels of polyunsaturated fatty acids (FIG. 1F). This is in line with limited capacity to produce polyunsaturated fatty acids. In conclusion this points to that the microbiota is involved in regulating the amount of fat, and the diet affects the lipid quality.

Hepatic lipid levels are the sum of stored dietary lipids, lipids mobilized from the periphery by adipose tissue lipolysis, and lipids produced in the liver by *de novo* lipogenesis[141]. Because the ZFD does not contain any lipids, this source can be excluded in those groups. Furthermore, CRZ mice display highest values of epididymal white adipose tissue weight (FIG. 1B), making extensive lipolysis and subsequent liver lipid uptake unlikely. These notions and RNA sequencing data (FIG. 2A, FIG. S1) led us to select genes involved in lipogenesis to be analyzed by qRT-PCR. We observed a specific increase in *Fasn* (encoding fatty acid synthase, FAS, the rate limiting enzyme in lipogenesis) mRNA levels in the liver of CR-Z mice (FIG. 2B). Furthermore, we observed the same pattern in the level of malonyl-Coenzyme A (malonyl

CoA), which is the substrate for FAS (FIG. 2C). However, malonyl-CoA is also a potent allosteric inhibitor of carnitine palmitoyltransferase-1 (CPT1), which serves as a shuttle for long-chain fatty acids into the mitochondria for β-oxidation. Inhibition of CPT1 could therefore contribute to the elevated liver lipid levels found in CR-Z mice. Hepatic β-oxidation is a key source of acetyl-CoA which is a substrate for ketone body production. By means of ketogenesis, the liver can dispose significant amounts of lipids, and it has been shown that ketogenesis is protective in diet-induced NAFLD[142]. However, circulating levels of acetoacetate and β hydroxybutyrate, two ketone bodies produced by the liver, are higher in CR-Z mice than any other group, potentially indicating higher ketogenesis (Appendix IV, 1A). Since ketone bodies can also be formed from ketogenic amino acids, we also measured the portal vein concentration of leucine and lysine to test if portal influx could explain the increased levels of ketone bodies. However, this revealed leucine levels that were increased in GF mice independently of diet, and lysine levels that were the lowest in CR-Z mice (Appendix IV, 1B). This indicates that ketogenesis driven by increased ketogenic amino acids is unlikely to explain the ketone body concentrations. These data suggest that β-oxidation might take place despite the high concentrations of malonyl-CoA. However, the actual rate of β-oxidation remains to be assessed in isolated mitochondria for conclusiveness. It should also be noted that mitochondrial uptake and β-oxidation of short- and medium-length fatty acids occur independently of CPT-1.

On mRNA level we also observed microbiota-independent increases in the levels of stearoyl-CoA desaturase 1 (SCD1) (FIG. 2F) and the elongation of very long chain fatty acids protein 6 (ELOVL6) (FIG. 2G). SCD1 is responsible to This is in agreement with the saturation profile observed (FIG. 1E, F).

4.3.2 SREBP-1c knockdown reduces steatosis development

To investigate the upstream events leading to steatosis (FIG. 1), and the observed lipogenic mRNA expression pattern (FIG. 2), we hypothesized that carbohydrate responsive element binding protein (ChREBP) or sterol regulatory element binding protein-1c (SREBP-1c) could be involved. These are transcription factors central to the regulation of hepatic metabolism[143]. While ChREBP mRNA expression was modestly increased in the liver of CR-Z mice, SREBP-1c expression was induced strongly in CR-Z mice (FIG. 2E). This prompted us to treat mice with antisense oligonucleotide to inhibit expression of SREBP-1c. CONV-R Mice were treated by intraperitoneal

injections before and during the switch to ZFD. To assess the treatment tolerance, a number of fundamental parameters were assessed. Body weight gain, liver fibrosis and liver TNFα mRNA expression were not changed by the treatment (FIG. S3), which is in line with a previous study. A 70% knock-down of the target gene was observed with a reduction in FAS mRNA expression to almost the same extent (FIG. 3A). This was associated with reduced liver lipids as observed by Oil Red O staining. Lipidomics analyses however displayed borderline significance (FIG. 3C) for reduction of total fatty acids as well as oleic acid.

Insulin is a known inducer of SREBP-1c activation and transcription of target genes. However, systemic glucose metabolism remained remarkably unaffected as measured by fasted blood glucose, fasting insulin, and intraperitoneal glucose tolerance test (Appendix IV, 2A, B, C). Interestingly, the insulin levels are highest in GF-Z mice, reaching statistical significance when compared with CR-Z mice, thus pointing to that insulin is unlikely to drive SREBP-1c expression and steatosis in these mice. It has been shown that ChREBP might act have a protective role in the development of insulin resistance during hepatic steatosis[144], and we observe a slight elevation of ChREBP in livers from CR-Z, but not GF-Z, mice. However, more work would be required to establish that this pathway is active. The small impact on insulin and glucose may thus indicate that other factors contribute.

4.3.3 The gut microbial composition is shifted by ZFD

Diet is strongly influences microbial composition in the gut[145,146]. Accordingly, we observe dramatic shifts in the microbial composition in ZFD fed mice. Based on a weighted Uni-Frac, which accounts for the relative differences by correcting for species abundance, a principal component analysis (PCA) demonstrated clear separation between CR-Z and CR-C mice (FIG. 4A), and diet is responsible for approximately 58% of the observed variation. Low species diversity has been associated with NAFLD[147] and metabolic syndrome[148] and there was a trend towards lower species diversity in the CR-Z group (FIG. 4B). This is in line with a less complex diet.

The largest increases in abundance induced by the ZFD are seen in the genera *Bilophila* and *Staphylococcus*. *Bilophila wadsworthia*, a prototypical member of *Bilophila*, has been shown to be increased after 12 and 16 weeks of HFD (45% calories from fat, 17% calories from sucrose) feeding in C57Bl/6 mice[149]. In this study, *B. wadsworthia* was also correlated with elevated blood triglyceridemia as well as some inflammatory markers in adipose tissue.

Interestingly, this bacterium has also been associated with IBD, and ascribed a pathobiontic role as it expands during development of IBD. This expansion and subsequent IBD phenotype can also be achieved by giving taurocholic acid to interleukin-10 deficient mice[150]. The genus *Staphylococcus* entails around 40 species of bacteria with potentially different roles.

The largest reduction of specific genera in CR-Z mice were observed in *Prevotella* and *Lactobacillus*. *Prevotella* appear in two studies to trend toward reduced abundance in NAFLD[151] and NASH[152]. Strains in the *Lactobacilli* genus are commonly used as probiotics, but these may differ significantly from the strains that are found naturally. However, a mixture of various *Lactobacilli* together with *Bifidobacterium bifidum* has been shown to reduce liver triglyceride levels in patients with NASH[153].

The genus *Parabacteroides* was found to be enriched in the ZFD-fed mice, and interestingly, one of the species representing this genus, *P. distasonis* was strongly enriched (4.4 – 28.4-fold) in ZFD-fed mice. This bacterium has previously been implicated in the modulation of steatohepatitis, also making it a candidate bacterium to explore in more detail, for instance by colonizing GF mice to test if it contributes to steatosis.

The observed alterations are in line with previously published results, although not conclusive at this point. While the composition of the microbiota is as an important factor in the development of NAFLD, studies are mainly correlative and causative microbes remain to be identified. The transfer of microbiota is implicated both in conferring positive health effects, and as a transmissible factor in disease. For instance, gastric bypass is the most efficient treatment for obesity and is associated with shifts in microbial composition, and transfer of this microbiota into GF mice has been shown to induce weight loss and decreased fat mass[154,155]. Furthermore, detrimental effects have been seen by transfer of microbiota from twins where one is obese and the other lean[156] to mice. Microbiota from the obese twin induced increased fat gain in the mice. We therefore next wanted to test the hypothesis that the altered microbiota is in fact a driver behind the steatosis observed in CR-Z mice. To this end, GF mice were colonized with caecal contents from either CR-Z mice or CR-C mice, while fed normal chow diet throughout the colonization period. The CR-Z microbiota induced a moderate but statistically significant increase in epididymal white adipose tissue weight (FIG. S5A) but had no effect on liver weight or liver lipids (FIG. S5B, C). The transcriptional response was blunted and even if hepatic mRNA levels of SREBP-1c were moderately increased, mRNA expression of FAS was slightly reduced (FIG. S5E). In conclusion, these results show that the

microbiota from CR-Z mice, although increasing adiposity in recipient mice, cannot recapitulate the steatosis phenotype when transferred to GF mice. This points to a more complex crosstalk between diet and microbiota and that microbial metabolite production might offer important insights.

4.3.4 Portal vein metabolome is altered by diet and microbiota

To assess the outcome of the microbial metabolism we next turned to analyses of the portal vein metabolites. In a recent publication, bile acid (BA) signaling via FXR was suggested to activate intestinal ceramide production. These ceramides would then be transported to the liver and increase SREBP-1c expression and thereby promote lipogenesis and steatosis[97]. This was prevented by antibiotic treatment which increased the tauro-conjugated β-muricholic acid (TβMCA) content in the intestine,[97] leading to inhibition of FXR signaling[95]. However, when assessing the portal vein ceramide content we observed a very heterogeneous response with few specific increases in the CR-Z mice (FIG. S6), indicating that other mechanisms are relevant. However, these previous studies point to a regulatory role for the intestine in the diet-gut microbiota-liver axis. It should be kept in mind that the epithelium is metabolically active and represent the first contact with microbial products. Exploring the epithelial response and production of bioactive compounds might hold clues to the shifts in the portal vein metabolome.

We next turned to an unbiased metabolomics analysis of the portal vein. This identified 537 compounds in total and revealed microbial (n = 74) and dietary (n = 94) regulation. Ten of the differentially regulated compounds were significant for interaction between microbiota and diet (FIG. 4C). Because we hypothesized that a microbially derived metabolite is responsible for the increased SREBP-1c mRNA levels, we next performed a pathway analysis of CR-C and CR-Z portal vein metabolomes (FIG. S5). This revealed several different pathways that were enriched in either CR-C or CR-Z portal vein metabolomes. We observed dramatic changes in the lysolipid sub pathway, with several compounds enriched in either CR-C or CR-Z mice. Also on the level of individual compounds we observe differences. Within the purine metabolism pathway several compounds were elevated in CR-Z mice, including inosine, deoxyinosine, inosine 5'-monophosphate, hypoxanthine, xanthine and xanthosine. These are involved in uric acid production, which is a biomarker for NAFLD[157]. Uric acid is however not altered, but it should be noted that uric acid is rapidly converted to allantoin, which is elevated in CR-Z mice.

Based on the results from the metabolomics analyses, we selected metabolites and attempted to stimulate primary hepatocytes. As of yet, these experiments have unfortunately not been fruitful. This could however be due to technical difficulties. Several possibilities to explain the lack of induction exist. For instance, primary mouse hepatocytes might be hyporesponsive as insulin and insulin in combination with glucose did not induce a strong SREBP-1c expression, which has been shown to be a key regulator *in vivo*[87]. Furthermore, the portal vein metabolome is complex and probably encompass more than the over 500 metabolites identified in this dataset, therefore it is not unlikely that either (I) a co-factor is required to induce SREBP-1c expression or (II) that several factors combined are required to recapitulate the observed SREBP-1c. These notions should be considered when designing future experiments.

4.3.5 Proposed mechanism

In summary, we can conclude that the ZFD alters the gut microbial ecology drastically, and that the portal vein metabolome is altered both by diet and the different microbiota. The liver responds to these changes by induction of lipogenesis, which is SREBP-1c dependent and leads to hepatic steatosis. More work is needed to establish the underlying factors that signal to the liver to induce its lipogenic profile in response to ZFD and in presence of the microbiota. Below is a schematic overview of the events related to this phenotype.

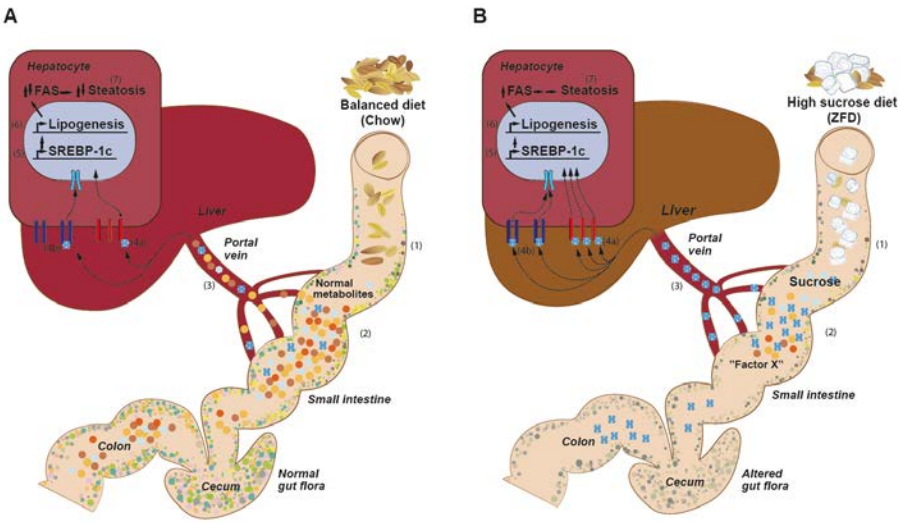

Figure 1. ***Proposed mechanism for sucrose-induced microbiota-dependent steatosis.*** *Under normal conditions (A) a balanced diet leads to normal microbial ecology (1) and a healthy mixture of microbially produced metabolites (2). The metabolites are transported through the portal vein (3) and signals to induce normal levels of lipogenesis (4-6) and no steatosis (7). In contrast, as depicted in B), the high-sucrose, zero-fat diet alters the composition of the microbiota (1). This leads to the production of, as of yet, unknown agonists (2, "factor X") that enter the liver via the portal vein (3). Here, the ligand could either signal via cell surface receptors (4a) or be taken up into the hepatocyte (4b) and signal to nuclear receptors. The signal cascade leads to increased mRNA expression of SREBP-1c (5) which in turn activates transcription of the lipogenesis programme, including FAS (6). The outcome of this is increased hepatic steatosis (7).*

5 CONCLUSIONS

- Vascular remodeling in the small intestine is microbiota-dependent.
- A new mechanism for microbiota-dependent vascular remodeling is proposed, involving tissue factor-thrombin-PAR1 signaling within the epithelium, leading to expression of angiopoietin-1.
- Vascular expansion is a prerequisite for villus maturation (i.e. villus widening in CONV-R mice).
- The gut microbiota induces increased small intestinal permeability, reduced mRNA expression of tight junction components, a widening of the paracellular space within tight junctions and a reduction of desmosomes.
- Microbiota-induced permeability is linked to FXR signaling but the exact mechanism remains to be elucidated.
- Sucrose-induced hepatic steatosis requires the gut microbiota and intact SREBP-1c activated lipogenic transcription.
- Zero-fat diet affects lipid saturation independently of colonization status.
- A high-sucrose, no-fat diet leads to dramatic alterations in gut microbial ecology.
- The portal vein metabolome is altered by both microbiota and diet, a notion that could hold clues regarding activating ligands leading to SREBP-1c stimulation and lipogenesis.
- Targeting the microbiota, or pathways driven by microbial activity during disease, may offer novel therapeutical options in numerous physiological systems.

6 FUTURE PERSPECTIVES

6.1 Paper II

The findings presented under 4.2 need to be expanded in some aspects. While the Ussing chamber has been indispensable in this and many other investigations, the differences in permeability should be verified also *in vivo*. In addition to strengthening the present data, the Ussing chamber set-up is limited by the fact that it is terminal. *In vivo* analyses where FITC-conjugated dextran is administered by oral gavage followed by measurement of blood plasma concentrations of FD4 has been described in literature[101] and could serve as a faster, physiologically relevant model. Furthermore, in principle, it would also be possible to maintain mice alive for interventions in a paired setting.

Furthermore, staining for TJ markers investigate a crucial aspect of a functional barrier: appropriate localization of TJ proteins. However, it is only semi quantitative and to better understand how transcriptional regulation translates to different abundances in protein amount, immunoblotting should be performed. This would also be a suitable method to investigate myosin light chain phosphorylation[54] to possibly associate with widening of the TJ.

Importantly, establishing a role for an FXR-regulated inhibitor will provide important mechanistic data. Analyzing the intestinal levels of miR-122 could serve as an initial readout.

Furthermore, reducing intestinal permeability specifically would be a useful tool to test the hypothesis that the gut microbiota induces permeability, and, in turn, directly contributes to the propensity to gain body fat in CONV-R mice by this mechanism. As shown in this and other publications, modulating permeability often involves other pathways, e.g. primarily regulating inflammation, and thus only secondary modulates permeability. As recently reviewed[158], these options, which could serve as treatment options for inflammatory bowel diseases or metabolic inflammation, are discouragingly sparse. Targeting the microbiota, or microbially regulated pathways, may give rise to novel treatment strategies.

6.2 Paper III

Several aspects of this project require more work. Determining the rate of β-oxidation in isolated hepatic mitochondria will provide additional information to the fate of liver lipids, for completeness. Furthermore, establishing any differences in liver glycogen content may provide additional information of the overall liver metabolism.

The ASO-mediated knockdown of SREBP-1c shows borderline significance. However, the liver fat content in the control-ASO group does not reach the level of CR-Z mice, whereas the *Srebf*-.ASO treated mice are very similar to CR-C mice. This points to a reduced induction of steatosis also in the control-ASO treated mice. This experiment should be repeated, and, importantly, the transcriptional response should be compared to CR-C levels to assure that the control-ASO does not affect the transcription profile.

Current efforts to decipher the metabolomics input to the liver via the portal vein have had limited results. This could be to technical difficulties. Plasma from the four groups of mice should be used, crude and fractionated, on hepatic cell lines, e.g. HepG2 to test their responsiveness. Another option could be to use reporter cell lines to test which receptors are activated by complete plasma. This would provide important information about factors upstream of SREBP-1c, and could narrow down possible candidates, which induce SREBP-1c expression, in the metabolomics dataset. Alternatively, the possibility that GF-Z (or CR-C) mice are in fact exposed to an inhibitor cannot be excluded, and testing this is tempting because it would provide a more direct link to treatment possibilities.

Another aspect is to identify causative microbes. By selecting bacteria enriched in CR-Z mice, either alone or in a cluster, to colonize GF mice it would be possible to identify steatosis-inducing bacteria. This would contribute greatly to the understanding of the interplay between diet, microbiota and host steatosis. However, also the opposite approach would be possible; by selecting bacteria enriched in CR-C mice to colonize GF mice fed the ZFD it could be tested if these have an inhibitory capacity. It could also be tested if these bacteria have “probiotic effect” by inoculating CR-Z mice repeatedly to suppress steatosis development.

ACKNOWLEDGEMENT

This work is the result of a great team effort. Without all these fantastic people around, nothing would have been possible. This section could probably have been made the longest in the entire thesis. However, I would like to extend my gratitude especially to:

Fredrik Bäckhed, I could not have asked for a better supervisor. Having the chance to be part of new discoveries is an amazing experience. I want to extend my deepest gratitude to you for giving me the great opportunity of being part of the research front. Your immense knowledge about everything concerning research has left me with the deepest respect and someone to look up to. I also want to thank you for your endless patience with my last-minute work. Thank you for showing what excellent leadership really means: Knowledge, kindness, good advices and discussions about how to catch the biggest fish.

Valentina Tremaroli, my co-supervisor. Thank you for all your input and advice regarding microbiota analysis, and all the work with the statistics. I will always be inspired by your meticulous way of working.

Jan Borén, my second co-supervisor. Thank you for helping me when deadlines moved faster than I did.

Members of group Bäckhed, past and present, thank you for creating such a fantastic and supportive environment. There is always a helping hand if you need it. I have had the best imaginable colleagues. Gunnel, thank you for being there when I took my first shaky steps into lab work and, together with Anna and Manuela, making the lab work smoothly. Thanks to Anna for the artwork in this thesis and elsewhere. Thanks to Christoph for being an inofficial supervisor during the first time. Lisbeth E, thanks for helping out with complicated administration.

The EBM staff – Carina, Carro, Sara, Zakarias – you're doing a fantastic job and without you the lab work would stop. Thank you.

My writing room buddies, past and present, Annika L, Mikael, Thomas, Martina, Volkan, Christina, Nadia, Martin, Abushek – there's really only one way to describe you guys: 100%! Thanks for all those crazy, awesome discussions about everything, from toilet habits to the world map and more often than not about bacon.

The Wallenberg lab staff; Magnus, Merja, Svenne, Ulrika – thanks for working hard to keep the entire lab going. Magnus and Merja, thanks for nice discussions about pretty much anything.

People in the lab that have grown from colleagues to friends… I've had a blast! Filipe, Steffi, Rozita, Marie-Christine, and lately Arianna – and of course my old friend Yohanna who turned out to be my only student. =) Thank you guys for being amazing! Honorable members of the sausage club; Antonio, Sam, Felix Sr, Felix Jr, Björn, Filipe – I have really enjoyed our evenings out, let's keep up Saray!

Thanks also to Gunnar H. for letting me use your lab for Ussing chamber analyses. And for providing a platform, in the form of a sofa and a coldroom, for social interactions (which turned out to have greater effects than expected). André, Christian, Hannah, George, Lisa, Jenny, Sjoerd and everyone elese "up there" – thanks for hikes and all kinds of adventures during many years.

Liisa, thank you for putting up with me during the past rather stressful times. You motivate me to work harder, but also to have more fun and there's always a new project to look forward to. Thank you also for your help with the thesis. Let's go somewhere nice!

Martina, lil' sis… Du har hunnit längre än mig de senaste fem åren och gått från liten till stor, från grundskola till att plugga till biotechmästare och/eller tandläkare. ;-) Jag är väldigt stolt över dig och önskar dig all lycka vilken väg du än väljer att gå!

Morsan, utan dig hade jag inte varit där jag är nu. Bokstavligt och bildligt talat. Du har lärt mig att det är viktigt att jobba för att komma framåt. Tack för att du alltid har stöttat mig och pushat mig åt ett håll som är spännande och utmanande.

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APPENDIX

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