Dietary fat and bile acids in the pathogenesis of gut barrier dysfunction

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ACADEMIC DISSERTATION

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**“True ease in writing comes from
art, not chance, as those who move
easiest have learned to dance”**

Alexander Pope

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (Studies I-IV) and some unpublished data.

I **Stenman, L.K.**, Holma, R., Korpela, R. (2012). High-fat-induced intestinal permeability dysfunction associated with altered fecal bile acids. World J Gastroenterol *18*, 923–929.

II **Stenman, L.K.**, Holma, R., Gylling, H., Korpela, R. Genetically obese mice do not show increased gut permeability or faecal bile acid hydrophobicity. Br J Nutr *In press*

III **Stenman, L.K.**, Holma, R., Eggert, A., Korpela, R. (2013). A novel mechanism for gut barrier dysfunction by dietary fat: epithelial disruption by hydrophobic bile acids. Am J Physiol Gastrointest Liver Physiol *304,* G227–234.

IV **Stenman, L.K.**, Holma, R., Forsgård, R., Gylling, H., Korpela, R. Increased fecal bile acid hydrophobicity is associated with exacerbation of DSS colitis by dietary fish oil on a high-fat diet in mice. J Nutr *Submitted*

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MAIN ABBREVIATIONS AND TERMS

CD14	Cluster of differentiation 14
CDCA	Chenodeoxycholic acid
Cr-EDTA	Chromium ethylenediaminetetraacetic acid
DCA	Deoxycholic acid
DSS	Dextran sodium sulphate
E%	Per cent of total dietary energy
FITC	Fluorescein isothiocyanate
FXR	Farnesoid X receptor
HIx	Hydrophobicity index
HF	High-fat diet
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
MLCK	Myosin light chain kinase
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
TER	Transepithelial electrical resistance
UDCA	Ursodeoxycholic acid
ZO	Zonula occludens

Endotoxin: A toxic heat-stable lipopolysaccharide substance present in the outer membrane of gram-negative bacteria that is released from the cell upon lysis

Intestinal permeability: Translocation of a molecule that is not actively absorbed through the gut epithelium, used synonymously with barrier function, since these two may not always be distinguished due to limitations in methodology

Metabolic endotoxemia: A low-grade but significant endotoxemia where serum endotoxin levels are approximately 2-fold compared to a healthy state

Translocation: Passage of a molecule through an epithelial layer by an undefined mechanism

ABSTRACT

Gut barrier function is impaired in several disorders such as inflammatory bowel diseases, diabetes and steatohepatitis. It is suspected that this is related to increased permeability to bacterial endotoxins from the lumen through the gut epithelium. Recent studies have shown a link between high-fat diet and endotoxemia, but the underlying mechanisms are unknown. One possible explanation is the contribution of other luminal substances, such as bile acids. Fat ingestion induces bile flow to the duodenum to facilitate the absorption of fat. At high concentrations, bile acids – especially very hydrophobic bile acids – are cytotoxic. The aim of this study was to investigate whether dietary fat or obesity causes barrier dysfunction, and whether bile acids play a role in its pathogenesis. The role of bile acid hydrophobicity in their capability of inducing barrier dysfunction was given special attention.

The effects of dietary fat and obesity on gut barrier function were investigated in the diet induced obesity and ob/ob -mouse models. Fecal bile acids were quantified and profiles calculated from these mice. The effects of bile acids on intestinal permeability were studied in an *in vivo* feeding trial with deoxycholic acid and *in vitro* in an Ussing chamber. *In vitro*, tissue preparations were incubated with deoxycholic acid and/or ursodeoxycholic acid - two bile acids greatly different in their hydrophobicity.

Gut barrier function was impaired by a high-saturated fat diet in mice, but not in genetically obese mice that were fed normal low-fat chow. Barrier dysfunction by dietary fat was especially prominent in jejunum and colon - no significant difference was seen in the permeability of duodenum or ileum. Fecal bile acid hydrophobicity was increased only by dietary fat, not by genetic obesity, and was positively correlated with intestinal permeability.

Deoxycholic acid alone increased gut permeability both *in vivo* and *in vitro*. The effect was more evident in colonic than jejunal tissue preparations, and the mechanism seemed not to be inflammation-dependent. Barrier impairment was reduced by the hydrophilic ursodeoxycholic acid, which was also reflected as improved tissue morphology. Deoxycholic acid -induced barrier dysfunction seemed to be aggravated by translocated lipopolysaccharides.

The present results suggest that dietary fat, but not obesity itself, impairs gut barrier function. The data imply that luminal bile acids are one mechanism for barrier impairment, with hydrophobic bile acids initiating tissue disruption and lipopolysaccharides likely playing the role of a second hit.

1 INTRODUCTION

The intestine has an essential function as a barrier between intestinal contents – the "outside world" – and the body (for review, see Arrieta *et al.*, 2006). The gut barrier is impaired in several pathologies, some of them severely affecting the quality of life: inflammatory bowel diseases (IBD) (for review, see Goyette *et al.*, 2007), irritable bowel syndrome (IBS) (for review, see Camilleri *et al.*, 2012), type 1 diabetes (for review, see Vaarala, 2008), non-alcoholic fatty liver disease (for review, see Valenti *et al.*, 2009) and allergy (for review, see Perrier and Corthésy, 2011).

Recently, impairment of gut barrier function has been linked to obesity and a diet high in fat (for review, see Teixeira *et al.*, 2012a), which are part of the Western lifestyle. In humans, a Western diet is suggested to lead to endotoxemia – an elevated level of bacterial surface molecules in the circulation (Pendyala *et al.*, 2012). These pathogenic compounds are expected to originate from the gut lumen and to reflect an impairment of gut barrier function. In the circulation, they are highly inflammatory and may in part promote the pathogenesis of type 2 diabetes and non-alcoholic fatty liver disease (for reviews, see Cani and Delzenne, 2009; Valenti *et al.*, 2009). Reducing the intestinal translocation of endotoxins may help prevent the metabolic disease burden associated with obesity. Therefore, understanding the

mechanisms underlying gut barrier dysfunction may help identify targets for future disease prevention and treatment.

It is yet unkown, whether it is the dietary fat or obesity that causes barrier dysfunction, and there are only suggestions upon possible mechanisms. Many research groups have addressed this field and proposed that alterations in the gut microbiome affect intestinal barrier function (Cani *et al.*, 2007a, 2008, 2009; Carvalho *et al.*, 2012; de La Serre *et al.*, 2010; Serino *et al.*, 2012). However, the gut holds a substantial number of other substances which may affect barrier function; for example bile acids. The excretion of bile acids is induced by ingestion of fat, as their main purpose is to solubilize dietary fat into a more readily absorbed form. Indeed, a high-fat diet increases their serum and fecal concentrations (Reddy, 1981; Suzuki and Hara, 2010). The fact that high levels of certain bile acids are cytotoxic to gut epithelium (for review, see Barrasa *et al.*, 2013) gave rise to the hypothesis that alterations in bile acid composition may affect gut barrier function in mice on a high-fat diet.

The purpose of this thesis was to investigate the role of dietary fat and bile acids in gut barrier dysfunction in mice. A specific focus was set on two bile acids, deoxycholic acid and ursodeoxycholic acid, which differ in hydrophobicity, a measure related to bile acid cytotoxicity.

2 REVIEW OF THE LITERATURE

2.1 What is intestinal permeability?

2.1.1 Components of the intestinal barrier

The intestinal barrier consists of several non-immunological defense mechanisms that together prevent luminal substances, such as microbes, viruses, antigens and toxins, from entering the body (for review, see DeMeo *et al.*, 2002). Together with the gut immune system they form the so-called intestinal barrier. These mechanisms are summarized in Figure 1.

In the small intestine, the gut epithelial lining sheds cells (1, Figure 1) and excretes fluid into the lumen (2) which dilute the intestinal contents and, assisted by motility, wash away potential toxins (for review, see Sarker and Gyr, 1992). Among luminal contents, pancreatic enzymes and bile have anti-bacterial effects (3) (Sarker and Gyr, 1992), and commensal microbes inhibit colonization of pathogens by competing for nutrients and secreting anti-microbials (for review, see Yu *et al.*, 2012). In addition, epithelial cells secrete vast amounts of mucus, that inhibits adherence of luminal bacteria onto the epithelium (4) (DeMeo *et al.*, 2002), and an array of antimicrobial peptides known as defensins secreted by epithelial Paneth cells (5) (for review, see Bevins *et al.*, 1999). The gut also secretes immunoglobulin A to bind

bacterial antigens into a complex that is excreted in feces (6) (DeMeo *et al.*, 2002). Together these mechanisms are called the first line of defense.

The second line of defense in gut barrier function consists of the epithelial cells and their junctional complexes, providing a physical barrier between the host and lumen (7) (DeMeo *et al.*, 2002). This is the most important protective component of the gut barrier. The epithelial barrier may be disrupted by either direct cell damage or through intracellular signaling mechanisms affecting cell-to-cell junctions (DeMeo *et al.*, 2002). The paracellular barrier consists of four protein junctions: desmosomes, gap junctions, adherens junctions and tight-junctions, of which desmosomes and adherens junctions have an important role in the mechanical linkage of cells (Anand *et al.*, 2008; for review, see Groschwitz and Hogan, 2009). The tight junction system controls the paracellular flux and prevents unwanted translocation of harmful substances, while it allows antigen-sampling by submucosal immune cells (DeMeo *et al.*, 2002). This mucosal immune system (8) forms the final line of defense in the gut barrier.

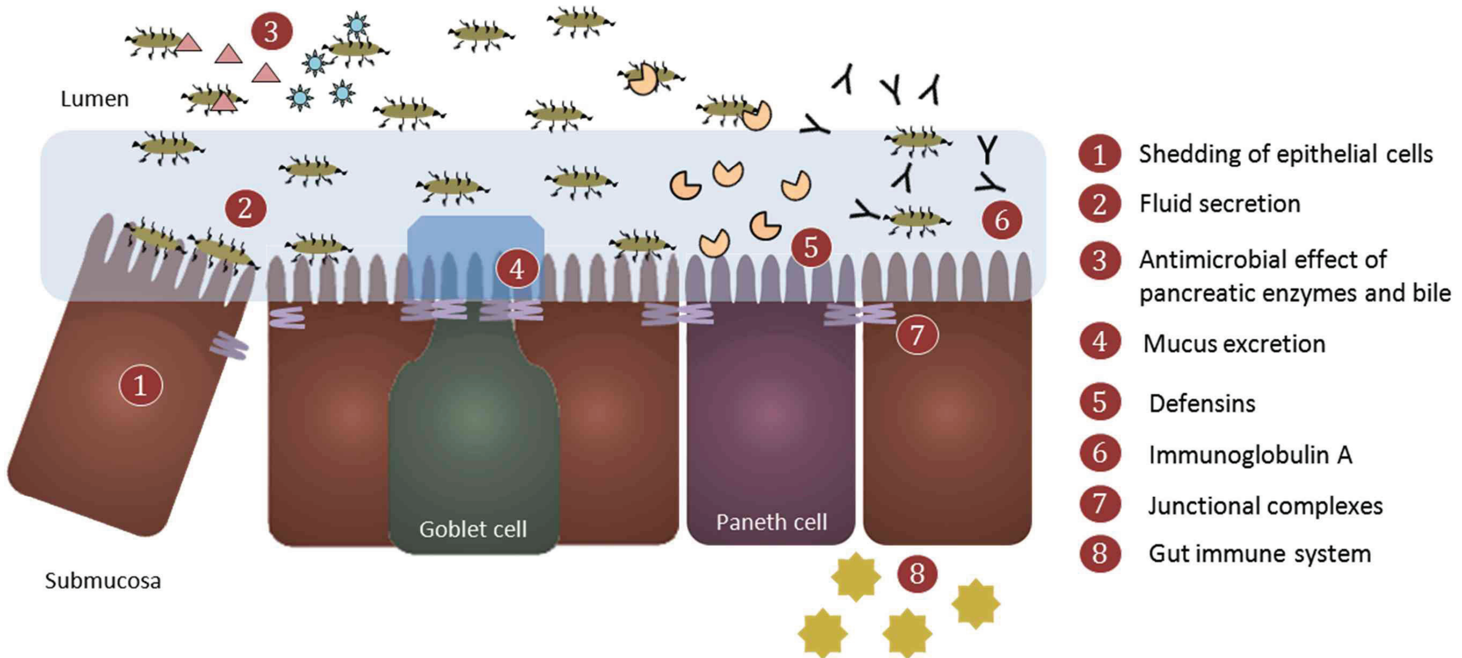

Figure 1. *Intestinal barrier mechanisms*

2.1.2 Pathways of endotoxin translocation

Intestinal permeability is described as the leakage of large molecules, such as endotoxins (i.e. lipopolysaccharide, LPS), through the gut epithelium, and should be distinguished from the carrier-mediated absorption of dietary ingredients. However, the concept of intestinal permeability is not very well defined, since the probes used in the assessment of intestinal permeability may translocate through different pathways. This translocation through gut epithelium may be divided into two possible pathways: the paracellular pathway and the transcellular pathway, which may be further divided into the aqueous and lipid transport routes (for review, see DeMeo *et al.*, 2002; Caesar *et al.*, 2010). It is generally assumed that small molecules translocate through the paracellular pathway, whereas large molecules require transcellular transport. The following evidence suggests that this is not always the case.

The paracellular pathway is guarded by tight-junctions (see 2.1.3), which do not normally allow passage of large molecules through the epithelium. However, there is some evidence indicating that even large molecules may translocate through the paracellular pathway, when induced by carbachol (Bijlsma *et al.*, 1996) or intestinal sensitization (Berin *et al.*, 1997, 1998) in the rat. Furthermore, interleukin-4 induces the translocation of large molecules up to 150 kDa through an epithelial cell layer, but is not reversed by an inhibitor of energy-dependent transport, which suggests an activation of the paracellular pathway (Mochizuki *et al.*, 2009). Evidence in human ileum shows that in healthy conditions, LPS translocation occurs only transcellularly whereas in tissue specimens of patients with Crohn's disease, LPS is also detected paracellularly (Keita *et al.*, 2008). These data demonstrate that a paracellular pathway for LPS translocation could be induced by pathological conditions.

Translocation of endotoxins is also suggested to be chylomicron-facilitated. This was shown as an increased translocation of

endotoxins, when Caco-2 cells were stimulated by fatty acids, which promote chylomicron formation (Ghoshal *et al.*, 2009). Endotoxin translocation was blocked by an inhibitor of chylomicron formation *in vivo*. In rat jejunum, fluorescence-labeled endotoxin has indeed been shown to be absorbed into brush-border membrane vesicles (Drewe *et al.*, 2001). In humans, this hypothesis is supported by findings that a fat-containing meal increases postprandial plasma endotoxin levels (Erridge *et al.*, 2007; Ghanim *et al.*, 2009; Laugerette *et al.*, 2011), which suggests a linkage to lipid absorption. However, these studies do not permit us to draw conclusions on whether the effect is specific to dietary fat, since results were not compared to a low-fat control meal. Only one study comparing cream, orange juice, glucose and water has demonstrated plasma endotoxemia only after fat, not carbohydrate ingestion (Deopurkar *et al.*, 2010).

> **In short, this evidence suggests that both pathways – paracellular and transcellular – are involved in endotoxin translocation. The paracellular pathway appears to be of particular importance in chronic disease, whereas the transcellular pathway likely contributes to postprandial endotoxemia.**

2.1.3 Tight-junction proteins as gate-keepers

The tight-junction is the most apical protein complex linking epithelial cells together. It is a structure comprising over 50 proteins, with transmembrane proteins interacting with the cytoskeleton through plaque proteins (for review, see Ulluwishewa *et al.*, 2011) (Figure 2). Tight-junction proteins include five families of transmembrane proteins, occludins, claudins junctional adhesion molecules (JAMs), the coxsackie virus and adenovirus receptor (CAR) and tricellulin, which is a tight-junction protein forming a linkage between three adjacent cells (for review, see Groschwitz and Hogan, 2009; Hossain and Hirata, 2008). Occludin, tricellulin and claudin are tetra-span proteins with four transmembrane domains and two extracellular

loops, whereas JAM and CAR are single-span proteins (Hossain and Hirata, 2008; Ulluwishewa *et al.*, 2011). The transmembrane proteins are connected to the cell cytoskeleton by intracellular proteins such as zonula occludens 1-3 (Ulluwishewa *et al.*, 2011). The roles of the most important tight-junctions are very briefly introduced below.

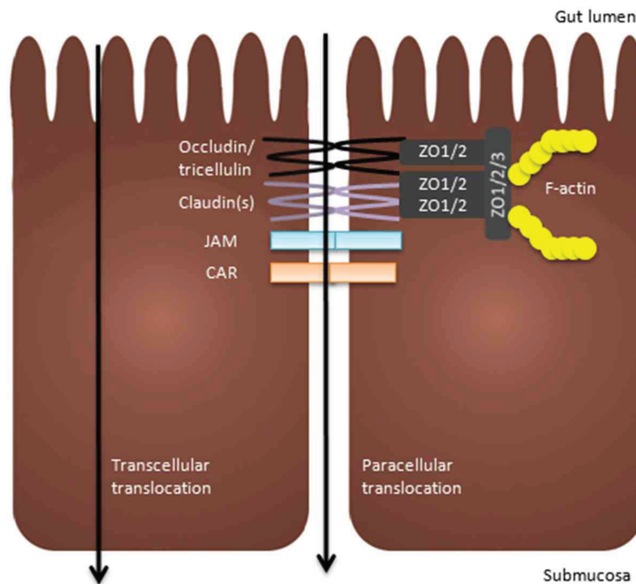

Figure 2. *Pathways of translocation through the epithelium with tight-junction proteins guarding traffic through the paracellular pathway. The size of the enterocytes is not scaled to the size of the tight-junction complex. JAM = Junctional adhesion molecule, CAR = Coxsackie virus and adenovirus receptor, ZO = Zonula occludens. (adapted from Ulluwishewa et al. 2011)*

Claudins are vital to the survival of an individual (for review, see Groschwitz and Hogan, 2009): claudin $1^{-/-}$ mice die within one day of birth due to the lack of a proper gut barrier. Humans bear 24 genes for different claudins, and the responses of those proteins to defects in barrier function are known to differ. For example in barrier dysfunction in Crohn's disease, expression of claudins-1 and -4 are unchanged, while claudin-5 and -8 are downregulated and claudin-2 even upregulated (for review, see Schulzke *et al.*, 2009). Claudins demostrate size- and charge specificity which enable strict control over

the paracellular flux of kations and anions (Groschwitz and Hogan, 2009).

Transgenic occludin$^{-/-}$ mice, in contrast, have normal barrier function despite the total lack of occludin (Groschwitz and Hogan, 2009). Occludin indeed seems to be more important in maintaining barrier function than in its formation, since occludin expression is inversely correlated with the translocation of FITC dextran from the gastrointestinal tract to serum (Cani *et al.*, 2008, 2009).

JAM$^{-/-}$ mice display compromised barrier function (Groschwitz and Hogan, 2009). JAMs seem to play a role especially in tight-junction formation, but not in barrier maintenance, because antibodies against JAM do not disrupt already formed tight-junctions (for review, see Assimakopoulos *et al.*, 2011).

Zonula occludens (ZO) proteins are cytosolic adaptor proteins linking together the cell skeleton and the transmembrane tight-junction proteins. There are three ZO types: ZO-1, ZO-2 and ZO-3. Of these, ZO-1 and -2 are critical in the formation of tight-junctions by recruiting claudins to the tight-junction complex (for review, see Marchiando *et al.*, 2010a).

Tight-junctions are regulated by several intracellular pathways including myosin light chain kinase (MLCK), mitogen-activated protein kinases (MAPK), protein kinase C (PKC) and the Rho family of small GTPases (Ulluwishewa *et al.*, 2011). Of these, the MLCK pathway is one of the most abundant in the gut, and is a crucial step in the regulation of tight-junctional permeability by several external stimuli, such as cytokines and pathogens (Scott *et al.*, 2002) - inhibition of MLCK prevented the deterioration of barrier function. It is well established that MLCK phosphorylates myosin light chain (MLC) leading to the reorganization of the actin cytoskeleton (for review, see Shen, 2012). Downstream events in tight-junction regulation are less well understood. It seems that MLCK induces the endocytosis of occludin from the tight-junction complex to intracellular vesicles, which is

triggered by cytokines or T-cell activation (Clayburgh *et al.*, 2005; Marchiando *et al.*, 2010b). MLCK may also affect permeability via ZO-1 mobility from the tight-junction to the cytoskeleton (Yu *et al.*, 2010). By these mechanisms, MLCK activation increases intestinal permeability. Another pathway for tight-junction regulation is the protein kinase C pathway, which is activated by zonulin, an epithelium-derived protein analogical to zonula occludens toxin (for review, see Fasano, 2000). Zonulin-induced protein kinase C alpha activation leads to actin polymerization and rapid opening of the tight-junctions, and is suggested to have a causal role in celiac disease (Fasano *et al.*, 2000).

> **To summarize, tight-junction proteins are undoubtedly crucial in maintaining the gut barrier. There are numerous different proteins that differently regulate intestinal permeability. To understand how tight-junction proteins are changed in barrier dysfunction, it is inadequate to analyze a single protein, since it is important to understand their interactions. Measuring the activation of regulatory pathways could also prove useful.**

2.1.4 Interplay of inflammation and barrier function

Intestinal inflammation is associated with impaired barrier function, but it is not always clear, which is the cause and which is the consequence. The intestinal barrier controls the passage of inflammatory substances to the submucosa, where they react with immune cells and cause damage via inflammation. For example in two mouse models of colitis, the IL-10 knock-out mouse and the dextran sodium sulphate (DSS) model, histological damage is associated with increased gut permeability (Kennedy *et al.*, 2000; for review, see Perše and Cerar, 2012). The association is likely caused by the increased leakage of inflammatory antigens (Perše and Cerar, 2012). Moreover, JAM$^{-/-}$-mice show increased susceptibility to DSS-induced colitis

(Groschwitz and Hogan, 2009), which implies that impairment of the epithelial barrier predisposes the epithelium to inflammation.

Inflammatory pathways also interfere with systems preserving the gut barrier, most notably tight-junctions (for review, see John *et al.*, 2011). During an inflammatory response, the epithelium releases reactive oxygen species, nitric oxide and a broad spectrum of inflammatory cytokines, which all contribute to barrier dysfunction. Reactive oxygen species are produced in the host response to intestinal bacteria, but an excess may lead to barrier leakage. For example, hydrogen peroxide can directly affect tight-junction distribution (Katsube *et al.*, 2007) or, together with nitric oxide, oxidate and nitrate the actin cytoskeleton, which further disrupts the intestinal barrier (Banan *et al.*, 2001). Hydrogen peroxide also activates nuclear factor kappa B, which leads to cytokine production (Schreck *et al.*, 1991). Moreover, cytokines induce the production of reactive oxygen species, leading to a vicious cycle.

Inflammatory cytokines are normally produced by submucosal immune cells in response to a luminally derived trigger. Modulation of barrier function by inflammatory cytokines is common to several inflammatory gastrointestinal diseases, although the specific cytokine profile varies (John *et al.*, 2011). Among the most important regulators are tumor necrosis factor (TNF)-α and interferon (IFN)-γ. They increase intestinal permeability and lead to a redistribution of tight-junction proteins, which seems to depend on endocytosis of these proteins. These cytokines are proposed to act through a pathway involving MLCK, which fosforylates MLC and leads to tight-junction disruption (Zolotarevsky *et al.*, 2002). Other proinflammatory cytokines include for example the interleukins (especially IL-13 and IL-8), whereas transforming growth factor (TGF)-β is immunosuppressive and has barrier-enhancing properties (John *et al.*, 2011).

2.1.5 How is intestinal permeability measured?

Different methods for the measurement of intestinal permeability may represent slightly different pathways of translocation. For direct measurement of barrier function, a molecular probe is introduced into the intestine and detected from either circulation or urine (for review, see Bjarnason *et al.*, 1995). Barrier function may also be indirectly assessed by the analysis of tight-junction proteins or the serological detection of substances that are assumingly gut-derived, such as bacterial lipopolysaccharides. The methods for assessment of intestinal permeability are summarized in Table 1.

Table 1. *Methods of measuring intestinal permeability, with molecular probes and their sizes*

Direct measurement	**Indirect measurement**
In vivo	
Sugar probes (0.16-0.34 kDa)	Tight-junction proteins
Cr^{51}-EDTA (0.34 kDa)	(Portal) LPS
FITC dextran (4 kDa)	LPS binding protein
In vitro	
FITC dextrans (4-2000 kDa)	
Fluorescein (0.38 kDa)	
Horseraddish peroxidase (44 kDa)	
Mannitol (0.18 kDa)	
Trans-epithelial resistance	

Direct measurement of permeability in vivo

Intestinal permeability may be directly defined by the permeation of a probe molecule. A suitable permeability probe is water-soluble, non-toxic, is not actively absorbed from the intestine, and not metabolized before, during or after translocation through the epithelium (for review, see Bjarnason *et al.*, 1995; DeMeo *et al.*, 2002). Originally, permeability was tested with single molecule probes (lactulose, polyethylene glycol [PEG], ^{51}Cr-labeled ethylenediaminetetraacetic acid [^{51}Cr-EDTA], ^{99m}Tc-

diethylenetriaminopentaacetare [^{99m}Tc-DTPA]). Single-probe tests, however, are influenced by factors unrelated to intestinal permeability, such as gastrointestinal motility and renal clearance (Bjarnason *et al.*, 1995). This led to the principal of dual probe tests, in which the urinary excretion of two sugar probes, lactulose and mannitol, forms an index that reflects mucosal permeability. Lactulose is a larger probe that only translocates through the paracellular pathway when mucosal barrier function is impaired. Mannitol is used as an internal control, since it readily translocates through both paracellular and transcellular pathways. Comparing the urinary concentrations of these two probes eliminates bias caused differences in gastric emptying and fluid ingestion. Despite being considered the gold standard for permeability testing in humans, the lactulose-mannitol test has its pitfall for use in diabetic subjects – chronic hyperglycemia is associated with increased enterocyte mass (Verdam *et al.*, 2011), which may lead to an increased transloaction of mannitol and false low values for intestinal permeability.

Probe size determines the location of translocation, since villus tips contain numerous small, aqueous channels in the tight-junction complexes, while crypts contain fewer but larger channels (for review, see Arrieta *et al.*, 2006). Thus larger probes such as lactulose, inulin and ^{51}Cr-EDTA would translocate the epithelium at the base of villi while small probes (i.e. mannitol) may permeate tips of villi. Very large probes like inulin and the smallest fluorescein isothiocyanate (FITC)-dextrans would only translocate from the crypts.

The metabolite-properties of a sugar probe determines the site of intestine that its permeation reflects (Arrieta *et al.*, 2006). Sucrose is rapidly hydrolyzed in the small intestine, which makes it a useful probe for studying gastric permeability. The small intestine probes lactulose, mannitol, rhamnose and cellobiose, on the other hand, are metabolized by gut microbiota. In conditions of small intestinal bacterial overgrowth, the evaluation of mannitol or lactulose loss is impossible, and introduces a large confounding factor into the method.

Only probes that are stable throughout the gastrointestinal tract, such as sucralose and ^{51}Cr-EDTA, are markers of colonic or whole intestinal permeability. Colonic permeability may be calculated by reducing small intestinal permeability from whole intestinal permeability.

In animal studies, the above-mentioned *in vivo* methods are entirely applicable, although sugar probes are more rarely used. In contrast, the most common probes used are the radiolabeled ^{51}Cr-EDTA and the fluorescence-labeled FITC-dextran, perhaps for their simple means of detection. FITC-dextran is gavaged into the animal and detected from plasma or serum after a certain time period, most often four hours. The dextrans are commercially available in sizes ranging from 4 kDa to 2000 kDa, but *in vivo* experiments have mainly utilized the 4 kDa dextran size.

Direct measurement of permeability in vitro

Intestinal permeability is also measured *in vitro* from tissue samples or cell inserts. These studies may be conducted in an Ussing chamber system (for review, see Clarke, 2009), which holds the sample in conditions mimicking those of the intact organism: water-jacketed for correct temperature in physiological buffer, which is gassed to a pH of 7.4 using a carbogen gas flow. Intestinal permeability may then be measured with molecular probes of various molecular weights (such as FITC dextran, horseraddish peroxidase, mannitol, fluorescein, cascade blue or lucifer yellow) or as transepithelial electrical resistance (TER), which can be used as a measure of the integrity of the paracellular pathway. In the Ussing chamber, TER is calculated from the Ohm's law ($U=R*I$) using tissue potential difference (U) and short-circuit current (I). Resistance (R) is then directly influenced by short-circuit current, which reflects the secretion of electrolytes from the tissue, and may increase deviation in TER between tissue preparations. Interestingly, cell cultures with truncated occludin mutants (Balda *et al.*, 1996) or occludin-targeted siRNA (Al-Sadi *et al.*, 2011), have an increased flux of various kinds of small probes, but show no difference in TER. These

studies suggest that the flux of molecular markers and TER are in some situations dissociated and may reflect different translocation pathways.

Indirect measurement of permeability

Intestinal tight-junction proteins or circulatory LPS are often measured to indirectly describe barrier function. Naturally, tight-junction proteins only reflect the integrity of the paracellular pathway. Nevertheless, decreased expression of occludin and ZO-1 are associated with increased permeability to FITC-dextran (Cani *et al.*, 2008, 2009), which supports their importance in regulating barrier function. The assessment of tight-junction integrity may be complex, since the proper function of tight-junctions is not only determined by protein or mRNA level, but also by tight-junction phosphorylation, structure and localization (for review, see Shen, 2012). A challenge for the future will be to address these issues with modern high-resolution histological techniques.

Impaired barrier function is often mistakenly perceived as a synonym to endotoxemia, because the gut is an important source of plasma endotoxins. Endotoxins from portal or peripheral serum/plasma are thus used as an indirect measure of permeability. However, the following concerns are related to circulating endotoxins as markers of barrier function (Teixeira *et al.*, 2012a): 1) Modifications in gut microbiota may change the luminal LPS concentration and lead to an increase in absolute translocation of LPS without a difference in epithelial permeability *per se*. 2) Altered liver clearance of LPS, for example due to advanced liver disease and defective Kupffer cell function, may affect circulatory levels and lead to false conclusions on the association of a disease with impaired barrier function. Because of these two confounding factors, circulating endotoxins do not necessarily describe gut permeability.

To summarize, intestinal permeability measurements would ideally be performed with two different methods.

Endotoxins or single tight-junctions may not reflect changes in gut permeability, and varying translocation pathways complicates data comparison. Nevertheless, in clinical studies, dual sugar probes, in particular the lactulose:mannitol test, are most often used for permeability measurements, whereas in vivo animal studies rely on FITC-dextran and ^{51}Cr-EDTA. In vitro, both probes and TER may be used quite reliably.

2.1.6 Impaired gut barrier function in disease

Impaired gut barrier function is a common characteristic of many diseases of systemic and local inflammation. This is because the intestinal barrier is a gate-keeper for the inflammatory luminal contents: bacteria and endotoxins. The mechanisms for impaired barrier function, however, have remained unknown.

Inflammatory response by endotoxins

The participation of bacteria-derived endotoxins in sepsis was well known back in the 60's (for review, see Lansing, 1963), but direct human evidence of an inflammatory response following endotoxin injection was not obtained until later (Fong *et al.*, 1989). It is now known that the activation of an endotoxin-induced inflammatory response requires the interaction of four proteins (for review, see Kitchens and Thompson, 2005): LPS binding protein (LBP), cluster of differentiation 14 (CD14), the lymphocyte antigen MD-2 and the endotoxin receptor toll-like receptor 4 (TLR-4). On the cell-surface, membrane-bound CD14 (mCD14) presents LPS to a complex of MD-2 and TLR-4, which together activate the signaling cascade. Soluble CD14 (sCD14) and LBP are serum proteins that influence the potency of LPS to activate TLR-4 (Kitchens and Thompson, 2005). LBP binds to LPS and may cleave it from bacteria to be presented to CD14. At low concentrations LBP promotes the transfer or LPS to the receptor complex and the induction of an inflammatory response. At high

concentrations, however, LBP is anti-inflammatory. Similarly, sCD14 participates in the inflammatory reaction by transfering LPS to mCD14 or the receptor complex. At high concentrations, however, sCD14 attenuates the ability of LPS to activate the receptor complex by transfering LPS into lipoporteins for clearance. LBP and sCD14 seem to be physiological control mechanisms that prevent harmful overreaction to LPS.

Endotoxin-associated gastrointestinal disease

In inflammatory bowel diseases, Crohn's disease and ulcerative colitis, systemic endotoxaemia is correlated with disease severity (Gardiner *et al.*, 1995; Pastor Rojo *et al.*, 2007). Endotoxaemia in these diseases is likely caused by increased intestinal permeability and inflammation of the gut wall (for review, see Arrieta *et al.*, 2006). Barrier dysfunction can also be observed in high-risk populations for Crohn's disease without any disease symptoms (Hollander *et al.*, 1986; Teahon *et al.*, 1992; May *et al.*, 1993; Munkholm *et al.*, 1994), indicating that impaired barrier function precedes disease onset. As one of the most used models of colitis, the interleukin-10 knock-out, does not develop colitis under germ-free conditions (Sellon *et al.*, 1998), inflammation could be suspected to be triggered by bacterial components.

Irritable bowel syndrome (IBS) is a functional gut disorder including diarrhea, constipation or both. It is usually diagnosed with a specific set of criteria called the Rome criteria (Engsbro *et al.*, 2013). There is consistent evidence indicating that intestinal permeability is increased especially in patients suffering the diarrhea-predominant form of IBS, facilitating mucosal inflammation (for review, see Camilleri *et al.*, 2012). Leaking of the gut is thought to contribute to symptom severity: Patients with increased intestinal permeability have both visceral and thermal hypersensitivity and they have a higher index of disorder severity (Zhou *et al.*, 2009a). It is unknown, however, whether gut endotoxins translocate into the circulation in these patients, or if inflammation is only local in the gut epithelium.

Gut-derived liver disease

One of the most investigated set of disorders related to gut barrier dysfunction are liver diseases, due to their direct relationship with the gut - the liver is the first organ to be hit by portal blood endotoxins. As reviewed by others in detail (Valenti *et al.*, 2009; Seki and Schnabl, 2012), increased intestinal permeability is hypothesized to be a key trigger to the onset of alcoholic liver disease and non-alcoholic steatohepatitis. As intestinal permeability is increased, translocated LPS bind to TLR-4 on the surface of Kupffer cells, hepatic macrophages, and induce the release of an array of cytokines causing liver damage. These preclinical observations are supported by data in humans. Patients with non-alcoholic fatty liver disease have elevated serum endotoxin levels (Harte *et al.*, 2010; Volynets *et al.*, 2012), and the degree of liver fat content is positively correlated with intestinal permeability (Miele *et al.*, 2009). However, increased intestinal permeability does not correlate with the presence of steatohepatitis (Miele *et al.* 2009). This suggests that there are further triggers determining the pathological change from steatosis to hepatitis.

Other diseases of low-grade inflammation

Circulatory endotoxins that have leaked from the gut are associated with severe inflammation in human sepsis (for review, see Vollmar and Menger, 2011). Unlike sepsis, low-grade inflammation involves a small but significant systemic inflammatory response. It is now increasingly evident that endotoxins, presumably gut-derived, are involved in the pathogenesis of several diseases. These include not only the inflammatory diseases exclusive to the gut and liver, but also diseases characterized by inflammation in other organs – the pancreas in type 1 diabetes (for review, see Vaarala, 2008) and joints in rheumatological diseases (Vaile *et al.*, 1999; Picco *et al.*, 2000), as shown in human studies. Although the role of gut barrier function in other diseases characterized by low-grade inflammation has not yet been thoroughly investigated, there is a possibility that intestinal permeability plays a

role in the pathogenesis of type 2 diabetes. The association of barrier dysfunction and endotoxemia with type 2 diabetes and obesity has been described in animal models (Cani *et al.*, 2007a, 2007b, 2008), and evidence in obese pregnant women points to an association between elevated serum endotoxins and serum inflammatory markers, as well as macrophage infiltration in adipose tissue (Basu *et al.*, 2011). As shown in large human cohort studies, low-grade inflammation is an independent risk factor for type 2 diabetes (Dehghan *et al.*, 2007; Liu *et al.*, 2007; Wang and Hoy, 2007). Endotoxins are assumed to derive from the gut, and circulating endotoxins are linked to low-grade inflammation. It is then hypothesized that increased gut permeability is related to low-grade inflammation in humans.

2.2 Factors that affect intestinal permeability

2.2.1 Fiber and other microbiome-modulating agents

Dietary fiber exerts beneficial effects in the colon through its bacterial metabolite, the short-chain fatty acid butyrate (for review, see Hamer *et al.*, 2008). Insoluble fibers such as cellulose and lignin are not very well fermented by the microbiota, whereas soluble fibers (e.g. oligofructose, inulin, oat bran) are more readily fermented and produce greater amounts of butyrate in the colonic lumen. Orally administered butyrate decreases intestinal permeability to ^{99m}Tc-DTPA in mouse mucositis (Ferreira *et al.*, 2012) and reduces permeability of a Caco-2 cell culture by facilitating tight-junction assembly (Peng *et al.*, 2007, 2009).

Modulation of the gut microbiota, either by prebiotic carbohydrates or using probiotic bacteria or antibiotics, has an impact on barrier function. Oligofructose and gluco-oligosaccharde, two prebiotic fibers, increase luminal bifidobacteria and decrease endotoxemia in both genetically obese and high-fat-fed mice (Cani *et al.*, 2007a, 2009; Serino *et al.*, 2012) - an effect that seems to be mediated through glucagon-like peptide 2 (GLP-2) (Cani *et al.*, 2009). Furthermore, oligofructose decreases permeability of the direct marker FITC dextran in genetically obese mice (Cani *et al.*, 2009). Although gut bifidobacteria are inversely correlated with endotoxemia (Cani *et al.*, 2007a, 2009), bifidobacteria have not been proven to improve gut barrier function. Thus it is unclear whether the prebiotic-induced improvement in barrier function is affected by increased bifidobacteria or some metabolite such as butyrate.

The gut microbiota itself is known to modulate barrier function. Antibiotic treatment blunts the detrimental effects of a high-fat diet on barrier dysfunction, endotoxemia and inflammation, as well as endotoxemia induced by genetic obesity (Cani *et al.*, 2008). The gut barrier may also be modulated by probiotics. Probiotics are viable, non-

pathogenic organisms that are able to reach the intestines in sufficient numbers to confer benefit to the host. They affect epithelial barrier function by various mechanisms (for review, see Ohland and MacNaughton, 2010): by induction of mucus secretion, promoting host defensin and secretory immunoglobulin excretion, production of antimicrobial factors against pathogens, competing with pathogens for binding sites on the epithelium, and, finally, directly influencing tight-junction structures. One of the most studied probiotics, *Lactobacillus rhamnosus* GG (LGG), was reported to improve barrier function as measured by the lactulose/mannitol test in children with abdominal pain (Francavilla *et al.*, 2010). LGG can also prevent cow milk -induced barrier dysfunction in suckling rats (Isolauri *et al.*, 1993).

2.2.2 Dietary fat and obesity

The emerging concept

The link between obesity, gut barrier dysfunction and endotoxemia was recently suggested in genetically obese mice (Brun *et al.*, 2007). The authors reported decreased TER and increased HRP flux in the small intestine of ob/ob mice, as well as an elevated level of portal endotoxins. During the same year, a pioneering group from Belgium reported metabolic endotoxemia in mice fed a high-fat, carbohydrate-free diet (Cani *et al.*, 2007a, 2007b). These reports established the concept of barrier dysfunction in obesity, and Cani *et al.* (2008) later showed that a high-fat, carbohydrate-free diet increases the flux of FITC-dextran from the gastrointestinal tract to the circulation.

Obesity or dietary fat?

Since gut barrier function was shown to be altered in both obesity and on a diabetogenic high-fat diet (Brun *et al.*, 2007; Cani *et al.*, 2008), it was still unclear whether barrier dysfunction is caused by obesity or a high-fat diet. A specific challenge in rodent obesity research is the diet-induced obesity model, where animals become obese by eating a high-fat diet (for review, see Hariri and Thibault, 2010). Although this model

is very useful, it has not permitted the distinction between the effects of diet and expanding fat mass. Several studies over the past few years have been conducted in the field of dietary fat, obesity, barrier function and endotoxemia. These are summarized in Table 2. As barrier dysfunction is thought to be strongly linked with endotoxemia, studies with only plasma endotoxin analyses are also included.

After the initial report by Brun *et al.* (2007), results on the link between obesity and barrier dysfunction have been unclear (Cani *et al.*, 2008, 2009; Suzuki and Hara, 2010; Haub *et al.*, 2011). On the other hand, several reports have shown endotoxemia or increased gut permeability in designs that cannot distinguish between the effects of diet and obesity (Cani *et al.*, 2007a, 2007b, 2008; Everard *et al.*, 2012; Kim *et al.*, 2012; Kirpich *et al.*, 2012; de La Serre *et al.*, 2010; Laugerette *et al.*, 2012; Serino *et al.*, 2012). Only two studies have attempted to rule out the effect of obesity, and have reported an increased Cr-EDTA flux (Suzuki and Hara, 2010) or elevated serum endotoxins (Carvalho *et al.*, 2012) in animals fed with a high-fat diet.

Another noteworthy matter is that although endotoxemia may reflect barrier dysfunction, the possible alterations in LPS clearance may bias results (Teixeira *et al.*, 2012a), as discussed previously on page 24. It is thus important to conduct studies using direct markers of intestinal permeability, and to specifically compare studies using permeability probes. Only seven studies have used direct markers of permeability. Most of them show a permeability-increasing effect for diet-induced obesity in rats or a diabetogenic diet in mice (Cani *et al.*, 2008; de La Serre *et al.*, 2010; Serino *et al.*, 2012), but the two studies on the effects of obesity, not dietary fat, have reported opposite results (Brun *et al.*, 2007; Suzuki and Hara, 2010) – Suzuki and Hara (2010) did not see any difference in permeability by genetic obesity. In rats, dietary lard seems to impair gut barrier function (De La Serre *et al.*, 2010; Suzuki and Hara, 2010), and in mice two studies using an extreme diabetogenic high-fat diet have showed increased dextran flux (Cani *et al.*, 2008; Serino *et al.*, 2012). Studies using this diabetogenic diet,

Table 2. *Animal studies on intestinal permeability (marked with bold) or endotoxemia in obesity and/ or high-fat feeding.*

	Animals	Diet	Duration	Methods	Main results	Effect	Reference
OBESITY ONLY	WT C57Bl/6J, ob/ob and db/db mice	SC	12 w*	TER, horseradish peroxidase *ex vivo*, portal endotoxins	TER↓, HRP flux↑, portal endotoxins↑ in obese animals	↑	**Brun *et al.*, 2007**
	WT C57Bl/6J and ob/ob mice	SC	10 w*	Plasma endotoxins, FITC gavage for other study design	Plasma endotoxins 8-fold, dextran not reported	↑/?	**Cani *et al.*, 2008**
	WT C57Bl/6J and ob/ob mice	SC and diets containing cellulose or prebiotics	11 w*	FITC gavage and detection after 1 and 4 h, plasma endotoxins, occludin and ZO-1 immunofluorescence	Tight-junction localization at apical border was decreased by obesity, but improved by dietary fiber; prebiotics decreased plasma endotoxins and plasma dextran, but values for control are not reported	?	**Cani *et al.*, 2009**
	Genetically obese and lean rats	SC and HF (mostly lard)	16 w	Cr-EDTA (weeks 9 and 15) and phenolsulfonphthalein (week 3), gavage	Genetic obesity had no effect on probe excretion at any time point	↔	**Suzuki and Hara, 2010**
	WT C57Bl/6J and ob/ob mice	Not reported	10-12 w*	Portal plasma endotoxins and duodenal occludin content	Portal endotoxins significantly elevated in first study set, but not in second; no difference in duodenal occludin	?	Haub *et al.*, 2011
HIGH-FAT DIET ONLY	Genetically obese and lean rats	SC and HF (mostly lard)	16 w	Cr-EDTA (weeks 9 and 15) and phenolsulfonphthalein (week 3)	HF diet increased urinary probe excretion at 3, 9 and 15 weeks (+30% in lean, +200% in obese at 15 weeks)	↑	**Suzuki and Hara 2010**
	Swiss mice	SC and pair-fed HF: 55 E% fat	12 w	Serum endotoxins	Serum endotoxins 3-fold in HF vs. control	↑	Carvalho *et al.*, 2012

C57Bl/6J mice	SC and HF: 72 E% fat (corn oil and lard), <1 E% carbohydrate	14 w	Plasma endotoxins	Plasma endotoxins 1.8-fold in HF vs. control and decreased by prebiotic treatment	↑	Cani *et al.*, 2007a
C57Bl/6J mice	SC and HF: 72 E% fat (corn oil and lard), <1 E% carbohydrate	4 w	Plasma endotoxins	Plasma endotoxins two-fold in HF vs. control mice	↑	Cani *et al.*, 2007b
C57Bl/6J mice	SC and HF: 72 E% fat (corn oil and lard), <1 E% carbohydrate	4 w	4 kDa FITC gavage and detection after 1 h, plasma endotoxins	HF vs. control: plasma endotoxins 2.5-fold, plasma dextran increased from zero to 0.3 µg/ml	↑	**Cani *et al.*, 2008**
Sprague-Dawley rats	10 E% and 45 E% fat, mostly lard	10-12 w	4 kDa FITC gavage, plasma endotoxins	Plasma endotoxins and dextran flux ~5-fold in high-fat obese vs. control lean rats, but not in obesity-resistant rats	↑	**De La Serre *et al.*, 2010**
C57Bl/6J mice	SC and 45 E% fat, mostly lard	8 w	Plasma endotoxins	Endotoxins ~2-fold in HF vs. control mice	↑	Everard *et al.*, 2012
C57Bl/6J mice	10 E% and 60 E% fat, mostly lard	8 w	Plasma endotoxins	Plasma endotoxins 1.9-fold in HF vs. control mice	↑	Kim *et al.*, 2012
C57Bl/6N mice	40 E% fat, MCT-oil+beef tallow or corn oil, no low-fat control	8 w	Blood LPS, 4 kDa FITC in ileum *ex vivo*, TJ expression	No differences in endotoxemia or permeability by fat quality, but TJ expression was lower after unsaturated fat	?	**Kirpich *et al.*, 2012**
C57Bl/6 mice	Milk fat, palm oil, rapeseed oil or sunflower oil diet, 37.7 E% fat, and SC	8 w	Plasma endotoxins	Plasma endotoxins were elevated by the unsaturated fatty acids, not by the saturated fatty acids, but systemic inflammation was highest in the palm oil group	↑/?	Laugerette *et al.*, 2012
C57Bl/6 mice	SC and HF: 72 E% fat (corn oil and lard), <1 E% carbohydrate	3 months	4 kDa FITC dextran in ileum, caecum and colon *ex vivo*, plasma LPS	Plasma LPS +8%, intestinal permeability 1.5-2-fold, although non-significant in colon	↑	**Serino *et al.*, 2012**

* Age of ob/ob-mice; SC = Standard chow, HF = High-fat, TER =Transepithelial electrical resistance, TJ = Tight-junction, WT = Wild-type, ↑ = Increased permeability or endotoxins by HF diet or obesity, ↔ = No change in permeability or endotoxins, ? = Effect not reported

however, have not been designed to study obesity and often do not report data for body weight. Thus, it cannot be ascertained that the reported effects are not attributable to the pathogenesis of diabetes.

Fat quality has not been studied using permeability probes. However, recent findings reveal the complexity of the effects of different fatty acids on endotoxemia and metabolic inflammation. Various serum factors are involved in LPS-induced signaling (see 2.1.6), namely LBP and sCD14. The relative proportions of these serum factors seem to determine the magnitude of the inflammatory response that LPS induces. In an 8-week dietary intervention with milk fat, palm oil, rapeseed oil or sunflower oil, the mice receiving palm oil had the highest levels of plasma and adipose tissue inflammatory markers, but no change in plasma endotoxins (Laugerette *et al.*, 2012). On the contrary, mice receiving rapeseed oil had the highest levels of plasma endotoxins, but lowest level of the plasma inflammation marker interleukin-6. These converse inflammatory responses to endotoxemia were attributable to the level of soluble CD14, a plasma protein that binds endotoxins into a form that cannot trigger an inflammatory response. The rapeseed oil diet resulted in a higher ratio of soluble CD14 to LPS compared to the palm oil diet. These results highlight the need to study plasma carrier proteins and inflammation in parallel with plasma endotoxins, in order to draw accurate conclusions of the effects of diet on pathophysiological mechanisms.

To summarize, animal studies show increased intestinal permeability in models of diet-induced obesity, where the effects of obesity and a high-fat diet cannot be distinguished. Results on the independent effects of obesity are contradicting, and the effect of dietary fat, irrespective of obesity, has only been shown once in rats. Most dietary intervention studies in animals have utilized a high-fat diet with lard as the

principle fat source. Not many studies have addressed the matter of fat quality in gut barrier function.

Human studies

Human studies in the field of dietary fat, obesity and barrier function are scarce – only five studies have addressed this field. No difference in small or large bowel permeability was reported in a small pilot study containing 13 obese and 11 control subjects (Brignardello *et al.*, 2010). A 24h food intake recall revealed that there were no differences in dietary fat quantity or quality, which suggests a lack of effect for obesity, not dietary fat. Another study on obese subjects demonstrated a borderline non-significant correlation for body weight and body mass index for lactulose/mannitol excretion ratio (Teixeira *et al.*, 2012b). Dietary intake was not recorded. Interestingly, a study on obese patients with metabolic syndrome reported that the translocation of direct probes for both small and large intestine was increased in obese patients (Leber *et al.* 2012). Again, the possible role of dietary fat cannot be excluded.

The effects of dietary fat and fiber were studied in an elegant study by Pendyala *et al.* (2012), in which eight subjects were kept in metabolic wards for the duration of a cross-over intervention trial. A 1-month Western-style diet containing 40 E% fat (20.8 E% saturated fat) and only 12.5 g/day fiber increased endotoxemia by 71% compared to baseline, whereas a 1-month prudent diet containing 20 E% fat (5.8 E% saturated) and 31 g/day fiber decreased endotoxemia by 31% compared to baseline. The trial periods were separated by a wash-out period of one month, and the energy contents of the diets were identical. The authors discussed that it is unclear whether the interventions affected intestinal permeability or merely changed the composition of gut microbiota (Pendyala *et al.*, 2012). Finally, a Chinese cross-sectional study suggested that obesity was associated with a marker of endotoxemia, LBP, in healthy men (Sun *et al.*, 2010).

To summarize, human studies do not clearly demonstrate a link between obesity or dietary fat and barrier dysfunction, although dietary changes appear to affect endotoxemia. In clinical settings it is more difficult to control for dietary fat intake, which may explain the small number of studies performed.

2.2.3 Other dietary components

Proteins and amino acids

The perhaps most well-known amino acid impacting gut function is glutamine, the primary energy source for enterocytes (Blachier *et al.*, 2009). In a rat model of acute pancreatitis, this amino acid improves barrier function when given as a component of parenteral nutrition (Foitzik *et al.*, 1997). Glutamine deprivation causes Caco-2 cell injury (Panigrahi *et al.*, 1997), whereas glutamine supplementation protects Caco-2 cells from barrier dysfunction induced by media change (Li *et al.*, 2003). In cancer patients, glutamine supplements may reduce barrier dysfunction induced by radiochemotherapy (Yoshida *et al.*, 1998). Several milk protein components have also been suggested to confer beneficial effects on the gut barrier, although mostly reported in cell models. These milk protein components include hydrolyzed casein, which decreases permeability in diabetes-prone rats (Visser *et al.*, 2010), a casein-derived peptide, Asn-Pro-Trp-Asp-Gln, which slightly but significantly increases TER and occludin levels in Caco-2 cells (Yasumatsu and Tanabe, 2010), and the major whey protein β-lactoglobulin, which increases TER when Caco-2-cell tight-junctions are destabilized by culturing in serum-free media (Hashimoto *et al.*, 1998). The arginine-rich nuclear protein protamine also improves barrier function in rat small intestine *in vivo* (Shi and Gisolfi, 1996). On the contrary, L-alanine and supraphysiological concentrations of tryptophan impair gut barrier in intestinal tissue of animals (Madara and Carlson, 1991; Sadowski and Meddings, 1993).

Alcohol

Alcoholic liver disease is well known to be characterized by gastrointestinal barrier dysfunction and endotoxemia in humans (for review, see Rao, 2009). It is clear that ethanol increases gastroduodenal permeability, but its role in barrier dysfunction in the distal intestine is unclear. Ethanol's primary metabolite, acetaldehyde, impairs intestinal barrier function at much lower concentration than ethanol itself. Such small concentrations are detected in rat feces. It is thus believed that barrier dysfunction in the distal gut is acetaldehyde-induced. Acetaldehyde accumulates in the colonic lumen, since the gut microbiota poorly metabolizes it further to acetate.

Zinc, flavonoids and gliadin

Zinc, flavonoids and gliadin have also been studied in terms of gut permeability, mostly in cell culture. Zinc has been proposed to have a role in barrier maintenance in cell cultures (Finamore *et al.*, 2008). Also, the flavonoid quercetin increases TER and decreases probe flux in Caco-2 cells (Amasheh *et al.*, 2008; Suzuki and Hara, 2009). Other flavonoids, epigallocatechin gallate in green tea (Watson *et al.*, 2004) and genistein in soy (Schmitz *et al.*, 1999), do not affect TER alone, but may be capable of preventing inflammation-induced barrier-disruption. Gliadin, the symptom-inflicting cereal component in celiac disease, decreases tissue resistance, causes reorganization of the cytoskeleton and compromises interactions of occludin and ZO-1 by inducing the production of barrier-toxic zonulin, as shown in patient tissue preparations and intestinal epithelial cell lines (Wang *et al.*, 2000; Clemente *et al.*, 2003; Drago *et al.*, 2006). These events are probable reasons for barrier impairment by gliadin in celiac disease.

2.2.4 Other factors

Age

Intestinal permeability varies during the human lifespan. Immediately after birth, the intestinal barrier is immature and highly permeable to

macromolecules, but begins its rapid maturation once oral feeding is started (Weaver *et al.*, 1984). This happens much faster in neonates receiving human milk compared to formula-fed infants (Taylor *et al.*, 2009). In the elderly, intestinal permeability does not seem to be changed (Saweirs *et al.*, 1985; Riordan *et al.*, 1997).

Psychiatric stress

Because early life stress is related to later onset of irritable bowel syndrome in humans, the maternal separation rat model has become a popular tool for irritable bowel syndrome investigation (for review, see O'Mahony *et al.*, 2011). Maternally separated rats have been demonstrated to show increased permeability of the colon (Gareau *et al.*, 2007) and barrier dysfunction induced by water avoidance stress (Söderholm *et al.*, 2002). A role for the hypothalamus-derived corticotrophin-releasing hormone and the neural muscarinic and nicotinic receptors were suggested by these reports. The gut-brain axis is bidirectional: psychological stress alters gastrointestinal function, and inflammation of the gastrointestinal tract sends signals to the brain via the vagus nerve or a humoral pathway (O'Mahony *et al.*, 2011). The brain thus seems to have an important role in mediating gut function and barrier integrity.

Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetyl salicylic acid and ibuprofein impair the gut barrier and may even cause ulcerations and perforation, accounting for the gastrointestinal side-effects of these drugs (for review, see Tachecí *et al.*, 2010). The drug may damage the epithelium several times: first locally upon administration, then systemically once absorbed, and again locally if the drug is eliminated with bile. Systemically, NSAIDs inhibit the cyclooxygenase enzyme, which produces barrier-protective prostaglandins. Locally, NSAIDs disturb mitochondrial energy metabolism and may induce nitric oxide release, which both contribute to gut barrier impairment in the rat.

Pathogens

The intestinal lumen is home to several commensal bacteria. Some strains of microbes, however, are pathogenic and may impair intestinal barrier function in preclinical studies by the disruption of tight-junctions and initiation of inflammatory cascades (for review, see Guttman and Finlay, 2009). These pathogens include bacteria such as *Escherichia coli*, *Salmonella, Shigella flexneri* and the common cause of food poisoning, *Clostridium perfringens,* most of which attack the cell using effector proteins or enterotoxins. The viruses hepatitis C, the respiratory and gastrointestinal pathogen reovirus, and the common cause of viral gastroenteritis rotavirus, usually specifically target tight-junction proteins. In contrast, coxsackieviruses and adenoviruses use the coxsackie virus and adenovirus receptor for internalization and breakdown of the epithelial barrier. Interestingly, *Vibrio cholerae* has been discovered to use a bacterial surface protein, zonula occludens toxin, which increases probe flux and reduces TER in rabbit small intestine, but not in colon (Fasano *et al.*, 1991, 1997).

Intestinal ischemia and radiation

Gut barrier function is impaired by several acute threats to the body, some of which increase the risk of sepsis. These include acute pancreatitis (for review, see Andersson and Wang, 1999), surgery (Roumen *et al.*, 1993; Bölke *et al.*, 2001) and burns (for review, see Magnotti and Deitch, 2005), which are all characterized by intestinal ischemia in human subjects. Ischemia leads to increased intestinal permeability, which precedes sepsis (for review, see Kong *et al.*, 1998). Another type of trauma to the gut is caused by radiation, which also impairs human gut barrier function (for review, see MacNaughton, 2000). Evidence from animal studies shows that this may occur even before cell cycle arrest and impaired cell renewal.

2.3 Luminal bile acids

2.3.1 Synthesis, transformation and absorption

Bile acid synthesis and regulation of the bile acid pool

Bile acids are synthesized from cholesterol in liver hepatocytes. The structures and synthesis of the most important bile acids are shown in Figure 3. Most bile acids, 75%, are synthesized by the classic or neutral pathway, in which the rate-limiting enzyme is the CYP7A1, which adds an α-hydroxyl group to carbon 7 in the sterol structure (for review, see Lefebvre *et al.*, 2009). The human liver synthesizes two primary bile acids: cholic acid and chenodeoxycholic acid (CDCA) (for review, see Houten and Auwerx, 2008). In addition to these, the rodent liver produces a third species of primary bile acids, the muricholic acids, of which β-muricholic acid (3α,6β,7β) is most prominent. The other stereoisomers are α-muricholic acid (3α,6β,7α) and ω-muricholic acid (3α,6α,7β). In rat liver, CDCA is metabolized into α-muricholic acid and, via a ketone intermediate, into β-muricholic acid (Botham and Boyd, 1983). Bile acids are secreted into bile as glycine and taurine conjugates, and stored in the gall bladder (for review, see Ridlon *et al.*, 2006). Upon fat ingestion, cholecystokinin stimulates contractions of the gall bladder and bile flow to the duodenum.

The bile acid pool is regulated by Farnesoid X receptor (FXR), which is activated by bile acids in both the intestine and the liver (for review, see Chiang, 2009). In the intestine of mice, but not humans, FXR induces the expression of the apical sodium-dependent bile acid transporter (ASBT). The lack of ASBT causes bile acid malabsorption. FXR also upregulates the ileal bile acid binding protein (IBABP) and fibroblast growth factor 19 (FGF19), which signals form the intestine to the liver to downregulate CYP7A1, the key enzyme in bile acid synthesis. FXR thus has an undeniable role in bile acid traffic in the intestine, mainly by increasing the reuptake of bile acids. In the liver its role is the opposite - it downregulates CYP7A1 and bile acid

synthesis via FGF19- and small heterodimer partner (SHP)-dependent pathways. FXR plays a critical role in the coordination of bile acid homeostasis. An unbalance in this homeostasis may lead to bile acid - related diseases such as cholestasis, where bile acids accumulate in the liver causing damage to liver cells. Interestingly, bile acids are also involved in metabolic regulation (for review, see Prawitt *et al.*, 2011) – bile acid sequestrants, such as cholestyramine, improve glycemic control in type 2 diabetic patients. The effects on glucose metabolism could be mediated through the bile acid receptor FXR, which guards liver glucose production. Another bile acid receptor, TGR-5, is suspected to take part in energy metabolism, possibly affecting weight gain in animals.

Modification of bile acids by intestinal microbiota

Bile acids are very efficiently absorbed from the ileum and transported to the liver for reuse (Houten and Auwerx, 2008). This cycle is called the enterohepatic circulation. However, a small number of bile acids flow to the large intestine, where the bile salt hydrolases of gut microbes cleave them into their deconjugated form (Ridlon *et al.*, 2006). Deconjugated bile acids are much more hydrophobic and on-go passive absorption from the colon.

Gut microbes contain enzymes capable of oxidation and epimerization of bile acids (Ridlon *et al.*, 2006). The most important of these is the 7α-hydroxylase, which produces the secondary bile acids deoxycholic acid (DCA) and lithocholic acid from their primary forms cholic acid and CDCA. This pathway is estimated to be found only in 0.0001% of colonic flora, in species of the *Clostridium* genus (Lefebvre *et al.*, 2009). Human liver is unable to 7α-hydroxylate the secondary bile acids back into their respective primary forms (Ridlon *et al.*, 2006). Thus, DCA accumulates in the bile acid pool. Lithocholic acid is sulphated and lost in feces, and does not accumulate in the enterohepatic circulation. In rodents, the secondary bile acids are rehydroxylated by the liver and are not present in primary bile (Lefebvre *et al.*, 2009).

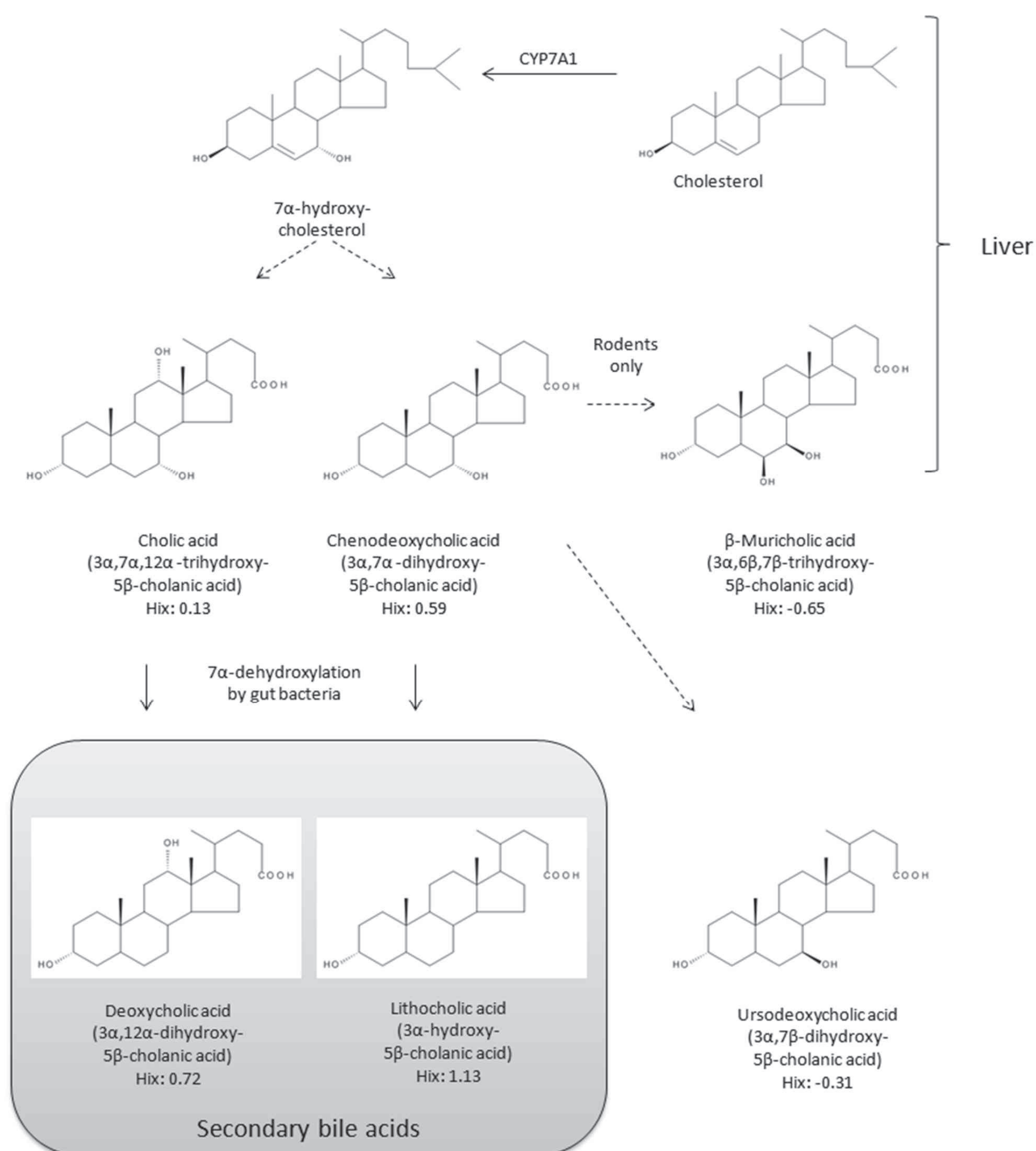

Figure 3. *Synthesis and metabolism of the most important bile acids. Dashed lines represent intermediate metabolites. The higher the hydrophobicity index (HIx), the more hydrophobic the structure is.*

Ursodeoxycholic acid (UDCA) is one of the most hydrophilic bile acids. It comprises only two per cent of both biliary and fecal bile acids in humans (Ridlon *et al.*, 2006). In bears it is one of the main bile acids (Houten and Auwerx, 2008), hence the name "urso". Because the enzymes producing UDCA are unelucidated, it is unclear whether this bile acid is solely produced by gut microbiota, or if liver may contribute to its synthesis. UDCA is a 7β-hydroxy epimer of CDCA, which also appears to be its precursor – the synthesis of UDCA is inducible by administration of CDCA (Salen *et al.*, 1974). Furthermore, intravenously injected radiolabeled CDCA results in the redistribution of the label between CDCA and UDCA, which provides evidence that CDCA is transformed into ursodeoxycholic acid via 7-ketolithocholic acid (Salen *et al.*, 1974). The bacterial species metabolizing CDCA to 7-ketolithocholic acid were only recently elucidated (Fukiya *et al.*, 2009). UDCA is sometimes called a tertiary bile acid, since it is not directly synthesized from the primary bile acids.

2.3.2 How does diet modulate the luminal bile acid pool?

There are several ways in which diet may affect the luminal bile acid pool. The most evident modifications are in the concentration and daily excretion of bile acids. Although useful, these parameters give only a rough estimate of how luminal bile acids could affect the gastrointestinal tract. The bile acid pool comprises several different bile acid structures that vary for example in hydrophobicity (Heuman, 1989) and their ability to bind receptor molecules (Lefebvre *et al.*, 2009). It is then important to not only study the quantity, but also the quality of luminal bile acids. Furthermore, the gut lumen contains agents such as fibers that bind bile acids (Huang *et al.*, 1978), possibly into a non-toxic form. If the bias caused by these agents cannot be ruled out in a study design, the cytotoxicity of fecal water (the aqueous phase in feces) should also be analyzed.

Dietary fat

An early report in the 70's concluded that increasing fat intake resulted in higher bile acid excretion in feces (Reddy *et al.*, 1977). Similar results were later obtained in a population study (Reddy, 1981), a controlled clinical trial (Cummings *et al.*, 1978) and in mice (Bianchini *et al.*, 1989). Unsaturated corn oil has been shown to increase bile acid excretion compared to saturated cocoa butter (Connor *et al.*, 1969). It was hypothesized that the bile acid excretion promoted by unsaturated fat is related to the serum cholesterol - lowering effect of the same fatty acids. However, Reddy *et al.* (1977) noted no such effect for fat quality (corn oil or lard).

Fat quality, in humans and monkeys, does not seem to affect bile composition in the gallbladder (Tanaka *et al.*, 1976; Scobey *et al.*, 1992; Yago *et al.*, 2005). It may, however, affect the profile of bile acids in feces (Reddy *et al.*, 1977, Sato *et al.* 1987; Monsma *et al.*, 1996; Reddy *et al.*, 1996). To compare results from the published papers in the present review, a hydrophobicity index was calculated from the data given by these reports. The index was calculated as a percentage-weighted mean of hydrophobicities, as previously reported by Heuman (1989). Reddy *et al.* (1996) did not report values for UDCA, thus the indices for that study were calculated without UDCA. A high hydrophobicity index reflects a high content of secondary bile acids, which are potentially harmful to the intestinal mucosa (Reddy, 1981). The calculated indices are reviewed in Table 3.

Table 3. *Effect of fat quality on fecal bile acid hydrophobicity in rats.*

Total dietary fat content (w/w)	Fat type	Hydrophobicity index	Reference
5% or 20%	5% corn oil	0.464	Reddy *et al.* 1977
	5% lard	0.482	
	20% corn oil	0.601	
	20% lard	0.650	
20%	Corn oil	0.719	Sato *et al.* 1987
	Rapeseed oil	0.627	
	Sesame oil	0.608	
	Soybean oil	0.299	
	Pork lard	0.797	
	Beef tallow	0.755	
16%	Corn oil	0.597	Monsma *et al.* 1996
	Pork lard	0.462	
	Beef tallow	0.265	
	Cocoa butter	0.235	
5% or 23.5%	Low fat corn oil	0.560	Reddy *et al.* 1996
	High-fat corn oil	0.657	
	High-fat fish oil	0.500	

The percentage-weighted hydrophobicity index (HIx) was calculated from data provided by the references using the hydrophobicities published by Heuman 1989 for lithocholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid, ursodeoxycholic acid and β-muricholic acid. As Reddy *et al.* (1996) did not provide data for ursodeoxycholic acid, it was not included in the calculations for that study.

Data of the effect of fat quality on fecal bile acid hydrophobicity is controversial. According to data by Reddy *et al.* (1977) and Sato *et al.* (1987), saturated fat feeding increased the hydrophobicity index of feces. In contrast, Monsma *et al.* (1996) reported a lower hydrophobicity index after saturated fat feeding. Data from Reddy *et al.* (1977; 1996) suggest that increased dietary fat increases fecal bile acid

hydrophobicity, while a high-fat fish oil diet did not affect hydrophobicity compared to the low-fat control. Without raw data and statistical analysis, these values should be reviewed only with caution and definite conclusions should not be drawn.

Dietary fat quality may also affect concentration of bile acids in fecal water. In a study by Lapré *et al*. (1993), rats fed with palm oil or milk fat excreted more bile acids and had a higher bile acid concentration in fecal water compared to rats fed with corn oil. Fecal water cytotoxicity was highest in the palm oil group.

> **To summarize, data on how dietary fat quality affects bile excretion are somewhat controversial. Increasing fat quantity results in higher bile acid excretion. The effect of fat quality on bile acid profile, however, is still debated.**

Cholesterol

In studies investigating the effects of animal fat and vegetable fat on bile acid excretion, the diet high in animal fat is also high in cholesterol. This does not, however, seem to confound the data since even a 30-fold increase in dietary cholesterol has no significant effect on fecal bile acid concentration in humans (Wilson and Lindsey, 1965). Excess cholesterol is mainly excreted as neutral sterols, not by conversion to bile acids (Quintão *et al.*, 1971).

Dietary fiber

The main action of dietary fiber on the luminal bile acid pool is its diluting effect. Bile acid excretion was investigated in two populations, Kuopio (Finland) and New York, with an equal fat and protein intake, but with a 3-fold higher fiber intake in Kuopio (Reddy *et al.*, 1978). Daily excretion of secondary bile acids was not altered, perhaps because of equal fat intake in the two study populations. The concentration of those bile acids, however, was decreased in the Kuopio population. Dietary fiber is thus associated with increased stool

bulk and decreased fecal bile acid concentration, suggesting a dilutive effect for secondary bile acids. In a controlled trial of 34 men, a high-fiber diet containing different sources of soluble and insoluble fiber was compared to a low-fiber diet with similar fat content (Kesäniemi *et al.*, 1990). The high-fiber diet increased bile acid excretion, when calculated per day. This supports the hypothesis that fiber binds luminal bile acids, enhancing excretion. The quality of dietary fiber may affect its impact on bile acid excretion in humans and animals (for review, see Huang *et al.*, 1978). Hemicellulose and pectin bind to bile acids and may increase their excretion which is proposedly accompanied by a decrease in serum cholesterol. In rats and hamsters, inulin, beta-cyclodextrin and psyllium enhance bile acid excretion, whereas guar gum and pectin have no effect (Levrat *et al.*, 1994; Trautwein *et al.*, 1998a, 1998b). Furthermore, fiber-rich food items may induce changes in bile acid profile. Bran feeding may reduce 7α-dehydroxylation of bile acid into their secondary forms, although not all studies support this result (Huang *et al.*, 1978). There is also some evidence that cellulose, pectin, konjac glucomannan and inulin reduce fecal water concentrations of secondary bile acids and its cytotoxicity to Caco-2 cells (Chen *et al.*, 2010). The effects of different fibers on luminal bile acid profile and cytotoxicity *in vivo* have not been studied.

Other dietary ingredients

Although animal products alter bile excretion, it seems to be the fat, not the protein that is effective - animal proteins have no effect on bile acid excretion (for review, see Reddy, 1981).

Dietary calcium has been suggested to protect from colonic cancer by precipitating toxic bile acids in the gut lumen. In a human placebo-controlled trial, high calcium intake decreased the concentration of secondary bile acids in fecal water and reduced fecal water cytotoxicity (Govers *et al.*, 1996). The daily output of bile acids was increased, which also suggests bile acid binding by calcium. Very similar results have been obtained in rats: Calcium phosphate supplementation

increased fecal bile acid excretion, but decreased fecal water cytotoxicity and concentration of bile acids (Lapré *et al.*, 1993). Another rat study with lower dietary calcium levels has shown contrasting results (Lupton *et al.*, 1994); increasing calcium phosphate intake reduced the excretion of total bile acids, while increasing fecal fat excretion. Fecal water was not analyzed for bile acids or cytotoxicity. A more recent human study shows that calcium increases excretion of bile acids, but has no effect on fecal water genotoxicity (Ditscheid *et al.*, 2009). There is, therefore, no consensus on whether calcium is able to precipitate luminal bile acids into a less cytotoxic form.

2.3.3 Bile acids and epithelial permeability

The earliest study on the effects of bile acids on intestinal permeability was published in 1972, but reported no difference in mucosal permeability to ^{14}C-thiourea (Sladen and Harries, 1972). Later studies with other molecular probes mostly disagree. Due to the surfactant effect of bile acids, it was no surprise that unconjugated bile acids, especially DCA and CDCA, reversibly increase intestinal permeability to various probes at 1-21 mM concentrations in the rat (Fihn *et al.*, 2003; Goerg *et al.*, 1980, 1983; Gyory and Chang, 1983; Sun *et al.*, 2004a, 2004b), the rabbit (Chadwick *et al.*, 1979; Fasano *et al.*, 1990) and the pig (Argenzio and Whipp, 1983; Henrikson *et al.*, 1989), with contrasting results on CDCA (Hollander *et al.*, 1989). In human biopsies of sigmoid colon, 1 mM CDCA or 0.5-1 mM DCA increased permeability to Cr-EDTA, but translocation of horseraddish peroxidase was unaltered (Münch *et al.*, 2007). Later the same group observed that colonic biopsies from colitis patients in remission responded to 100 µM DCA or CDCA by an increased translocation of *E. coli* bacteria, but without any change in TER (Münch *et al.*, 2011). In human subjects *in vivo*, a 750 mg bolus of CDCA increased lactulose/rhamnose ratio indicating an increased intestinal permeability (Van Nieuwenhoven *et al.*, 1999). The barrier disrupting effect of bile acids, especially DCA and CDCA is thus well shown at various concentrations ranging between 0.5-21 mM.

Comparisons of bile acid structures in their potency to impair barrier function have mainly been studied in the Caco-2 cell model. In this cell model, the applied concentrations, 50-250 µM, are much smaller than for whole tissue. At 200 µM, all bile acids irrespective of hydrophobicity, seem to decrease TER (Araki *et al.*, 2005), but at 50 µM some differences between bile acid species were seen: cholic acid, CDCA and DCA increased dextran flux, but UDCA had no effect (Raimondi *et al.*, 2008). Hughes *et al.* (2008) have also shown a clear barrier-disruption by the secondary bile acids DCA and lithocholic acid, whereas data for cholic acid and CDCA are not as consistent. In rabbit colon, only DCA and CDCA increased permeability at 5 mM, whereas cholic acid and ursodeoxycholic acid did not (Chadwick *et al.*, 1979).

Translocation pathway activation has been investigated by a mixture of cholic acid, DCA and taurocholic acid at equimolar concentrations (Catalioto *et al.*, 2008). The mixture was applied onto Caco-2 cells at concentrations ranging between 0.5-4.5 mM, and three markers of permeability were used: lucifer yellow for paracellular transport, propranolol for transcellular transport and phenylalanine for active transport. The bile acid mixture was shown to upregulate the paracellular pathway only.

There are some indications of the mechanism of bile acid -induced barrier dysfunction. Whereas some studies have indicated an important role for the enteric nervous system (Fihn *et al.*, 2003; Sun *et al.*, 2003, 2004a) and nitric oxide (Sun *et al.*, 2004b) in bile acid -induced barrier dysfunction in rat *in vivo*, cell studies have investigated the direct effects of bile acids on the epithelial layer. In Caco-2 cells, barrier disruption has been suggested to be mediated through production of reactive oxygen species (Araki *et al.*, 2005) or epithelial growth factor receptor activation and occludin dephosphorylation and rearrangement (Raimondi *et al.*, 2008), but not via changes in occludin content (Hughes *et al.*, 2008).

Increased bile flow has been proposed as a mechanism for barrier dysfunction in high-fat feeding (Suzuki and Hara, 2010). However, the group could show increased permeability in Caco-2 cells only at a high concentration of gallbladder bile. The effects of different luminal bile acids, also colonic bile acids, and the possible alterations of bile acid profile in high-fat feeding and barrier dysfunction were not investigated.

To summarize, the concept of individual bile acids increasing epithelial permeability is old, but little is still known of the effects of different bile acid structures and their mechanisms of action in intact tissue. Data comparing the effects of different bile acids are limited to cell studies, and an approach to study the possible barrier-disrupting effects of alterations of the entire bile acid pool is still missing.

3 AIMS OF THE STUDY

Impaired gut barrier function predisposes to several inflammation-related pathologies, such as inflammatory bowel diseases and fatty liver disease, which are proposed to develop when increased gut permeability allows leakage of endotoxins into circulation. In this study, the possible link between dietary fat and obesity, bile acids and barrier dysfunction was investigated in mouse models of diet-induced obesity and genetic obesity, as well as *in vitro*.

The specific aims were:

i. To study whether barrier dysfunction is caused by obesity or dietary fat only, using diet-induced obese mice on a high-fat diet and genetically obese mice on a normal chow diet. (Studies I and II)

ii. To investigate whether dietary fat quantity or quality, or obesity as such, affect luminal bile acid profile and hydrophobicity. (Studies I, II and IV)

iii. To define the possible role of hydrophobicity/hydrophilicity of bile acids in altering intestinal permeability using tissue preparations in an Ussing chamber and a feeding trial in mice. (Studies I and III)

4 MATERIALS AND METHODS

4.1 Experimental animals and study designs

All animals were obtained from Charles River: mice of the C57Bl/6J strain (Studies I-IV; Sulzfeld, Germany) and of the ob/ob strain (Study II; Calco, Italy). Mice were acclimatized for one week prior to experiments. They were housed in a 12 h/12 h light/dark environment with food and water *ad libitum*. Animal experiments were approved by the National Animal Experiment Board of Finland (ESAVI-2010-03684/Ym-23 and ESAVI-6806-04.10.03-2011). Main research questions, mouse strains and diets for the individual studies are summarized in Table 4.

In Study I mice were on a low-fat control diet or a high-fat diet for 15 weeks until intestinal permeability was measured from tissue segments in the Ussing chamber with 4 kDa FITC dextran. Fecal samples for bile acid analysis were collected at week 13.

In Study II fecal samples from ten 13-week-old C57Bl/6J mice and from seven ob/ob mice of the same age and background were collected for bile acid analysis. Mice were euthanized at week 15 for sample collection and measurement of intestinal permeability in the Ussing chamber with 4 kDa FITC dextran.

Study III included *in vitro* experiments, where DCA and/or UDCA were incubated with tissue preparations in the Ussing chamber before measurement of intestinal permeability (designs described in detail on page 57). Also, a 10-week DCA feeding experiment *in vivo* was included in this study. In the feeding trial, mice were divided into two groups: control and control diet with 0.2% DCA. Since the mice receiving DCA lost weight during the first week of the experiment, the dose was halved to 0.1%. Mice were euthanized at two time points: week 5 (n = 6+6) and week 10 (n = 6+6). Intestinal permeability was measured *in vivo* at the time of euthanasia.

In Study IV mice were divided into the following four groups:

- Control: control diet, no colitis
- DSS control: control diet, DSS colitis
- Lard DSS: high-lard diet, DSS colitis
- Fish oil DSS: high-fish oil diet, DSS colitis

DSS colitis was induced with dextran sulphate sodium (MP Biomedicals, Santa Ana, CA, USA) as a 5% solution in drinking water for two days, followed by two days of tap water. The fat sources of the study diets were as follows:

- Control diet, 10 E% fat: 5.5 E% soy oil, 4.5 E% lard
- Lard diet, 60 E% fat: 5.5 E% soy oil, 54.5 E% lard
- Fish oil diet, 60 E% fat: 5.5 E% soy oil, 27.2 E% lard, 27.2 E% menhaden oil

Table 4. *Summary of the study designs*

Study	Main research questions	Duration	Group	Animals	N	Diet
I	Does fat-induced obesity increase gut permeability? Is bile acid profile altered?	15 w	Control	♂ C57Bl/6J mice	24	10 E% fat, D12450B, Research Diets, USA
			High-fat	"	26	60 E% fat, D12492, Research Diets, USA
II	Does obesity without a high-fat diet impair barrier function?	15 w	Wild-type	♂ C57Bl/6J mice	10	CRM(E), SDS, Essex, United Kingdom
			Obese	♂ ob/ob mice	7	"
III	Do bile acids disrupt the gut barrier *in vitro*?	-	Designs of *in vitro* studies, page 57	♂/♀ C57Bl/6J mice, ♂ Wistar rats		CRM(E), SDS, Essex, United Kingdom
	Does DCA impair barrier function *in vivo*?	5 / 10 w	Control	♂ C57Bl/6J mice	6 per time point	Lantmännen lantbruk R36 (Stockholm, Sweden)
			0.1% DCA	"	6 per time point	As control, suplemented with 0.1% DCA
IV	Are there differences in the effects of lard and fish oil on fecal bile acid profile? Do they affect susceptibility to experimental colitis?	4 w	Control	♂ C57Bl/6J mice	10	10 E% fat, D12450B
			DSS-Control	"	10	10 E% fat, D12450B
			DSS-Lard	"	10	60 E% fat, D12492
			DSS-Fish oil	"	10	60 E% fat, D09020506

For the fish oil diet, half of the fat not accounting for soy oil was lard and half menhaden oil, to be able to pellet the diet. Feces were collected in metabolic cages before colitis induction. At the end of the study, intestinal permeability was measured *in vivo* and *ex vivo* with fluorescein in an Ussing chamber. Jejunum and colon were collected for further analysis of TNF-alpha. The study design is illustrated in Figure 4**.**

Figure 4. *The design of Study IV. Animals were fed with low-fat and high-fat (lard/fish oil) diets for the entire duration of the study. DSS colitis was induced during the final four days. The round-headed arrow represents the time point where fecal samples were collected in metabolic cages.*

4.2 Tissue collection

Mice were euthanized with a gas mixture of CO_2 (70%) and O_2 (30%) (AGA, Riihimäki, Finland) and cervical dislocation (Studies I-II) or decapitation, in the experiments with terminal blood sampling (Studies II-IV).

Fresh segments of intestine for Ussing chamber experiments were collected by carefully removing mesenteric fat. No muscle layers were removed for the Ussing chamber experiments. The first two segments of the small intestine after the pylorus were determined to be duodenum and the last two segments ileum. Jejunum was collected by folding the small intestine into a loop with the proximal duodenum and ileum side-by-side, and dissecting the two middle-most segments for the Ussing chamber. The two most proximal segments of the colon were called proximal colon and the two most distal segments distal colon, however, the colon is only long enough for three Ussing chamber segments. In Studies I, II and III, segments from each area of the intestine were collected in duplicate. Since only four Ussing chambers were available for Study I, permeability could only be measured from

two intestinal locations from each mouse. Segments from the following locations were collected:

- Study I: duodenum, jejunum, ileum and proximal colon
- Study II: jejunum and distal colon
- Study III: jejunum and distal colon
- Study IV: proximal colon

Tissue specimens for protein or gene expression analysis were snap-frozen in liquid nitrogen and stored at -80°C.

4.3 Ussing chamber experiments

Intestinal segments were opened along the mesenteric border, pinned onto 0.3cm^2 sliders and mounted into an EasyMount Ussing chamber system with a voltage-clamp apparatus (Physiologic Instruments, CA, USA). Tissues were surrounded by 5 ml Ringer solution on each side (120 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose) and water-jacketed to 37°C. The system was carbonated with a carbogen (95% O_2 , 5% CO_2, AGA, Riihimäki, Finland) gas flow to a pH of 7.4. The system was allowed to equilibrate for a minimum of 10 min before experiments.

For measurement of intestinal permeability, 4 kDa FITC-dextran (TdB Cons, Uppsala, Sweden) was added to the luminal side to a final concentration of 2.2 mg/ml (Studies I, II and III), or for fluorescein (Study IV) measurements to a concentration of 2 µg/ml. Serosal samples were taken at 45 min. Serosal fluorescence was detected with a Wallac Victor^2 1420 Multilabel counter (Perkin Elmer, Waltham, MA, USA) using an excitation/emission of 493/518 nm. Results were obtained by comparing serosal fluorescence to luminal fluorescence and expressed as per mille (1/1000) translocated dextran. Tissue resistance and short-circuit current were monitored throughout the experiments.

4.4 In vitro bile acid experiments

The barrier-altering effects of two bile acids different in hydrophobicity, DCA and UDCA, were investigated *in vitro* in an Ussing chamber (study III). The designs of the *in vitro* studies are presented below. Both jejunum and colon were used in designs 1-3.

Design 1: Dose-dependence studies

The effects of sodium deoxycholic acid (Sigma-Aldrich, St. Louis, MO, USA) and ursodeoxycholic acid (Leiras Ltd, Helsinki, Finland) on barrier function were tested with five concentrations for each bile acid: 0, 0.03, 0.3, 1 and 3 mM. Bile acids had no effect on the pH of Ringer solutions, nor did they induce cell death, measured as lactate dehydrogenase activity, which could have caused misinterpretations of the data. Intestinal permeability was measured with 4 kDa FITC dextran. Using a larger molecular weight dextran (20 kDa FITC dextran, TdB Cons), six experiments were conducted with the concentrations 0, 1 and 3 mM for only DCA. The bile acid -induced changes in tissue resistance were calculated from all experiments containing 0, 1 and 3 mM DCA.

Design 2: UDCA in DCA-induced barrier dysfunction

To test the possible protective effect of UDCA in DCA-induced barrier defect, luminal DCA (3 mM) was used with four different concentrations of UDCA: 0.1, 0.3, 0.6 and 1 mM. Permeability was measured with 4 kDa FITC dextran.

Design 3: Dexamethasone treatment

To investigate whether the permeability-inducing effect of DCA is inflammation-dependent, we used 1 μM water soluble dexamethasone (D2915, Sigma-Aldrich) on the luminal side of the tissue segment. The drug was co-incubated with 3 mM DCA for 20 min before a wash and permeability measurements with 4 kDA FITC dextran.

Design 4: LPS and intestinal permeability

To test the hypothesis that LPS aggravates DCA-induced barrier dysfunction, but does not increase intestinal permeability alone, a barrier defect was induced with 3 mM DCA for 20 min. The bile acid was washed from the chambers twice with Ringer solution before adding LPS from *Escherichia coli* O55:B5 (L2880, Sigma-Aldrich) at the concentrations 0.5, 1.5 and 4.5 µg/ml for 60 min. To test whether LPS has an effect on intestinal permeability independently of DCA, 0.5 and 4.5 µg/ml LPS was incubated with segments preincubated with Ringer solution only. Similar experiments were performed with 1 and 5 µg/ml in colon segments from Wistar rats. Permeability was measured with 4 kDa FITC dextran.

Tissue preparation for protein analyses

Distal colon was dissected from male and female 12-13-week-old C57Bl/6J mice (Charles River). The mice were euthanized with a gas mixture of 70% CO_2 / 30% O_2 (AGA) and cervical dislocation. The distal colon was cut into four segments, each of which were placed in one of four solutions: 1) Ringer, 2) Ringer with 3 mM DCA, 3) Ringer with 3 mM DCA and 0.3 mM UDCA, and 4) Ringer with 3 mM DCA and 0.6 mM UDCA. Segments were incubated for 20 min at 37°C, until either snap-frozen in liquid nitrogen and stored in -80°C for Western blot analysis of COX-2, or fixed in 10% neutral buffered formaldehyde (Sigma-Aldrich) for 24-48 h and embedded in paraffin for immunohistochemistry or hematoxylin-eosin staining.

4.5 Measurement of intestinal permeability *in vivo*

Permeability of the whole intestine was measured *in vivo* in Studies III and IV. Mice were fasted overnight and gavaged with 500 mg/kg FITC dextran (4000 Da; TdB Cons). After exactly 4 h the mouse was euthanized and blood was collected by decapitation into an Eppendorf tube. Serum was separated by centrifugation (15 min, 1000 g) and diluted to 1/5 into phosphate-buffered saline. Fluorescence was

detected with a Wallac Victor^2 1420 Multilabel counter (Perkin Elmer), using excitation/emission of 493/518 nm.

4.6 Fecal bile acid analyses

Feces were collected from each mouse in individual metabolic cages (Studies I, II and IV). Feed and water was provided *ad libitum*. Feces were carefully separated from all other material and frozen at -20°C. Upon analysis, fecal samples were dried overnight with a nitrogen gas flow and pulverized. Bile acids were extracted and analyzed for all fecal bile acids by gas-liquid chromatography according to a previously described method (Grundy *et al.*, 1965). Internal standards were run to identify isolithocholic acid, lithocholic acid, epideoxycholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid, β-muricholic and ursodeoxycholic acid. The proportions of these bile acids were calculated as a percentage of total bile acids, both identified and unidentified. An index for fecal bile acid hydrophobicity was calculated (Studies II, IV and unpublished data from Study I) as a percentage-weighted mean of the hydrophobicities of six bile acids – lithocholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid, ursodeoxycholic acid and β-muricholic acid – according to a previous report (Heuman, 1989), using estimated values for lithocholic acid (1.13) and muricholic acid (-0.65). A value for daily bile acid excretion was calculated from bile acid concentrations and metabolic cage data for daily fecal excretion.

4.7 Biochemical analyses

Gene expression analyses (Study II)

RNA was extracted from tissue with Trizol reagent. RNA was converted to cDNA with an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, using 1µg of RNA in a reaction volume of 20µl. Reactions for quantitative polymerase chain reaction were run using TaqMan chemistry (Applied Biosystems,

Carlsbad, CA, USA) for FXR (Mm00436420_m1), and LBP (Mm00493139_m1). Gene expressions were normalized to beta-actin. Reactions were run on a CFX96 (Bio-Rad) in triplicate. Gene expression was calculated with Bio-Rad CFX Manager software using the normalized expression ΔΔC(t) method.

Western blot analysis (Study III)

Western blot -analyses were performed using the following antibodies: mouse occludin 1:350 (33-1500, Invitrogen, Life Technologies, Paisley, United Kingdom), anti-mouse 1:200 (HAF007, R&D Systems, Minneapolis, MN, USA), rabbit β-actin 1:300 (#4967S, Cell Signaling Technology, Danvers, MA, USA), anti-rabbit 1:500 (#170-6515, Bio-Rad), goat COX-2 1:200 (sc-1747, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-goat 1:2000 (sc-2020, Santa Cruz Biotechnology). Occludin was blotted from four tissue preparations per group, whereas COX-2 was blotted from seven tissue preparations per group.

Immunohistochemistry (Study III)

Immunohistochemistery was performed using the following antibodies: mouse anti-occludin (33-1500, Invitrogen), anti-mouse FITC (Fisher Scientific, Waltham, MA, USA). Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI).

Tissue TNF-alpha analysis (Study IV)

Tissue TNF-alpha was quantified using tissue homogenates in a commercial enzyme-linked immunosorbent assay (ELISA) kit (RBMS607/2R, BioVendor, Brno, Czech Republic) in a single run (CV 4.3%), and data were expressed as pg/mg protein.

Serological analyses

In Study II, serum LBP was analysed using a commercial ELISA for mouse LBP (HK205, Hycult biotech, Uden, The Netherlands) in a single

run (CV 3.6%). Serum cholesterol precursors desmosterol and lathosterol, which reflect whole body cholesterol synthesis, and cholestanol and campesterol, which reflect cholesterol absorption efficiency also in animal studies (Pirinen *et al.*, 2010), were quantified with gas liquid chromatography on a 50-m long capillary column (Ultra 2; Agilent Technologies; Wilmington, DE) using 5α-cholestane as internal standard (Miettinen, 1988). The values are expressed as ratios to cholesterol of the same run (10^2 µg/mg cholesterol).

4.8 Scores for diarrhea and colonic inflammation

In Study IV, the severity of DSS colitis was evaluated during the last four days of the study with a subjective score for the severity of diarrhea in mice. The score was defined using the following five classes: 0 = no symptoms, 1 = blood at rectal area, 2 = bloody excrement, 3 = massive bloody diarrhea, 4 = animal deceased.

At euthanasia, the entire colon was dissected, opened and flushed free of intestinal contents. The preparation was pinned onto a silicon dish and photographed. Three experienced evaluators blindedly scored the samples for a general inflamed appearance using a VAS line (0-100).

4.9 Data analysis

All differences between groups were analyzed non-parametrically with Mann-Whitney U test. In the case of several groups, global differences were first analyzed with Kruskall-Wallis test. If global $P < 0.05$, differences were analyzed with Mann-Whitney U test.

Intestinal permeability could only be measured from two locations in each mouse. To correlate all bile acid analyses to intestinal permeability, an average of intestinal permeability in the two segments measured from each mouse was calculated and correlated to hydrophobicity index. All correlations were calculated as the non-parametric Spearman's rho.

For data on *in vivo* feeding of DCA, results were analyzed with a 2x2 factorial test (Univariate analysis of variance), since the study contained two fixed factors: diet (control and DCA) and duration (5 and 10 weeks). This test shows the independent effect of these two factors and any possible interaction.

For experiments with DCA and LPS on rat intestine, the data were analyzed by a professional statistician with the Generalized Estimating Equations -method. DCA+LPS groups were compared to the DCA control, and LPS groups were compared to Ringer only. For mouse tissue, differences for all pairs were analyzed with the non-parametric paired Wilcoxon test.

Statistical analyses for all studies were performed with PASW Statistics version 18.0.2 (IBM, Armonk, NY USA). All data are expressed as median (interquartile range) unless otherwise stated, and all P-values are expressed two-sided.

5 RESULTS

5.1 Body weight of mice in dietary interventions

After a 15-week high-fat feeding (Study I), the high-fat mice weighed approximately double compared to control mice [50.5 g (47.2,51.6) for high-fat, 28.8 g (27.4, 30.3) for control, P < 0.001]. In Study II, 15-week-old ob/ob mice were approximately the weight of the high-fat-fed mice [54.0 g (51.9, 54.7)], also double that of the wild-type mice [26.3 g (24.1, 28.5), P < 0.001). In the four-week feeding trial with a high-fat lard diet, high-fat lard-fish oil diet and a low-fat control diet (Study IV), high-fat groups weighed slightly more than the control groups at week three, the time of bile acid collection [25.4 g (23.7, 26.6) for control, 29.2 g (27.2, 31.6) for lard, 28.6 g (27.4, 29.4) for fish oil, P < 0.01 for both high-fat vs. controls]. There was no difference in weight between the two high-fat groups (P = 0.24).

5.2 Effects of dietary fat and obesity on gut barrier function

Dietary fat -induced obesity increased dextran translocation in jejunum and colon (Study I)

High-fat-feeding increased intestinal permeability significantly in jejunum (median 0.334 per mille translocated dextran for control vs.

0.393 for high-fat, P = 0.03) and colon (median 0.335 for control vs. 0.433 for high-fat, P = 0.01), but not in duodenum (P = 0.33) or ileum (P = 0.69, Figure 5).

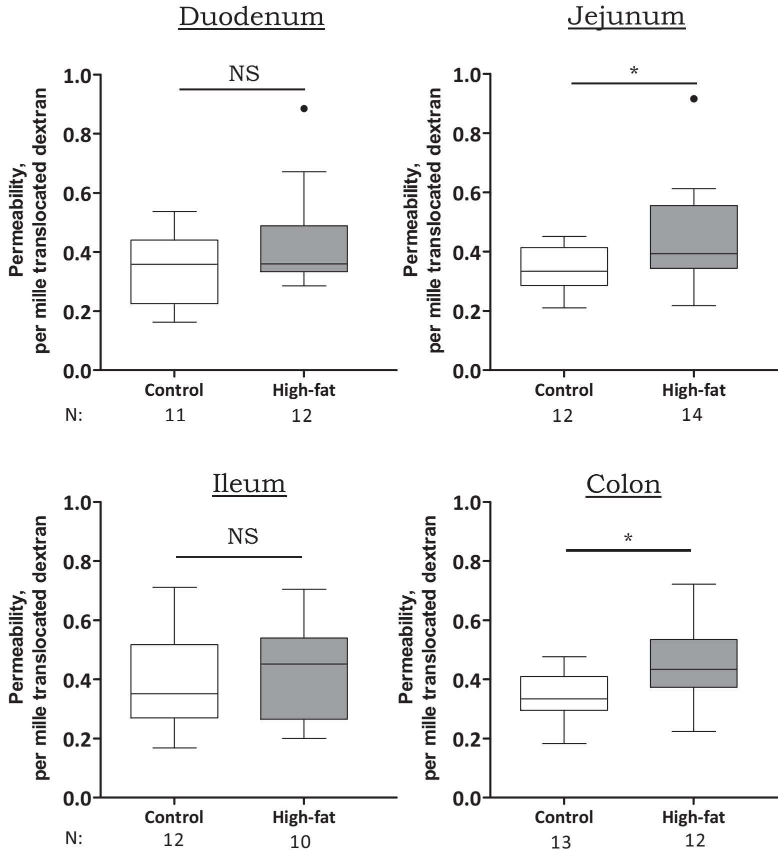

Figure 5. *The effect of a high-fat diet on intestinal permeability in duodenum, jejunum, ileum and colon. Permeability was measured in an Ussing chamber. Boxplots show median with upper and lower quartiles. Whiskers show min and max with outliers (Tukey) expressed as black dots. * P < 0.05, NS = Non-significant.*

Obesity had no effect on barrier function (Study II)

There were no signs of altered permeability to FITC dextran in either jejunum (P = 0.93) or colon (P = 0.86) in obese vs. wild-type mice (Figure 6).

Liver LBP expression was measured as a marker of portal endotoxin. There was no difference in liver LBP expression between obese and wild-type mice [0.47 (0.41, 0.60) for obese, 0.53 (0.19, 0.84) for wild-type, P = 0.70]. In contrast, serum LBP was increased 2.5-fold in obese mice vs. wild-type [30.5 μg/ml (24.8, 41.8) for obese, 11.4 μg/ml (8.8, 16.3) for wild-type, P = 0.001].

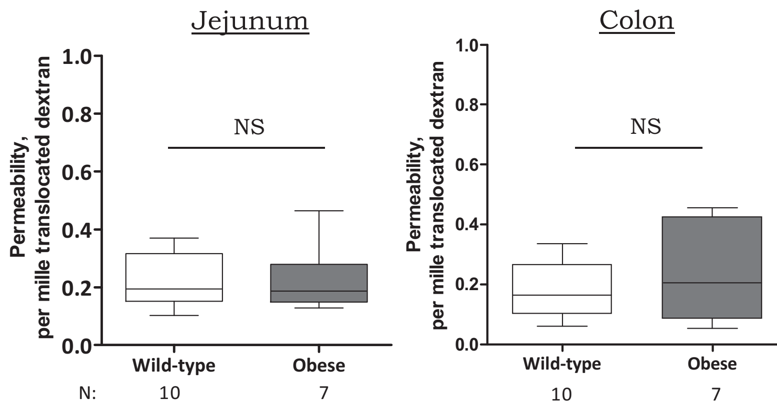

Figure 6. *The effect of genetic obesity on permeability of jejunum and colon. Permeability was measured in an Ussing chamber. Boxplots show median with upper and lower quartiles. Whiskers show min and max. NS = Non-significant.*

5.3 Effects of dietary fat and obesity on fecal bile acids

Fecal bile acid excretion was increased by dietary lard, and even more by dietary fish oil (Studies I, II and IV)

High-fat feeding increased fecal bile acid concentration 3.6-fold in a 15-week feeding trial (Study I, Table 5). This was reflected as a statistically significant elevation of each bile acid concentration except isolithocholic acid and β-muricholic acid. A non-significant tendency for a similar effect for lard feeding was seen in the much shorter, 3-week trial (Study IV, Table 5). Total fecal bile acid concentration was nearly doubled by fish oil. The fish oil diet resulted in a significantly greater concentration of DCA and total bile acids in feces compared to lard-fed mice.

In genetically obese mice, daily excretion of fecal bile acids was increased 36% compared to wild-type mice (3908 μg/day vs. 2870 μg/day, P = 0.001), however, the concentration of total fecal bile acids was decreased by 33% (Study II, Table 5). The concentrations of cholic acid and β-muricholic acid were decreased in obese mice, whereas the concentration of isolithocholic acid was increased (Table 5). There were no differences in other bile acids. The increased daily excretion of bile acids was not reflected in liver FXR expression, which was unchanged [relative expression 0.337 (0.177,1.000) for obese vs. 0.607 (0.516,1.089) for wild-type, P = 0.42].

Table 5. *Fecal bile acid concentrations in dietary intervention studies.*

	Study I			Study II			Study IV					
	Control N = 6	HF Lard N = 6		Wild-type N = 10	Obese N = 7		Control N = 12	HF Lard N = 10	HF Fish oil N = 10	Fold-change		
	Median, µg/g dry feces		Fold-change	Median, µg/g dry feces		Fold-change	Median, µg/g dry feces			Control to Lard	Control to Fish oil	Lard to Fish oil
ILCA	6.5	9.6	**1.5**	21.7	28.9	**1.3***	10.5	6.8	7.8	**0.6**	**0.7**	**1.1**
LCA	24.5	163	**6.6****	37.0	45.7	**1.2**	60.2	89.0	112	**1.5***	**1.9****	**1.3**
EDCA	21.9	164	**7.5****	75.3	89.9	**1.2**	81.4	125	129	**1.5**	**1.6**	**1.0**
DCA	200	1159	**5.8****	372	258	**0.7**	288	560	927	**1.9****	**3.2****	**1.7***
CDCA	14.6	35.4	**2.4***	50.9	92.3	**1.8**	23.5	26.4	28.0	**1.1**	**1.2**	**1.0**
CA	76.8	535	**7.0****	210	101	**0.5***	239	587	558	**2.5***	**2.3****	**1.0**
UDCA	9.3	28.2	**3.0****	3.4	9.1	**2.7**	10.7	10.6	15.8	**1.0**	**1.5**	**1.5***
β-MCA	76.0	116	**1.5**	100	32.2	**0.3****	128	172	233	**1.3**	**1.8***	**1.4**
Total fecal bile acids	1442	5167	**3.6****	3412	2289	**0.7***	2812	3440	4683	**1.2**	**1.7****	**1.4***

HF: High-fat; ILCA: Isolithocholic acid; LCA: Lithocholic acid; EDCA: Epideoxycholic acid; DCA: Deoxycholic acid; CDCA: Chenodeoxycholic acid; CA: Cholic acid; UDCA: Ursodeoxycholic acid; β-MCA: Beta-muricholic acid; *P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney U)

Fecal bile acid hydrophobicity was increased by dietary lard and fish oil, but not by obesity (Studies I, II and IV)

Dietary fat feeding (Studies I and IV) increased the fecal proportion of one of the most hydrophobic bile acids, DCA, whereas obesity alone had no effect (Study II) (Table 6). The proportion of β-muricholic acid was decreased by both long-term dietary fat feeding and obesity, but not by short-term high-fat feeding. Neither obesity nor dietary fat affected UDCA, although obesity tended to increase its proportion in fecal bile acids 4.6-fold ($P = 0.051$). The proportion of isolithocholic acid was decreased by dietary fat, but increased by obesity. A similar trend was seen for CDCA. Conversely, the proportion of cholic acid was increased by dietary fat and decreased by obesity.

The ratio of UDCA to DCA was halved by both high-fat diets in even short-term feeding, but was not significantly affected by obesity, despite a 3.2-fold higher ratio compared to wild-type mice. Long-term feeding of dietary fat increased fecal bile acid hydrophobicity 2.7-fold ($P = 0.004$, Study I), and short-term 1.4-fold by lard and 2.0-fold by fish oil ($P = 0.027$ and $P = 0.003$, respectively, Study IV), whereas obesity had no effect ($P = 0.205$) (Table 6).

Table 6. *Fecal bile acid profile and hydrophobicity index (HIx) in dietary intervention studies*

	Study I			Study II			Study IV					
	Control N = 6	HF Lard N = 6		Wild-type N = 10	Obese N = 7		Control N = 12	HF Lard N = 10	HF Fish oil N = 10	Fold-change		
	Median, % of all bile acid structures		Fold-change	Median, % of all bile acid structures		Fold-change	Median, % of all bile acid structures			Control to Lard	Control to Fish oil	Lard to Fish oil
ILCA	0.46	0.30	**0.7****	0.72	1.10	**1.5***	0.40	0.20	0.18	**0.5**	**0.5****	**0.9**
LCA	1.2	4.0	**3.3****	1.1	1.7	**1.6**	2.1	2.8	2.3	**1.3**	**1.1**	**0.8**
EDCA	1.5	3.1	**2.0****	2.3	3.3	**1.4***	2.7	3.6	2.9	**1.3**	**1.1**	**0.8**
DCA	13.0	25.1	**1.9****	11.3	11.7	**1.0**	9.5	13.2	21.8	**1.4***	**2.3****	**1.7**
CDCA	1.0	0.74	**0.7***	1.4	3.8	**2.7**	0.84	0.68	0.53	**0.8**	**0.6**	**0.8**
CA	5.6	10.0	**1.8****	6.1	4.1	**0.7***	8.2	13.5	10.5	**1.6***	**1.3***	**0.8**
UDCA	0.62	0.55	**0.9**	0.08	0.37	**4.6**	0.38	0.29	0.31	**0.8**	**0.8**	**1.1**
β-MCA	5.6	1.9	**0.3***	3.0	1.3	**0.4****	4.0	5.1	4.6	**1.3**	**1.2**	**0.9**
HIx	0.086	0.230	**2.7****	0.090	0.123	**1.4**	0.069	0.113	0.179	**1.4***	**2.0****	**1.5**
UDCA/ DCA	0.048	0.024	**0.5****	0.010	0.032	**3.2**	0.045	0.023	0.015	**0.5****	**0.3****	**0.7**

HF: High-fat; ILCA: Isolithocholic acid; LCA: Lithocholic acid; EDCA: Epideoxycholic acid; DCA: Deoxycholic acid; CDCA: Chenodeoxycholic acid; CA: Cholic acid; UDCA: Ursodeoxycholic acid; β-MCA: Beta-muricholic acid, HIx: Hydrophobicity index; *P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney U)

5.4 Effects of fecal bile acids on gut barrier function and inflammation

Fecal bile acid hydrophobicity was correlated with intestinal permeability (Study I and unpublished results)

A value for total intestinal permeability was calculated as a mean permeability of the measured segments, because permeability could not be measured from all segments from all mice. Fecal bile acid hydrophobicity was correlated with total intestinal permeability so that those mice with a higher hydrophobicity index displayed higher probe permeability (ρ = 0.75, P = 0.008; Figure 7). A trend for a similar positive correlation was noted in jejunum, ileum and colon, but not in duodenum (Figure 8).

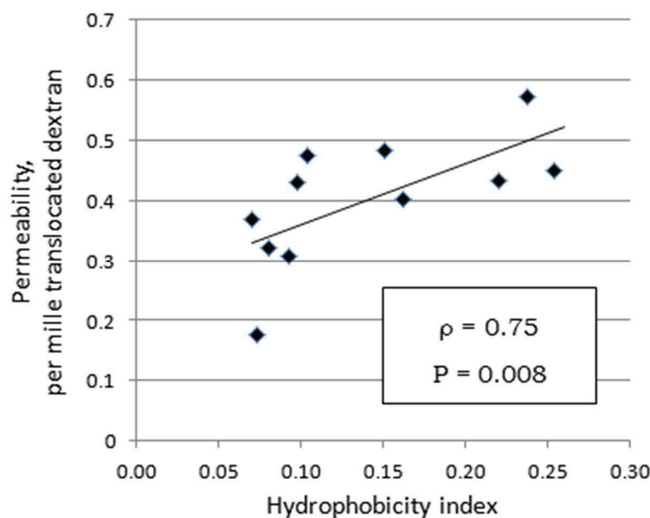

Figure 7. *Correlation between total intestinal permeability and fecal bile acid hydrophobicity index. Each dot represents an individual animal.*

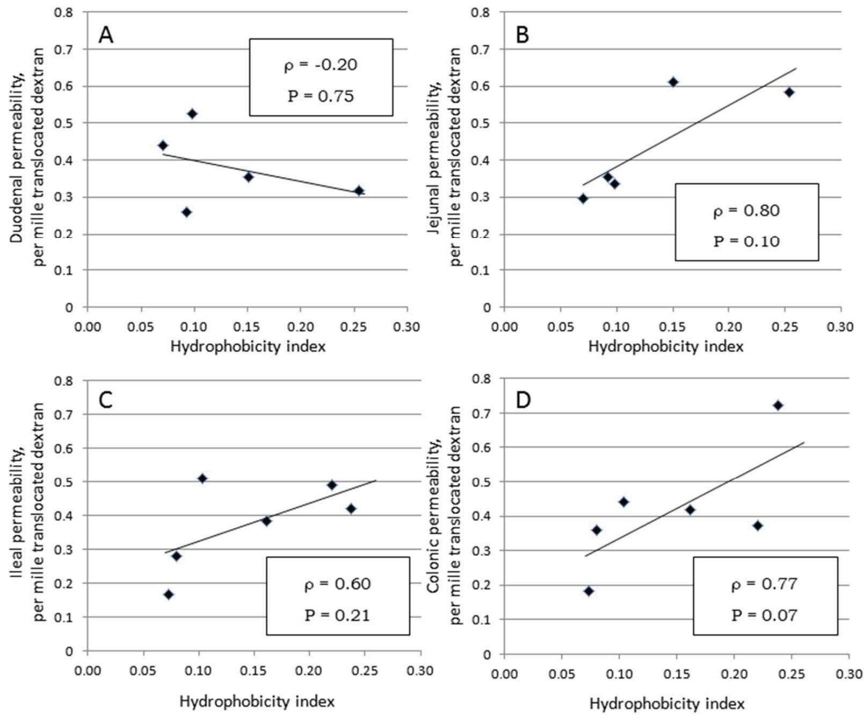

Figure 8. *Correlation between intestinal permeability of duodenum (A), jejunum (B), ileum (C) and colon (D) and fecal bile acid hydrophobicity index. Each dot represents an individual animal.*

DCA impaired gut barrier function in vivo and dose-dependently in vitro (Study III)

In a 10-week *in vivo* feeding trial, a diet containing 0.1% DCA impaired gut barrier function (factorial test, *P* for diet = 0.016, *P* for duration = 0.27; Figure 9). *In vitro*, DCA increased intestinal permeability to 4 kDa FITC dextran dose-dependently in both jejunum and colon (Figure 10 A-B). A significant barrier impairment was noted at concentrations that are found in feces of high-fat fed mice: 1 mM (1.8-fold increase in median in jejunum, 1.9-fold in colon) and 3 mM (2.2-fold increase in median in jejunum, 3.5-fold in colon), whereas no effect was seen at lower concentrations: 0.03 and 0.3 mM. Permeability to the larger 20 kDa dextran was also increased by 3 mM DCA in colon (Figure 10 D), but not in jejunum (Figure 10 C). Tissue resistance was decreased by DCA in colon, but not in jejunum.

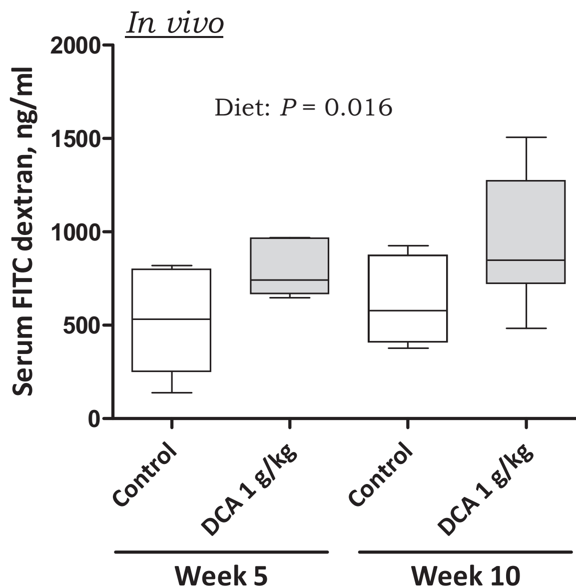

Figure 9. *Intestinal permeability after 5 or 10 weeks of dietary deoxycholic acid (DCA) feeding. Serum 4 kDa FITC dextran concentrations 4 h after dextran gavage. Boxplots show median with upper and lower quartiles. Whiskers show min and max. Results were analyzed with a factorial test. N = 5-6 per group.*

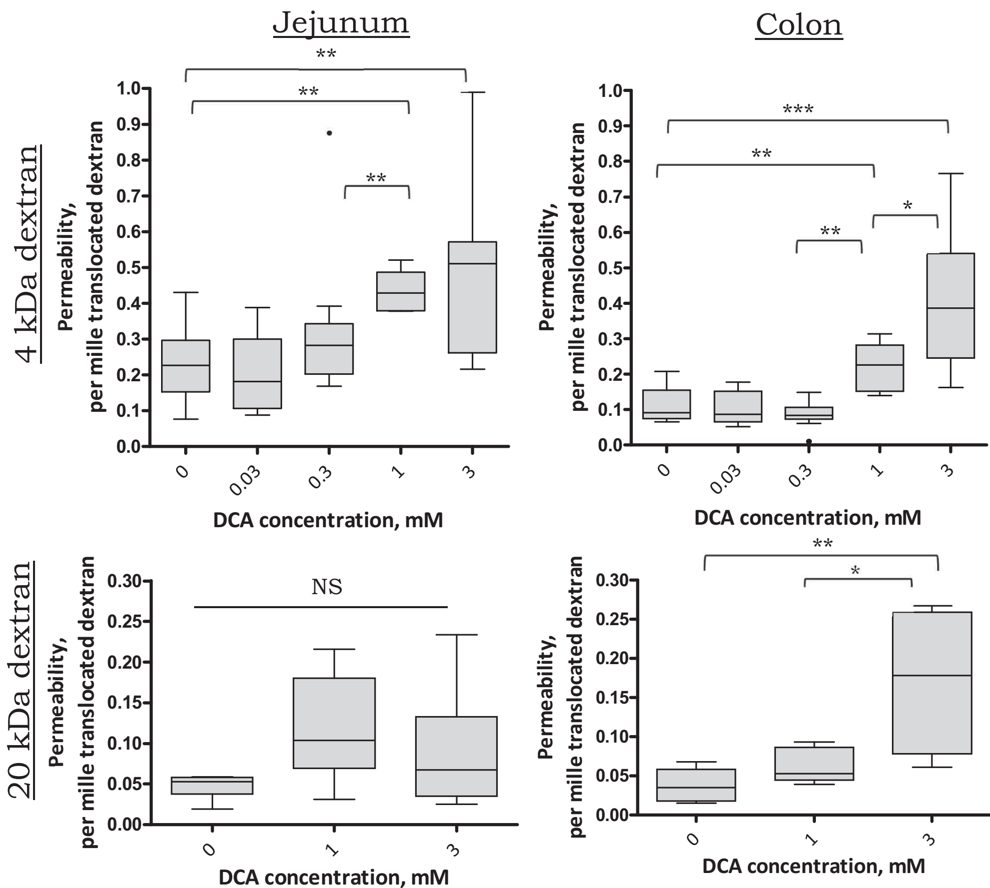

Figure 10. *Effect of deoxycholic acid (DCA) on the permeability of 4 (A-B) and 20 kDa FITC dextran (C-D) in jejunum and colon preparations. Boxplots show median with upper and lower quartiles. Whiskers show min and max with outliers (Tukey) expressed as black dots. A and B: 0, 0.3 and 3 mM: N = 12; 0.03 and 1 mM: N = 6. C and D: N = 6 per group. * P < 0.05, ** P < 0.01, *** P < 0.001, NS = Non-significant.*

UDCA protected the gut from DCA-induced barrier dysfunction (Study III)

UDCA did not affect permeability in either jejunum (global $P = 0.10$) or colon (global $P = 0.75$) (Figure 11 A-B). UDCA had no effect on tissue resistance in either jejunum or colon (data not shown).

UDCA protected colon against DCA-induced barrier dysfunction at 0.6 mM ($P < 0.05$) with a more than 50% decrease in the median value of permeability, and there was a decreasing trend at concentrations 0.1 mM and 0.3 mM (Figure 11 D). UDCA had no significant protective effect against DCA in jejunum (Figure 11 C). UDCA had no effect on the DCA-induced changes in tissue resistance (data not shown).

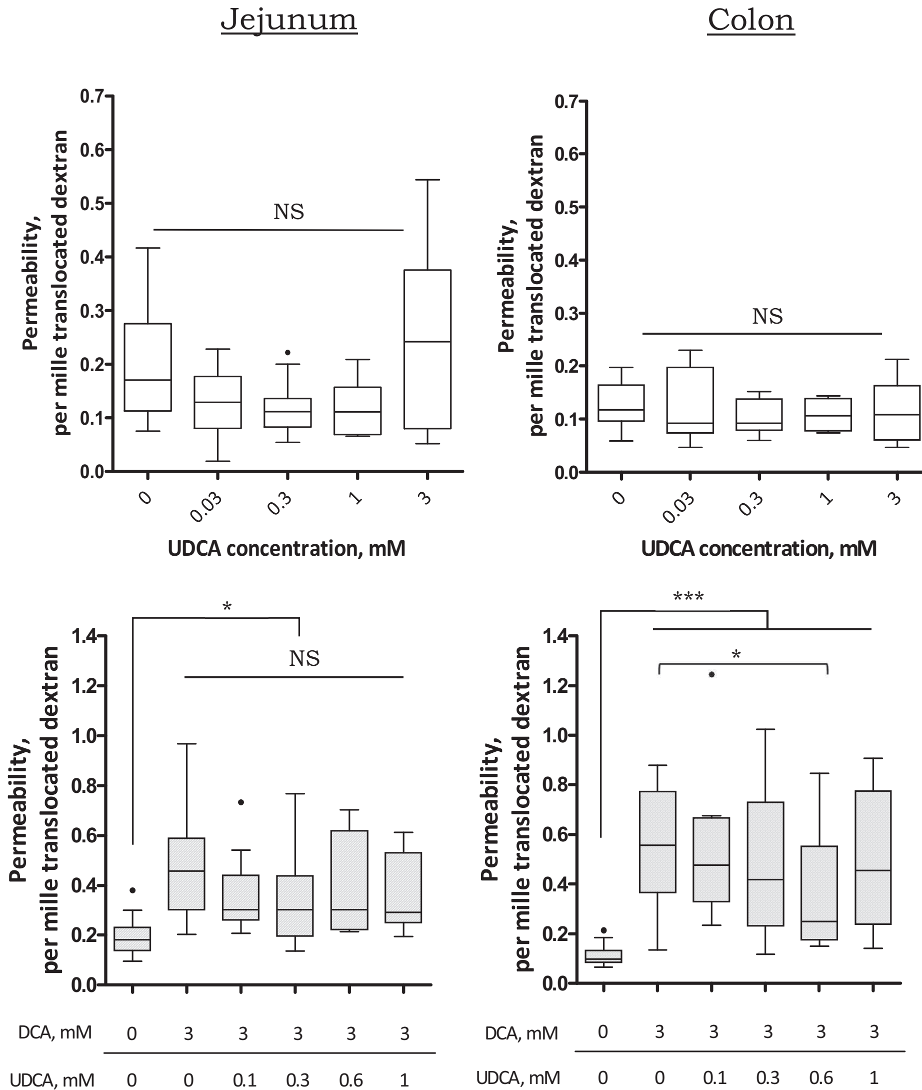

Figure 11. *Effect of ursodeoxycholic acid (UDCA) on permeability of jejunal and colonic tissue preparations (A-B), and its interaction with 3 mM deoxycholic acid (DCA) (C-D). Boxplots show median with upper and lower quartiles. Whiskers show min and max with outliers (Tukey) expressed as black dots. A and B: 0, 0.3 and 3 mM: N = 12; 0.03 an 1 mM: N = 6. C and D: N = 20 for control and DCA only, N = 10 for groups with DCA + UDCA. * P < 0.05, ** P < 0.01, *** P < 0.001, NS = Non-significant.*

DCA disrupted colonic morphology, but not via inflammation (Study III)

Epithelial morphology was dramatically altered by deoxycholic acid. Both hematoxylin-eosin staining and immunofluorescence staining for occludin revealed that the colonic epithelium was disrupted by 3 mM DCA. This effect was inhibited by 0.6 mM UDCA (Figure 12). Tissue disruption was not reflected as an elevated COX-2 content (P = 0.57, Figure 13), nor did dexamethasone treatment inhibit barrier impairment.

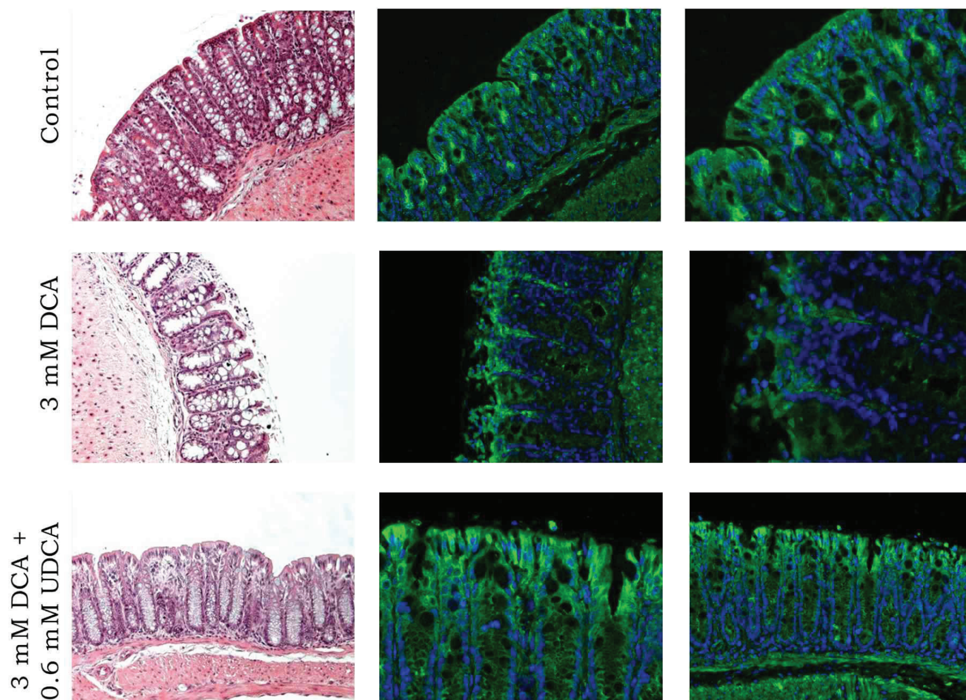

Figure 12. *Tissue morphological changes by deoxycholic acid (DCA) and DCA with ursodeoxycholic acid (UDCA). Slides were stained with hematoxylin-eosin (A) and occludin (B: 20x magnification, C: 40x magnification). Green shows occludin, blue shows DAPI staining.*

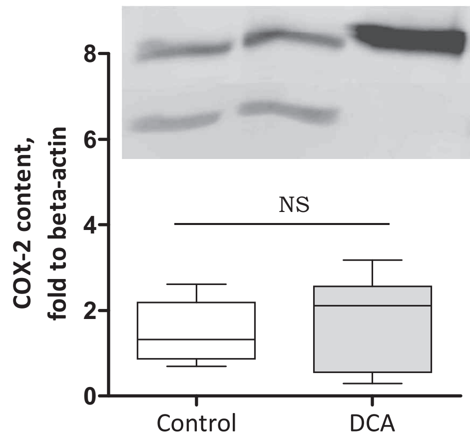

Figure 13. *COX-2 content, as measured by Western blot after incubation in 3 mM deoxycholic acid (DCA). Boxplots show median with upper and lower quartiles. Whiskers show min and max with outliers (Tukey) expressed as black dots. N = 7 per group.*

LPS may aggravate DCA-induced barrier dysfunction in colon (Study III)

LPS treatment seemed to aggravate DCA-induced barrier dysfunction by doubling permeability at a concentration of 4.5 µg/ml (P = 0.075, Figure 14 A). LPS had no effect on the permeability of intact intestinal tissue. Similar results were seen in rat colon (Figure 14 B). Median colonic permeability to dextran was slightly higher in both LPS+DCA groups compared to DCA only, and in a Generalized Estimated Equations analysis 5 µg/ml LPS with DCA tended to aggravate barrier dysfunction (P = 0.05). LPS had no effect on the permeability of intact rat intestine.

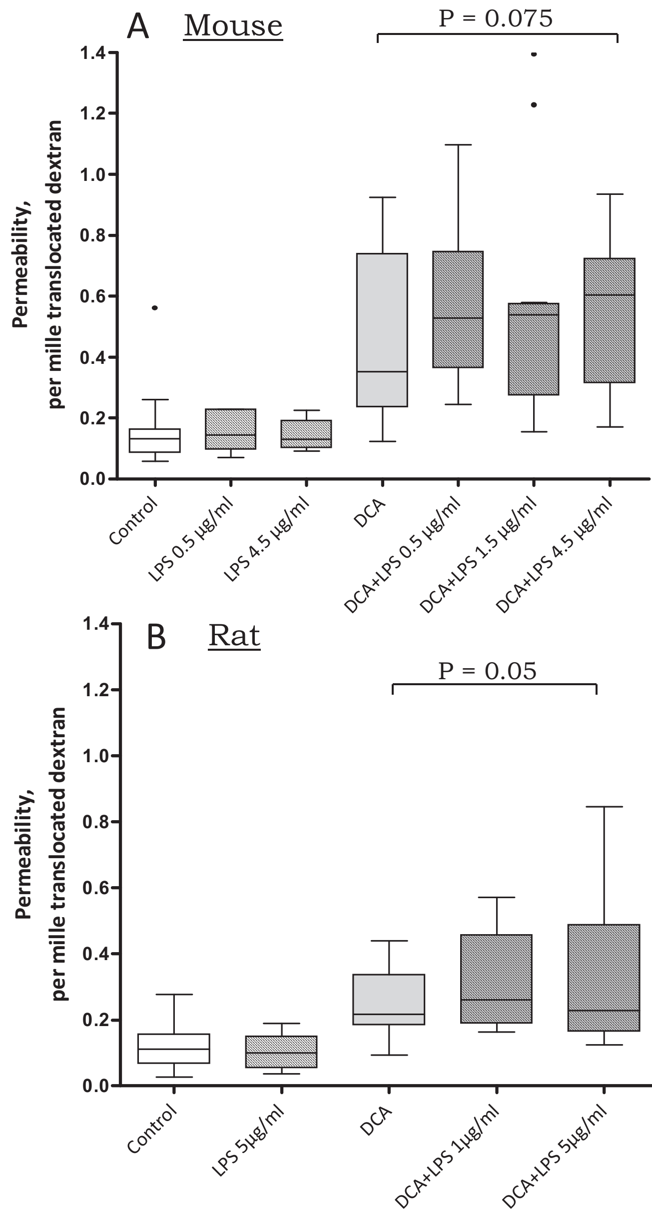

Figure 14. *Effect of LPS on DCA-induced barrier dysfunction in mouse (A) and rat (B) colon. Boxplots show median with upper and lower quartiles. Whiskers show min and max with outliers (Tukey) expressed as black dots. N = 7-24 per group.*

5.5 Dietary fat and susceptibility to experimental colitis

Upon DSS administration (Study IV), mice in the DSS groups began to lose weight and the general well-being of all DSS groups was greatly impaired compared to the healthy control [Symptom score 0 (0, 0) for Control, 7.0 (1.8, 10) for DSS-Control, 1.0 (0.0, 7.3) for DSS-Lard, 7.5 (5.8, 9.0) for DSS-Fish oil, $P < 0.01$ for all DSS groups vs. healthy control]. However, there were marked differences between the DSS-groups in symptom development. Whereas most DSS-Fish oil mice deceased after the second day of DSS administration and had severe bloody diarrhea, the DSS-Lard group seemed to have less bleeding than either of the other DSS groups ($P = 0.03$ DSS-Lard vs. DSS-Fish oil, $P = 0.07$ DSS-Lard vs. DSS-Control). These differences were also reflected in the large intestinal inflammation score [Score 4 (1.0, 5.5) for Control, 58 (41, 86) for DSS-Control, 27 (9.0, 79) for DSS-Lard], although only two DSS-Fish oil mice could be scored for large intestinal inflammation and were not included in the statistical analysis (values 19 and 77). All DSS-groups scored higher than control mice ($P < 0.05$ for all comparisons), and the gut inflammation score was, although non-significantly, halved in DSS-Lard mice compared to DSS-Control mice ($P = 0.32$). The symptom score AUC was significantly correlated with fecal bile acid hydrophobicity (Spearman's rho 0.44, $P = 0.026$, $N = 26$), as well as with fecal DCA concentration (Spearman's rho 0.39, $P = 0.046$, $N = 26$) in DSS mice.

In intestinal TNF-α, there were differences between groups in colon ($P = 0.001$), but not in jejunum ($P = 0.48$). Although DSS groups had marked macroscopic inflammation, tissue levels of TNF-α were lower in both DSS groups compared to healthy control [78 pg/mg protein (69, 87) for Control, 51 pg/mg protein (46, 53) for DSS-Control, 36 pg/mg protein (32, 41) for DSS-Lard, $P < 0.01$ for both comparisons]. As with inflammatory markers, intestinal permeability could only be measured from those mice that survived until the last day of the experiment. The

data concerning these markers could be biased by the missing individuals, which may explain why no difference in permeability was caused by the DSS colitis model.

The main findings of this thesis are summarized in Table 7.

Table 7. *Summary of the main findings*

	Study	Result
Do obesity and dietary fat impair gut barrier function?		
High-fat diet	I	↑
Obesity	II	↔
Do dietary fat, obesity, or dietary fish oil compared to lard, increase fecal bile acid hydrophobicity?		
High-fat diet	I, IV	↑
Fish oil vs. Lard diet	IV	↗
Obesity	II	↔
Do specific bile acids different in hydrophobicity increase intestinal permeability?		
Deoxycholic acid (DCA) *in vivo*	III	↑
DCA *in vitro*	III	↑
Ursodeoxycholic acid (UDCA) *in vitro*	III	↔
UDCA in DCA-induced barrier impairment	III	↓
By which mechanisms do bile acids cause barrier impairment?		
Increased COX-2 protein content	III	↔
Histological disruption of tissue structure	III	↑
Changes in occludin content or distribution	III	↔
Do dietary fats increase susceptibility to DSS colitis?		
Dietary lard	IV	↘
Dietary fish oil	IV	↗
Is fecal bile acid hydrophobicity associated with increased susceptibility to DSS colitis?	IV	↑

Arrows indicate yes (↑), no (↔) or contrary effect (↓) in answer to the question stated. Diagonal arrows indicate statistically non-significant tendencies.

6 DISCUSSION

Impaired barrier function allows the translocation of toxic substances from the gut lumen into the circulation. Some of these substances, i.e. endotoxins, may cause metabolic inflammation, which is a risk factor for cardiovascular disease and diabetes. This series of studies investigated the role of dietary fat and obesity in the pathogenesis of gut barrier dysfunction. Luminal bile acids and their properties were studied as a mechanism for barrier impairment.

6.1 Methodological aspects

6.1.1 Methods for measuring intestinal permeability

In this series of studies intestinal permeability was primarily measured as the translocation of fluorescent probes through intestinal preparations in an Ussing chamber and into serum *in vivo*. These are direct measures of intestinal permeability, and thus more reliable than the biochemical analysis of tight-junction proteins.

According to the hypothesis, increased intestinal permeability enables the increased translocation of LPS, which causes systemic low-grade inflammation (Cani and Delzenne, 2009). A permeability probe in this setting should then preferably simulate the translocation of LPS. In the present set of experiments, intestinal permeability was measured with FITC-labeled dextran in two different sizes, 4 and 20 kDa, and

fluorescein (0.38 kDa). Whereas the smallest of these, fluorescein, likely translocates paracellularly from villous tips (Arrieta *et al.*, 2006), the largest, 20 kDa FITC dextran, may translocate either transcellularly or paracellularly (see chapter 2.1.2). These experiments suggest that bile acids increase translocation of both 4 and 20 kDa FITC dextran, which may contribute to the pathogenesis of gut barrier dysfunction in high-fat feeding. LPS has a lipid A component bound to a core sugar and a polysaccharide repeat. The molecular size of LPS varies tremendously, but for example the lipid A component of LPS from *E. Coli* is 1.80 kDa in size (Que *et al.*, 2000), or 2.01 kDa when containing the core sugar (PubChem database). An average LPS molecule has a molecular size of approximately 4.29 kDa (PubChem database), which implies that it corresponds well to the size of FITC dextrans, and that more than 50% of its molecular mass is comprised of polysaccharide. The polysaccharide component, called O antigen, determines the serotype of the LPS molecule, as well as its hydrophobicity. The longer the O antigen, the more hydrophilic the LPS molecule is. Since the average LPS molecule contains more than 50% hydrophlilic polysaccharide, the hydrophilic FITC-dextrans used in these studies, 4 and 20 kDa, could be expected to somewhat represent the translocation of LPS.

The Ussing chamber method allows measurement of permeability from selected tissue segments, whereas the *in vivo* gavage of FITC dextran does not distinguish between segments. Serum samples for the *in vivo* method used in this study were taken 4 h after gavage, because at this time point FITC-dextran is visible by eye in the colon. Although it was suspected on these grounds that the *in vivo* method may measure permeability from also the large intestine, there is no evidence to confirm this assumption. The *in vivo* method may also be affected by gastrointestinal transit time, whereas the Ussing chamber method holds no such bias.

It should be stressed, however, that the Ussing chamber method cannot fully represent a physiological environment. Although care has

been taken to set pH, temperature and ion concentrations in the Ringer solution, the tissue segment is no longer connected to blood circulation. Despite carbogen gas flow in the chamber system, the tissue segment is inevitably ischemic to some degree. Moreover, tissue handling causes damage in cut areas. Given these circumstances, tissue segments cannot be held in the Ussing chamber for more than a few hours, depending on incubation conditions.

Although a diet high in fat elevates serum levels of LPS (Cani *et al.*, 2007a, 2007b, 2008; de La Serre *et al.*, 2010; Everard *et al.*, 2012; Kim *et al.*, 2012; Laugerette *et al.*, 2012; Serino *et al.*, 2012), it has been questioned whether this indicates increased intestinal permeability, because high serum LPS levels may also result from decreased clearance or high prevalence of LPS-containing gut bacteria in the lumen (Teixeira *et al.*, 2012a). Instead of measuring serum LPS levels, the potential of LBP as a marker of intestinal permeability was addressed in this study. LBP was measured in Study II from serum and as liver expression. Although LBP is a marker mostly produced by the liver in response to LPS, these two parameters were surprisingly not correlated: although serum LBP level was increased in genetic obesity, liver LPB expression was unchanged. Previous studies have shown a relationship between serum LBP and obesity or fat mass (Sun *et al.*, 2010, Moreno-Navarrete *et al.*, 2011). Adipose tissue expression of LBP correlates with adipocyte size (Dr. Robert Caesar, personal communication), which suggests that LBP may also be excreted from adipose tissue. These reports indicate that serum LBP does not necessarily reflect serum LPS levels in obesity.

6.1.2 Mouse models

Obesity

The C57Bl/6J mouse strain is a well-established healthy control that is often used as the basis for genetic modification (Rivera and Tessarollo, 2008). In this study, the C57Bl/6J mouse strain was used

for two obesity-models: the diet-induced obesity model and the commercially available genetically obese ob/ob model, which is bred into a C57Bl/6J background. There is a substantial difference in the mechanism of becoming obese between these two animal models. Whereas the diet-induced obese mice become obese on an energy-dense high-fat diet, the ob/ob mouse is leptin-deficient and characterized by hyperphagia (Carroll *et al.*, 2004), and thus becomes obese eating regular chow. The diet-induced obese mouse model better describes human obesity when compared to genetically obese models with single mutations. However, in the diet-induced obese model it cannot be verified whether any noted physiological changes are caused by dietary fat or obesity, i.e. expanding adipose tissue and alterations in its hormonal function. The genetically obese ob/ob model was used in this study to see whether obesity without dietary fat causes the modifications that are seen on a high-fat diet.

In the ob/ob mouse model, it should be noted that effects of obesity and leptin deficiency cannot be entirely distinguished from each other. Not much is known of how leptin affects gut barrier function, but leptin pre-treatment is known to protect from intestinal ischemia-reperfusion-induced mucosal damage in the rat (Sukhotnik *et al.*, 2007). It could then be speculated that leptin is protective to the epithelium, and leptin deficiency would predispose to barrier dysfunction. However, since permeability was not increased in ob/ob in the present study, leptin deficiency seems not to have affected the data.

Experimental colitis

Study IV aimed to investigate whether dietary fat quantity or quality affects susceptibility to experimental colitis by a mechanism related to bile acid hydrophobicity. Colitis was induced by dextran sodium sulphate (DSS) in drinking water. The DSS-induced colitis model is commonly used as a model of inflammatory bowel disease, to study disease pathogenesis and therapeutic options (for review, Perše and

Cerar, 2012). The pathogenesis of DSS colitis is based on DSS-induced disruption of colonic epithelial cells and their tight-junctions, which enables translocation of luminal antigens (Perše and Cerar, 2012). A similar pathogenesis is proposed for human inflammatory bowel disease (see 2.1.6). In DSS mice, a loss of tight-junction proteins is observed after only one day of treatment, accompanied by an increased expression of inflammatory cytokines (Perše and Cerar, 2012). Later on, increased apoptosis and decreased proliferation aggravate barrier dysfunction. The DSS colitis model has been validated for its use in studies on IBD (Melgar *et al.*, 2008). The severity of DSS colitis is regulated by the duration of DSS administration, and the concentration of the drinking solution (Perše and Cerar, 2012). In the present study, the 5% DSS solution caused severe inflammation, and in some mice premature death, in only two days of administration, although this dose has been used successfully in studies using the same method or in a pilot study performed for the present study (Liu *et al.*, 2009; unpublished data). This may be due to differences in diet compositions, since in earlier studies mice were fed standard chow, which usually contains approximately 15% fiber, whereas the diets used in the present study contained only 6.5% fiber. Insoluble dietary fiber has been suggested to protect from the development of DSS colitis (Nagy-Szakal *et al.*, 2013).

6.1.3 Experimental diets

This thesis included two dietary intervention trials in mice (Studies I and IV). These trials utilized commercially available open source diets. In Study I, a high-fat diet containing 60 E% fat (mostly lard) was used for the diet-induced obesity model. The alternative diet containing 45 E% fat was not chosen, because it was unknown at the time of designing the study whether dietary fat has an effect at all. Thus the more extreme diet was chosen for the experiments. The diet used in the present study was much less extreme, however, than the previously used high-fat carbohydrate-free diets that are used to initiate diabetes (Cani *et al.*, 2007a, 2007b, 2008; Serino *et al.*, 2012). In Study IV, the

60 E% fat diet was problematic, since lard had to be included in the fish oil diet to be able to form a pelleted diet. For future studies on the quality of dietary fat in inducing barrier dysfunction it could be beneficial to use a 45 E% fat diet. This improves the pelletability of diets containing oils.

Fish oil is generally perceived as anti-inflammatory, and because the present results are against this asssumption, they are easily critisized. However, a Cochrane systematic review and meta-analysis of nine studies on IBD concluded that there is insufficient data to recommend the use of fish oil to maintain remission in IBD (Turner *et al.* 2011). The high concentration of fish oil in the diet of Study IV may have increased intestinal bleeding in the fish oil group, because fish oil reduces platelet aggregation (for review, see Vanschoonbeek *et al.* 2003). The aforementioned review claimed, however, that fish oil does not increase bruising or bleeding time in humans. With the present evidence it cannot be excluded that the anticoagulation tendency brought on by fish oil may have caused bias in data on DSS severity.

The 10 E% fat control diet for Studies I and IV was paired to the high-fat diet in all but fat and carbohydrate content. Instead of excess fat as lard, the control diet contained sucrose. It can be debated whether this is metabolically the best control in permeability research. Sucrose contains 50% fructose, which is suspected to increase translocation of endotoxins (Bergheim *et al.*, 2008). At present, a low-sucrose diet paired for the high-fat diet used here is also available. From the view-point of obesity research, however, the control diet used here could be more ideal in *ad libitum* studies than those with lower sucrose contents, because a diet high in sucrose forms a very hard pellet. As reported, diet texture is essential in weight development (Desmarchelier *et al.*, 2013) - only pelleted diets resulted in a difference in weight gain, whereas with soft diets all mice developed obesity. As a compromise for permeability studies in obesity models, it could be advisable to use a low-sucrose control diet and slightly restrict food intake if a large weight-difference is desired.

6.2 Effects of dietary fat and obesity on intestinal permeability

These data show that a diet high in saturated fat increases the permeability of jejunum and colon to a fluorescent probe (Study I). It cannot be concluded with the present sample sizes, however, whether permeability of duodenum or ileum was increased or not. Previously, only four studies have shown with direct markers of permeability that high-fat feeding increases intestinal permeability in animals. Cani *et al*. (2008) showed that a carbohydrate-free (i.e. high-fat) diet increases permeability, when measured with *in vivo* gavage of FITC dextran. Both Suzuki and Hara (2010) and De La Serre (2010) have shown with direct probes *in vivo* that dietary fat increases gut permeability in rats, although the later only saw a difference in obesity-prone, but not obesity-resistant rats. Only Serino *et al*. (2012) have measured permeability from different segments of the distal gut after feeding with a high-fat, carbohydrate-free diet. They reported a 1.5-2-fold permeability of colon in high-fat-fed mice compared to control, although the difference was non-significant. The present study is the first to measure permeability in four locations along the gut in mice on a high-fat diet.

These data show that obesity without dietary fat does not affect intestinal permeability (Study II). This is supported by a finding in genetically obese rats (Suzuki and Hara, 2010). Cani *et al*. (2008, 2009) have studied ob/ob mice and report decreased tight-junction protein levels in obese mice compared to wild-type, but do not give gut permeability data for wild-type mice to be compared with the obese mice. Another report from Brun *et al*. (2008) shows increased intestinal permeability in ob/ob mice. All mice were fed regular chow, and there are no major differences in the method of measuring permeability, but the mice used in that study were three weeks younger and obtained from a different supplier than in the present study. This raises the question of whether impaired barrier function is a feature specific to

some distinct strain of ob/ob mice, but would not be related to obesity itself. Several studies report elevated blood endotoxins in genetically obese mice (Brun *et al.*, 2007; Cani *et al.*, 2008, 2009; Haub *et al.*, 2011). It cannot be concluded, however, whether this is due to increased permeability, impaired hepatic or intestinal clearance, or increased luminal production of LPS.

Although several studies report that obese animals on a high-fat diet demonstrate increased permeability, the present results suggest that this is due to dietary fat, not obesity itself.

6.3 Bile acids as a possible mechanism

These data suggest that alterations in luminal bile acid profile have a role in the pathogenesis of barrier dysfunction by dietary fat. Study I shows that fecal bile acid hydrophobicity and intestinal permeability are associated and are both increased by a high-fat diet. Although Suzuki and Hara proposed a role for bile acids in fat-induced barrier dysfunction (2010), the present study is the first one to show a correlation of bile acid profile alterations to gut permeability. Data from Study IV suggest that dietary fish oil may increase fecal bile acid hydrophobicity even further than dietary lard. The concentration of DCA was higher in feces of fish oil -fed mice compared to lard-fed mice. Although fecal bile acid hydrophobicity correlated with disease symptom severity, the impact of dietary fat quality on intestinal barrier function is yet to be determined.

Study III compared the luminal DCA concentrations of control mice to those of diet-induced obese mice in inducing barrier dysfunction. It is well known that DCA increases intestinal permeability (Chadwick *et al.*, 1979; Goerg *et al.*, 1980; Argenzio and Whipp, 1983; Goerg *et al.*, 1983; Gyory and Chang, 1983; Henrikson *et al.*, 1989; Fasano *et al.*, 1990; Fihn *et al.*, 2003; Sun *et al.*, 2004a, 2004b), as also shown both *in vivo* and *ex vivo* in Study III. However, this study is novel in finding that intestinal permeability was increased by DCA concentrations that

are found in the feces of diet-induced obese mice, but not by those found in the feces of control mice.

The present data suggest that the mechanism by which DCA increases intestinal permeability is rather related to disruption of tissue structure than inflammation, since tissue COX-2 content was unchanged, although histological staining showed clear disruption of the epithelium. Moreover, suggestive data show that treatment with the anti-inflammatory dexamethasone did not reduce epithelial disruption, nor was tissue occludin content affected. Henrikson *et al.* (1989) have shown that DCA reversibly alters porcine colonic morphology at 3-15 mM concentrations, with an irreversible disruption of the basal lamina at 21 mM. An earlier study suggested, however, that 2-8 mM DCA does not disrupt the integrity of rat colon, when imaged with electron microscopy (Goerg *et al.*, 1982). Instead, it was proposed that shedding of colonic cells is an artefact caused by the loosening of tight-junctions, and that changes in permeability are caused by tight-junction modifications, not by disruption of cell membranes. In the present study, the possibility for such an artefact cannot be ruled out. Tissue occludin levels were not changed by DCA incubation (Study III), which does not support the suggestion by Goerg *et al.* (1982), although sample-size was small and other tight-junctions or their localization were not analyzed. These data also do not support an inflammatory mechanism, since tissue COX-2 content was unchanged. The co-incubation of DCA with dexamethasone suggests that anti-inflammatory treatment does not inhibit barrier dysfunction, although it is possible that the incubation time was too short for this drug to have an effect. Thus, this study does not prove a mechanism of action for barrier-disruption by DCA. The present results tentatively suggest, however, that the induction of barrier leakage is not inflammation-dependent.

To compare the effects of DCA to a more hydrophilic bile acid, experiments were conducted with UDCA. This bile acid had no effect on permeability itself, but prevented DCA-induced barrier dysfunction at a

0.6 mM concentration. UDCA has shown other gut-protective effects in several previous studies: it has protected against chemically or pharmacologically induced colitis (Bernardes-Silva *et al.*, 2004; Kullmann *et al.*, 1997a, 1997b) and colitis-associated adenocarcinoma (Loddenkemper *et al.*, 2006) in rodents.

UDCA protects lipid membranes from disruption and perturbation by DCA *in vitro* (Heuman and Bajaj, 1994; Rodrigues *et al.*, 1998). Possible mechanisms of action include UDCA replacing DCA in the cell membrane or UDCA binding to the cell membrane and inducing a repelling negative charge towards the negatively charged DCA. These hypotheses are inadequate, however, since in the lack of cholesterol UDCA is as cytotoxic as DCA (Zhou *et al.*, 2009b). Zhou *et al.* (2009b) showed that UDCA protects cell membranes from DCA, but only in the presence of cholesterol. These results may partly explain the large variation in response to DCA-UDCA-mixtures in the present Ussing chamber studies. Since UDCA requires membrane cholesterol to be cytoprotective, any possible variation in epithelial cholesterol between individuals could have affected the results of the present study. Moreover, UDCA was only protective at 0.6 mM, with 1 mM having no effect. It could be hypothesized that with a high UDCA concentration a depletion of membrane cholesterol prevented any further cytoprotection, and additional UDCA was cytotoxic. This would lead to a cut-off concentration of 0.6 mM for the barrier-protective effect of UDCA in the present study design.

UDCA protected from DCA-induced barrier dysfunction in colonic, but not jejunal preparations. It is unknown whether this is only a matter of concentrations or incubation conditions. Before arriving to the ileum, where most bile acids are absorbed, the luminal concentration of bile acids in jejunum is 2-3-fold compared to that in colon (Hagio *et al.*, 2009). This difference may have an impact on physiological defense mechanisms such as mucus excretion. Moreover, there are substantial differences in the microbial concentration of jejunum and colon, which may affect the host’s response to external stimuli.

It is probable that in the physiological conditions in the intestine there are various factors protecting the intestine from the cytotoxicity of bile acids. Besides gut mucus and other factors that are lost in the *ex vivo* setting of the Ussing chamber, there may be other bile acids capable of cytoprotection. β-muricholic acid is another hydrophilic bile acid, which is primarily produced in the rodent liver (Lefebvre *et al.*, 2009). Although it has not been tested in intestinal permeability, it has a similar beneficial effect against cholesterol gallstones, as that of UDCA (Wang and Tazuma, 2002). As a very hydrophilic bile acid, the efficacy of β-muricholic in protecting from DCA cytotoxicity should be examined in further studies.

Besides UDCA, there may be many other luminal factors participating in the pathogenesis of barrier dysfunction by DCA. Due to the gut microbiota, the gut lumen contains LPS, which constantly translocates to the submucosa at a low rate (for review, see Kelly *et al.*, 2012). To the human immune system, LPS is highly inflammatory. DCA has been shown to provoke mucosal sensitivity to β-lactoglobulin, perhaps by increasing translocation to the lamina propria (Baird and Cuthbert, 1985). Analogically, it was hypothesized in this study that cytotoxic bile acids sensitize the gut to LPS-induced inflammation by promoting the translocation of LPS to the lamina propria. The results show a tendency for LPS to exacerbate DCA-induced barrier dysfunction. LPS alone, on the contrary, had no effect on intestinal permeability. Previous reports have also shown apically administered LPS not to increase permeability in colon (Emmanuel *et al.*, 2007) or intestinal Caco-2 cells (Hanson *et al.*, 2011) even at concentrations 100-fold compared to the ones used in the present study. However, bile acids have been reported to facilitate the translocation of LPS into human gut mucosa, as examined with confocal microscopy (Münch *et al.*, 2007). The present data suggest that once in the submucosa, LPS may increase gut permeability. This is supported by a previous study showing that basolateral administration of LPS to Caco-2 cells increases permeability (Hanson *et al.*, 2011).

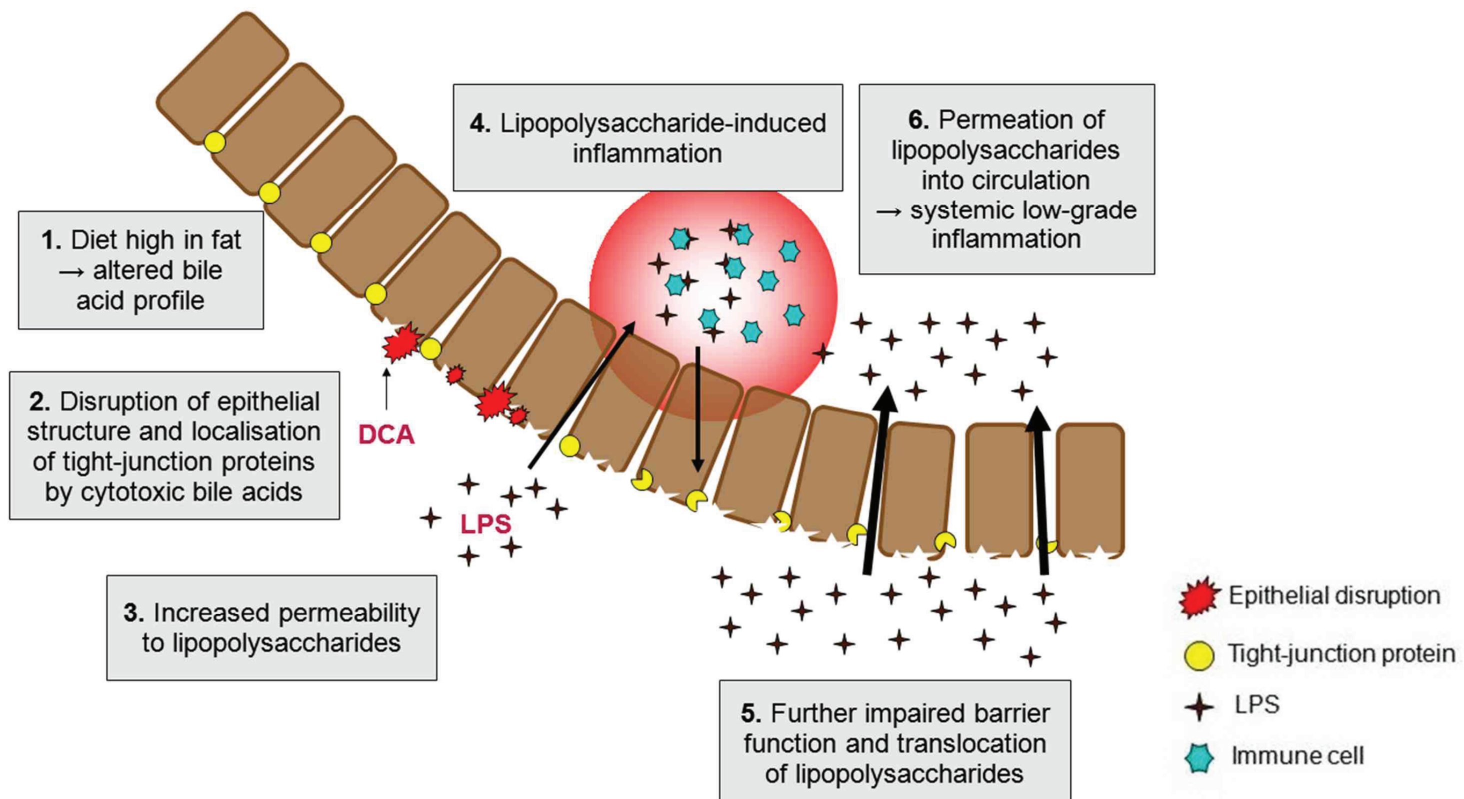

Figure 15. *Hypothesis on the role of bile acids in the pathogenesis of barrier dysfunction by dietary fat.*

Together the findings of this study imply that bile acids may have an initiating role in the pathogenesis of gut barrier dysfunction, where LPS or other luminal antigens possibly play a secondary role. As illustrated in Figure 15, the novel hypothesis of the pathogenesis of gut barrier dysfunction, derived from the results of this study, suggests that dietary modifications in the fecal bile acid profile result in increased gut permeability and permit increased translocation of LPS. This LPS, in turn, causes submucosal inflammation and initiates a vicious cycle, which leads to metabolic endotoxemia.

6.4 Clinical relevance

Intestinal barrier dysfunction is related to an increasing array of diseases common in the Western countries: inflammatory bowel diseases (IBD) (for review, see Goyette *et al.*, 2007), irritable bowel syndrome (IBS) (for review, see Camilleri *et al.*, 2012), type 1 diabetes (for review, see Vaarala, 2008), non-alcoholic fatty liver disease (for review, see Valenti *et al.*, 2009) and allergy (for review, see Perrier and Corthésy, 2011). This thesis investigated the role of dietary fat, obesity and bile acids as causes for intestinal barrier dysfunction. The present results suggest that increased bile acid hydrophobicity in the large intestine impairs mucosal barrier function. Ursodeoxycholic acid appeared to be a potential therapeutic agent for the prevention or treatment of these pathophysiological events in mice. Options for other membrane-strengthening agents should be investigated, and their efficacy should be tested in humans.

The results of these animal and *in vitro* studies cannot be directly extrapolated into humans. There are differences in for example the bile acid metabolism, microbiota and gut mucosal immunology of mice and humans, which demonstrate a clear need for similar experiments in humans. One of the largest differences in murine and human bile acid metabolism is in the murine ability to metabolize DCA back into its primary bile acid. Surprisingly, this does not pose such a large

difference in their small intestinal DCA concentration, because of coprophagia in mice (Groen *et al.*, 2006).

6.5 Future directions

Although the present series of studies suggests that a diet high in fat modifies luminal bile acid profile, it is still unknown how dietary fat elicits these changes. Because luminal hydrophobicity was increased mostly due to changes in secondary or tertiary bile acids, it could be hypothesized, that these changes are caused by alterations in gut microbiota. Although preliminary evidence shows that a high-fat diet increases fecal 7α-hydroxylase activity (Reddy *et al.*, 1996), suggesting a difference in microbial metabolism, this assumption should be fully investigated in future research. It is also imperative to know how different dietary fats affect the bile acid pool, and how these changes alter gut epithelial function. In the present study, mice eating fish oil were more susceptible to death of DSS colitis than those eating lard. This interesting finding should be further investigated to see if the effect can be replicated on lower doses of fish oil and DSS.

Another recently published paper proposed that increased biliary excretion of taurocholic acid in mice favors the growth of *Bilophila wadsworthia*, which is suspected to be a colitis-predisposing gut microbe (Devkota *et al.*, 2012). The authors did not discuss the fact that taurocholic acid is mostly unconjugated in colon. It is thus unknown whether the effect was attributable to taurocholic acid itself or its metabolites. Nevertheless, it is important to know how bile acids affect metabolism and gut mucosal function in all its forms – from biliary bile salts to serum and fecal bile acids. Peter J. Turnbaugh, a pioneer in research on obesity and gut microbiota, also emphasizes the need to continue research on the effects of diet on gut microbiota and bile acids (Turnbaugh, 2012). Bile acids have indeed raised interest in gut microbiota research and it is time to combine these two areas of obesity research.

7 CONCLUSIONS

The present series of studies investigated the role of dietary fat, obesity and bile acids in the pathogenesis of gut barrier dysfunction using mice on a high-fat diet, genetically obese mice and tissue preparations *in vitro*. The main findings are as follows:

i. Intestinal permeability is increased, especially in jejunum and colon, by dietary fat, but not by obesity itself.

ii. Dietary fat alters not just fecal bile acid concentration, but also fecal bile acid profile. Lard and fish oil severely increase fecal bile acid hydrophobicity compared to control. Dietary fish oil seems to induce a more hydrophobic bile acid profile than dietary lard. Obesity has no effect on bile acid hydrophobicity.

iii. Fecal bile acid hydrophobicity is positively correlated with intestinal permeability. A very hydrophobic bile acid, deoxycholic acid, impairs barrier function, whereas the more hydrophilic ursodeoxycholic acid may even protect gut epithelium from bile acid cytotoxicity. The mechanism of bile acid -induced disruption of the epithelium seems inflammation-independent.

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