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Methods to determine intestinal permeability and bacterial translocation during liver disease

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Abstract

Liver disease is often times associated with increased intestinal permeability. A disruption of the gut barrier allows microbial products and viable bacteria to translocate from the intestinal lumen to extraintestinal organs. The majority of the venous blood from the intestinal tract is drained into the portal circulation, which is part of the dual hepatic blood supply. The liver is therefore the first organ in the body to encounter not only absorbed nutrients, but also gut-derived bacteria and pathogen associated molecular patterns (PAMPs). Chronic exposure to increased levels of PAMPs has been linked to disease progression during early stages and to infectious complications during late stages of liver disease (cirrhosis). It is therefore important to assess and monitor gut barrier dysfunction during hepatic disease. We review methods to assess intestinal barrier disruption and discuss advantages and disadvantages. We will in particular focus on methods that we have used to measure increased intestinal permeability and bacterial translocation during experimental liver disease models.

Keywords

endotoxin; intestinal injury; microbiome; microbiota; steatohepatitis; gut-liver axis; gut barrier; intestinal leakiness

Introduction

The luminal side of the intestine is lined by epithelial cells, which promote water and nutrient absorption; the epithelium also provides a dynamic and semi-permeable barrier between the luminal microbiota and the host. The barrier is formed by individual epithelial cell membranes and tight junction proteins that seal the paracellular space between adjacent

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cells. Thus, the permeability of this barrier is regulated by the integrity of cellular plasma membranes and tight junctions, as well as by epithelial cell processes mediating secretion and absorption. Small molecules (<300 Da) and electrolytes passively cross the tight junction barrier (Sun et al., 1998). Both physiological and pathological stimuli change the barrier permeability. During homeostasis the intestinal epithelium absorbs nutrients while effectively preventing translocation of intraluminal bacteria. However, pathological conditions (e.g. toxins or intestinal inflammation) can increase the paracellular pathway and adversely affect barrier permeability, which poses the risk of an ineffective nutrient absorption and a failure to prevent the translocation of luminal bacteria and their products (also called pathogen associated molecular patterns or PAMPs). This can result in chronic intestinal diseases, but it might also affect other distant organs that drain and filter translocated bacteria and associated PAMPs (Sun et al., 1998; Turner, 2006; Marchiando et al., 2010a; Fouts et al., 2012).

The majority of the intestinal venous blood reaches the liver via the portal vein. Due to this unique blood supply system, the liver is vulnerable to exposure of bacterial products translocated from the gut lumen when intestinal epithelial barrier functions are disrupted (Seki and Schnabl, 2012). The liver represents therefore the first organ in the body that encounters not only nutrients from the diet, but also other molecules that are able to translocate from the intestinal lumen to the blood stream. The amount of translocated PAMPs is usually low during health (Bode et al., 1987; Fukui et al., 1991; Lin et al., 1995). However, liver diseases are associated with increased intestinal barrier permeability in humans (Bode et al., 1987; Fukui et al., 1991; Lin et al., 1995) and animal models (Yan et al., 2011; Hartmann et al., 2012; Hartmann et al., 2013). Increased levels of lipopolysaccharide (LPS or endotoxin) and bacterial DNA resulting from increased intestinal barrier permeability are elevated in the serum of patients with liver diseases (Fukui et al., 1991). Translocated bacterial products contribute to liver disease progression by binding to specific pathogen recognition receptors. In particular, Toll-like receptor 4 (TLR4), the major receptor for LPS has been implicated in the progression of many liver diseases and induces hepatic inflammation (Schnabl and Brenner, 2014). Increased translocation of microbial products due to a disrupted intestinal barrier will also lead to an activation of the mucosal immune system and secretion of inflammatory mediators, which in turn might increase barrier dysfunction. Such an inflammatory process might eventually also affect the quantity (overgrowth) and composition of the luminal microbiota (Marchiando et al., 2010a). Although intestinal permeability can increase with a rise in transcellular transport processes, the relevance and importance of transcytosis for liver disease have not been determined.

In addition, enhanced translocation of viable bacteria to mesenteric lymph nodes and extra-intestinal sites is commonly seen in patients with end-stage liver disease (cirrhosis) (Schnabl, 2013). The hepatic immune system might also be compromised in liver cirrhosis, so that translocated viable bacteria cannot be effectively cleared (Balmer et al., 2014). However, whether and how viable bacteria affect liver disease progression require further investigation.

Given the significance of monitoring intestinal permeability in the setting of acute and chronic liver diseases, we will review methods to assess gut permeability in mostly animal models.

1. Evaluation of intestinal integrity and mucosal tight junctions

Histology is important to initially evaluate the integrity of the intestinal barrier. Standard light microscopy of Hematoxylin & Eosin (H&E) stained intestinal sections is able to detect intestinal pathology including ulcerations of the mucosa and severe intestinal inflammation that will cause and contribute to increased intestinal permeability.

As mentioned above, tight junctions play an essential role in maintaining the integrity of the membrane barrier in the intestine. Tight junctions locate in the apical end of the lateral membrane and are composed of couples of transmembrane proteins such as occludins and claudins interacting with intracellular anchor proteins such as zonula occludens proteins which in turn are connected to the actin cytoskeleton. Tight junctions are rate-limiting for the paracellular leakage pathway (Menard et al., 2010).

Electron microscopy led to the discovery of tight junctions in epithelial barriers. The zonula occludens (tight junction) is characterized by fusion of the adjacent cell membranes with a dense outer leaflet of the adjoining cell membranes, which converge to form a single intermediate line. A diffuse band of dense cytoplasmic material is often associated with this junction (Farquhar and Palade, 1963). In addition, many reports have employed mostly immunofluorescent staining methods for visualization using specific antibodies directed against various tight junction proteins (Hartmann et al., 2012; Chen et al., 2014a; Chen et al., 2014b). Protein and mRNA transcript levels of tight junction molecules can be assessed with western blotting and quantitative PCR, respectively (Chen et al., 2014b). Tight junction complexes are composed of multiple proteins. Because the importance of single molecules for the integrity and function of tight junctions is rather obscure at this moment, functional assays might be necessary to elucidate their role.

An example for the importance of evaluating gut barrier integrity in culture and *in vivo* is alcoholic disease. A direct cytotoxic effect of high concentrations of ethanol (>40%) increases intestinal permeability by causing vascular and mucosal damage, which can be best seen on H&E stained slides (Szabo et al., 1985). However, subsequent Caco-2 monolayer cell based studies showed that even lower, non-cytotoxic doses of ethanol may alter the structure and function of tight junctions through activating myosin light chain kinase (MLCK) (Ma et al., 1999). Differentiated intestinal epithelial cells such as Caco-2 cells are commonly used to functionally analyze tight junction dynamics. Although ethanol has been reported to disrupt tight junctions in Caco-2 cells, acetaldehyde, a product of ethanol metabolism, is a much stronger inducer of tight junction dysfunction. Detailed protocols using acetaldehyde have been published (Rao, 2008; Chen et al., 2014b). Ethanol and acetaldehyde have also been used in three dimensional Caco-2 cell culture systems to disrupt tight junction integrity (Elamin et al., 2012). Other polarized cell culture systems are currently being developed to overcome the use of colon cancer cell lines. Isolated primary intestinal stem cells can be differentiated into crypt forming enterocytes that are also called enteroids (Foulke-Abel et al., 2014).

Ethanol is oxidized to acetaldehyde in the intestine and accumulation of acetaldehyde has been reported to be associated with alcohol related tissue injury (Salaspuro, 1996). Bacterial overgrowth may increase the oxidation of ethanol to acetaldehyde and result in accumulation of acetaldehyde in intestine. Because the colonic mucosa and microbiome have a low capacity in oxidizing acetaldehyde, acetaldehyde accumulates in the colon (Salaspuro, 1996) and acetaldehyde may redistribute tight junction proteins mediated by a tyrosine kinase-dependent mechanism with a subsequent increase in intestinal permeability.

Patients with cirrhosis show a decreased expression in tight junction proteins in duodenal biopsy. Patients with decompensated cirrhosis had less tight junction protein expression than patients with compensated cirrhosis (Assimakopoulos et al., 2012). However, another study showed that patients with compensated liver cirrhosis showed no alteration in tight junction protein expression in gastroduodenal and small intestinal mucosa but down regulation of these proteins in the colon (Pijls et al., 2014). This again suggests that functional assays are required to determine intestinal permeability.

2. Functional methods to assess intestinal permeability

2.1. Methods assessing the flow from the intestinal lumen to the blood—

Intestinal permeability can be assessed through enteral administration of non-digestible markers, which ideally should cross the mucosal barrier by non-mediated diffusion (Sun et al., 1998). The principle of this method is based on assessing the flow from the intestinal lumen to extraintestinal space such as blood, specific organs or urine. There are several types of markers including sugars, radioisotopes (e.g. ^{51}Cr -EDTA) and polyethylene glycols (PEG).

The obvious advantage is that intestinal permeability can be tested under *in vivo* conditions. However, the location of gut barrier dysfunction cannot always be accurately assessed. In addition, there are factors that might affect the absorption, the metabolism and the excretion of the sugars, e.g. gastrointestinal motility including intestinal transit time and surface area, mucosal blood flow, the distribution of the markers in the body, use of interfering drugs and kidney function (Bjarnason et al., 1984a; Bjarnason et al., 1984b; Peeters et al., 1994). Other factors might affect the urinary excretion of ingested molecules, such as the urine volume and/or the duration of the collection. A careful monitoring of the test and precise measurement of the parameters is therefore warranted (Mattioli et al., 2011).

We have used fluorescent-labeled dextrans for assessment of intestinal permeability during liver disease. Dextrans are polysaccharides and are available in different molecular sizes (3kD to 2,000kD) and conjugated to various fluorophores. Using a larger size will mimic bigger endogenous macromolecules, although dextran is still an inert test probe. It is important that tested tissue or blood does not have an autofluorescence that interferes with the emission of the fluorescent labeled probe. For example, during cholestatic liver disease, increased bilirubin in the plasma has a similar emission wavelength as fluorescein isothiocyanate-conjugated (FITC)-dextran. Choosing different fluorophores might overcome this problem. We are typically administering 200 μl of FITC-dextran 4kD (600mg/kg body weight) to mice by gavage, and the blood is collected 4hrs later. Varying the time after harvesting will depend on which part of the intestinal tract is to be investigated. The serum

concentration of the FITC-dextran is then determined using a fluorimeter with an excitation wavelength at 490 nm and an emission wavelength of 530 nm. Serially diluted FITC-dextran is used to establish a standard curve, and the concentration of serum FITC-dextran can then be calculated. Ideally, dilutions of FITC-dextran should be performed with non-hemolytic serum from healthy, non-gavaged mice. Intestinal permeability will be presented as the concentration of serum FITC-dextran (Napolitano et al., 1996; Furuta et al., 2001; An et al., 2007; Fouts et al., 2012; Hartmann et al., 2013).

The role of other sugars was first investigated in 1899 by Hober who found that dogs absorbed galactose faster than glucose (Hober, 1899). Since then many changes have occurred. In the 1970s, non-metabolizable oligosaccharides were introduced to develop reliable methods to assess the gut permeability (Menzies, 1974). The combined administration of a larger and a smaller molecule yields a specific large/small molecule ratio in the urine which is a reflection of the intestinal permeability and has greater clinical value than the administration of one marker alone (Menzies, 1974; Menzies, 1984). The most common dual-sugar test in clinical practice is the lactulose-mannitol test (van Elburg et al., 1995; Dastyk et al., 2008), however L-rhamnose is sometimes used instead of mannitol (van Nieuwenhoven et al., 1999; van Wijck et al., 2013). Common characteristics of these sugars are that they are passively absorbed from the gut without considerable metabolism and that they are excreted in an unaltered form into the urine in a direct correlation to their absorbed amount from the intestine (Sequeira et al., 2014). Mannitol is a monosaccharide with a molecular weight (MW) of 182Da and demonstrates a transcellular permeation with high appearance in urine after oral application, and a decrease in the presence of villous atrophy as it occurs in celiac disease (Cobden et al., 1978; Juby et al., 1989). Similarly, L-rhamnose – another monosaccharide of 164Da – shows a reduced absorption in celiac disease, whereas lactulose is absorbed at an increased rate hence leading to a heightened lactulose/L-rhamnose excretion ratio (Menzies et al., 1979). This is believed to occur since the larger disaccharide lactulose (MW 342Da) is transported via a paracellular route through the gut wall versus the transcellular route of the aforementioned monosaccharides (Bjarnason et al., 1986; Maxton et al., 1986). The routinely employed method of the lactulose-mannitol test consists of the simultaneous ingestion of the sugars in water, and – after fasting for 2 hours – the collection of the urine over a 24-hour period. The lactulose/mannitol ratio from the urine collection of the first six hours is used to measure the small intestinal permeability (Spiller et al., 2000). More rarely, the urine collections at 0-3, 3-5, and 5-24 hours are carried out to assess the permeability of the proximal small intestine, distal small intestine, and colon, respectively (Bjarnason et al., 1983a; Bjarnason et al., 1983b; Maxton et al., 1986).

Sugar absorption tests are employed in the diagnostic workup of several diseases, such as irritable bowel syndrome (IBS) (Rao et al., 2011), inflammatory bowel disease (IBD) (Munkholm et al., 1994; Halme et al., 2000), and autoimmune diseases (van Elburg et al., 1993; Vogelsang et al., 1995). However, studies using small inert markers to assess intestinal permeability *in vivo* do not necessarily correlate with the uptake of larger macromolecules (Menard et al., 2010). And indeed an intestine that is permeable to small sugar molecules can be impermeable to larger molecules (Vojdani, 2013).

Creating isolated intestinal loops is a powerful model to assess intestinal permeability in individual intestinal segments. The concept again relies on the flow of markers from the intestinal lumen of the loop to extraintestinal space. For this reason, non-digestible markers as described above, labeled bacterial products or even live bacteria are injected into an isolated gut segment that has blind ends on each side. Translocation of these markers is then measured in plasma or other tissues that the reagent/bacteria might penetrate into. Several studies used this method to investigate intestinal permeability:

Example A: We have assessed intestinal permeability in various intestinal segments of alcohol- or isocaloric diet-fed mice (Chen et al., 2014a). Mice were fully anaesthetized, and a midline laparotomy incision was made. Gentle manipulation of the intestine using sterile cotton swabs helped with the identification of each intestinal segment (e.g. jejunum, ileum, cecum, and colon). An approximately 4 cm long segment of the intestinal tract was isolated with two sterile vascular hemoclips (Jorgensen Laboratories) without disrupting the blood supply. Care must be taken that the mesenteric vascular arcades are not injured (Figure 1). FITC-dextran 4kD (50µl, 100mg/ml) is then carefully injected into the isolated loop of the intestine using an insulin syringe, and the abdomen is closed with sutures. One hour later, mice are sacrificed and fluorescence in the plasma is measured as described above. By using different fluorescent-labeled dextrans, this method can assess intestinal permeability simultaneously in multiple intestinal segments of the same mouse.

Example B: Besides fluorescent-labeled markers, live bacteria can be injected into the loop to quantify bacterial translocation. For example, we used green fluorescent protein (GFP)-plasmid transfected *Escherichia coli* (*E. Coli*) serotype O74:K:H39 (carrying a chloramphenicol resistance) to show that disruption of the colon barrier caused labeled bacteria to translocate to mesenteric lymph nodes following bile duct ligation (BDL) as compared with sham treatment. Loop surgery was performed as described above. The bacteria suspension (approximately 10^6 CFUs in a total volume of 50µl) is injected into the loop and mice were sacrificed 4 hours later. Mesenteric lymph nodes (MLN) are then harvested in a sterile fashion, homogenized and cultured on nutrient-broth agar plates (containing chloramphenicol) for 48-72 hours. GFP expression is confirmed in colonies using fluorescence microscopy (Hartmann et al., 2012).

Example C: We also use this model to assess survival rate of bioluminescent *E.coli in vivo*. Non-pathogenic *E. coli* were transfected with the pXen13 plasmid (Caliper), which is a vector carrying the original *Photorhabdus luminescens* luxCDABE operon for engineering bioluminescent bacteria. The bacteria suspension (approximately 10^6 CFUs in a total volume of 50µl) is then injected into the isolated intestinal loop. Bioluminescence imaging was performed using IVIS Spectrum (Caliper). Mice were kept anaesthetized, the abdominal cavity was left open and bioluminescence was recorded serially over time (Figure 2) (Hartmann et al., 2013). Since the abdominal cavity is left open in the heated (37°C) IVIS imager, intestinal surfaces exposed to the air require intermittent mild moisturization. Survival in percentage and effective killing of injected bacteria can be calculated. Although the purpose of our experiment was to investigate survival of bacteria in the isolated intestinal loop, this model could possibly be altered to image translocation of bioluminescent bacteria into the adjacent tissue like mesenteric lymph nodes. And indeed, after injecting

labeled LPS into the loop we were able to assess translocation and phagocytosis by macrophages (Grivennikov et al., 2012). Similarly, intravital microscopy was able to visualize the transit of GFP-tagged *E.coli* from the lumen into the mucosal stroma and muscularis of the terminal ileum in rats with developing cirrhosis (Palma et al., 2007).

Taken together, this intestinal loop model is a very powerful method to assess intestinal permeability and it can serve with modifications a wide variety of purposes. It is also independent from intestinal motility as compared with gavage of macromolecular tracers.

2.2. Methods assessing the flow from the blood to the intestinal lumen—The intestinal permeability is influenced not only by the epithelial but also the endothelial barrier. Capillary permeability is restricted to large solutes, such as red blood cells (40,000 Å radius). The capillary endothelium partly restricts molecules such as albumin, which has a molecular radius of 36 Å (Granger and Taylor, 1980).

The use of models that assess the flow from the blood to the intestinal lumen offers a variety of advantages. Ideally, one would like to use endogenous markers that are restricted to the blood compartment during healthy conditions and are not present in the intestinal lumen. Following the onset of a barrier dysfunction this endogenous marker moves from blood vessels across the mucosal barrier into the intestinal lumen by non-mediated diffusion. Using such a method is non-invasive, does not require any manipulation of the animal and could be assessed serially over time provided that concentrations can be measured in fecal pellets.

Measurement of albumin in fecal pellets is an example for such a flow directed towards the intestinal lumen. Albumin represents approximately 50% of the total protein content in human blood. Albumin is a small globular protein (molecular weight: 66.5 kDa) consisting of a single chain of 585 amino acids, produced by the hepatocytes with none or very low intracellular storage (Nicholson et al., 2000; Evans, 2002). 30-40% of the albumin is maintained in the blood stream, while the remainder is distributed in the interstitial space, where its concentration is low (1.4 g/dL). The protein leaves the circulation, returning to it via the lymphatic system. Albumin can be catabolized in many tissues, but mainly in the muscles, liver and kidneys (Nicholson et al., 2000; Evans, 2002; Fanali et al., 2012; Garcia-Martinez et al., 2013). Enhanced capillary permeability increases the release of albumin into the interstitial space (Peters, 1984). However, for this test to be accurate, serum albumin levels need to be normal. Conditions with low serum levels of albumin due to decreased synthesis (e.g. end-stage liver disease) or due to albumin losing diseases (e.g. kidney disease) might result in false negative results.

Usually an impermeable macromolecule in the healthy being, isotope-labeled albumin has been used to measure intestinal permeability in disease states. A healthy intestinal epithelial and endothelial barrier prevents the spilling over of albumin into the interstitial space. However, in conditions where the intestinal barrier integrity may be injured, endothelial and epithelial permeability is increased (Iqbal et al., 1996). As mentioned above, a prominent feature of alcohol abuse is disruption of the intestinal barrier. Animal models of alcoholic liver disease lead to leaky gut, (Hartmann et al., 2013) and patients with alcohol abuse

display an impaired intestinal barrier (Bode et al., 1987). Thereby, measurement of albumin in fecal samples is a good indicator of a disrupted intestinal barrier. We have measured albumin concentrations in freshly collected fecal pellets from mice by a standard ELISA test (Bethyl Lab). Mice are single housed in cages without bedding for several hours to collect fecal pellets from individual mice (Hartmann et al., 2013). We compared intestinal permeability results using a fecal albumin ELISA to the plasmatic fluorescence of orally administered FITC-Dextran 4kD (as described above) in mice fed an alcohol or isocaloric control diet. The results indicative of a gut barrier dysfunction in ethanol fed mice were comparable, and correlated well between the two methods (Hartmann et al., 2013).

Intestinal permeability can also be measured by an *in vivo* perfusion system assessing the flow from the blood to the intestinal lumen. Mice are subjected to the model or treatment that is relevant to assess intestinal permeability. Then the mice are anesthetized and injected intravenously with Alexa 488-conjugated bovine serum albumin (BSA). The abdomen is opened by a midline incision, and a 5-cm loop of jejunum is cannulated at the proximal and distal ends with 0.76-mm internal diameter polyethylene tubing. Then the flushing solution (NaCl 140 mM, HEPES 10 mM, pH 7.4) and test solution (NaCl 50 mM, HEPES 5 mM, sodium ferrocyanide 2 mM, KCl 2.5 mM, glucose 20 mM, pH 7.4) will be perfused through the jejunal loop using a peristaltic pump. Aliquots of the test solution are collected at the beginning and end of the perfusion. The intestinal permeability which is reflected by the BSA flux from the blood into the lumen of a perfused segment of small intestine will be measured by the concentration of fluorescent-tagged BSA in the perfusate. The BSA concentration in the perfusate is detected using a fluorescent microplate reader (Clayburgh et al., 2005; Clayburgh et al., 2006; Marchiando et al., 2010b). This *in vivo* perfusion system could also be used to test potential effects of drugs on intestinal permeability.

3. Measurement of translocated microbial PAMPs and bacteria in extraintestinal space

Liver diseases are commonly associated with increased intestinal permeability. Translocation of gut-derived PAMPs has been implicated into the pathogenesis of several chronic liver diseases including alcoholic liver disease and NASH (Schnabl and Brenner, 2014). It is therefore important to directly assess levels of gut-derived and translocated PAMPs in extraintestinal space such as the portal and systemic blood circulation, mesenteric lymph nodes, liver and spleen. It is important to harvest animals in a sterile fashion. In addition, all instruments used for harvesting need to be sterile, DNA- and RNA-free. Various ELISA assays and other kits are available to determine serum or tissue levels of specific PAMPs. For example, LPS can be measured by the traditional Limulus assay, but a competitive ELISA with increased sensitivity is now also available (Hartmann et al., 2012; Chen et al., 2014a). Microbial proteins can be detected with western blotting from sterile tissue extracts. We have used an *E. coli* antibody (Dako) to determine direct hepatic translocation of PAMPs (Chen et al., 2014b). Similarly, bacterial DNA can be amplified from tissue using common 16S ribosomal primers (unpublished results) or deep 16 rRNA sequencing (Cuenca et al., 2014).

Although the importance of translocation of viable bacteria from the gut lumen to extraintestinal space for the progression of liver disease is not completely understood,

translocated living bacteria play an important role in advanced stages of liver disease and cirrhosis. A significant percentage of patients with alcoholic hepatitis succumbs to bacterial infections with infection-attributed mortality of 12% to 54% (Coffin and Sharpe, 2007) underscoring the importance of a leaky gut with subsequent translocation of bacteria to extraintestinal sites. To detect viable bacteria in blood and tissues standard aerobic and anaerobic culture techniques are used (Fouts et al., 2012). We typically use beads (Zirconia/Silica, 1.0mm; Spectrum Laboratory Products) and a mechanical beads beater to release bacteria from tissue specimens (MagNA Lyser (Roche); speed setting: 6,000; time setting: 30 sec to 60 sec). Given that the minority of intestinal bacteria can be cultured by traditional culture techniques, this method will obviously not be able to detect non-culturable translocated bacteria.

Liver disease, in particular advanced stages of liver disease, results in an impairment of the immune system. The diseased liver is not able to clear bacteria and bacterial PAMPs as effectively as a healthy organ does (Balmer et al., 2014). Increased levels of translocated bacteria and bacterial products might therefore not necessarily be the result of increased intestinal permeability alone, but rather a combination between a dysfunction of the gut barrier and the immune system. Methods to detect bacteria and PAMPs in the blood and tissues are therefore considered more indirect evidence for gut leakiness. Nevertheless, they are important to estimate the risk of liver disease progression and bacterial infections.

The above mentioned methods with their advantages and disadvantages are summarized in Table 1.

4. Biomarkers mainly used in humans to assess intestinal inflammation and permeability

Zonulin is a 47 kDa protein which modulates intestinal permeability by disassembling intercellular tight junctions between epithelial cells in the digestive tract (Wang et al., 2000; Fasano, 2001; Vanuytsel et al., 2013). The effect of zonulin on increased intestinal permeability is mediated through activation of EGF receptor (EGFR) via proteinase-activated receptor 2 (PAR2) activation (Tripathi et al., 2009). Patients with type 1 diabetes, an autoimmune disease in which the finely tuned regulation of intestinal tight junctions is lost, have increased serum zonulin levels. Serum zonulin correlates with increased intestinal permeability. Moreover, this study also revealed that zonulin upregulation seems to precede the onset of the disease, suggesting the important role of increased intestinal permeability in the pathogenesis of this disease (Sapone et al., 2006). Other diseases with increased levels of zonulin are celiac disease and obesity (Fasano, 2012). Although further studies are needed for the application of serum zonulin in the human diseases, an important role for zonulin in regulation of intestinal permeability has been established and will hold much promise for future applications.

Calprotectin is a 36-kDa calcium- and zinc-binding protein complex which consists of one light and two heavy polypeptide chains (Dale et al., 1983). It constitutes up to 60% of the cytosolic proteins in human neutrophil granulocytes (Johne et al., 1997) but it is also expressed in activated macrophages and monocytes (Dale et al., 1985; Johnne et al., 1997). In the condition of intestinal diseases, activated granulocytes migrating into the intestinal wall will overexpress and release calprotectin into feces (Bjerke et al., 1993; Costa et al., 2005).

There is substantial evidence to prove that the fecal calprotectin is a sensitive marker of intestinal inflammation (Konikoff and Denson, 2006; Langhorst et al., 2008; Xiang et al., 2008; Schoepfer et al., 2009). Fecal calprotectin has shown excellent diagnostic accuracy in distinguishing inflammatory bowel disease (IBD) from irritable bowel syndrome (IBS), and has been applied to monitor therapy and assess treatment response (Sipponen, 2013; Burri and Beglinger, 2014).

Alpha-1-Antitrypsin (A1AT) is a protease inhibitor, which protects tissues from enzymes of inflammatory cells, especially neutrophil elastase. A1AT is one of the principal serum proteins and has a reference range in serum of 1.5–3.5 g/L, but the concentration can increase to a very high level during acute inflammation. A1AT is highly resistant to proteolysis in the intestine and can be excreted intact in the feces (Sharp, 1976). A1AT can extravasate from serum into the gut in the condition of increased intestinal permeability, and finally be detected in the feces. This supports fecal A1AT as a biomarker of intestinal permeability (Crossley and Elliott, 1977; Laine et al., 1993; Alam et al., 1994). There is a commercial kit to measure the fecal A1AT and researchers are exploring the application of this assay especially in infants with intestinal disorders (Keller et al., 1997; Kosek et al., 2013).

Fatty acid binding proteins (FABP) are small (14–15kDa) cytosolic proteins which can bind and transport fatty acids. There are several immunologically distinct types of FABP depending on the different tissues, e.g. heart, intestine, liver, muscle, and adipocyte (Niewold et al., 2004). Intestinal fatty acid binding protein (IFABP) is a 15-kDa protein uniquely located in mature small-intestinal enterocytes. The location of IFABP in the mature epithelium of villi facilitates its leakage into the circulation from enterocytes when intestinal mucosal damage occurs (Kanda et al., 1992). Therefore, it is easily detected in plasma or urine in the setting of intestinal ischemia (Gollin et al., 1993). Measurement of plasma I-FABP concentrations was a highly specific and sensitive method for assessing the severity of mucosal injury in rats (Kanda et al., 1992). Recent studies also suggested that IFABP in serum or urine might be a useful biochemical marker for the diagnosis of intestinal ischemic injury in humans (Kanda et al., 1996; Thuijls et al., 2011). Compared with uninfected individuals, individuals with chronic hepatitis B and C infection had higher plasma levels of IFABP indicative of enterocyte death. IFABP levels became undetectable in patients with successful treatment of the hepatitis infection (Sandler et al., 2011). It is expected that the availability of commercial kits detecting the IFABP in serum or urine might lead to a wider application of IFABP measurement in the human intestinal diseases.

Diamine oxidase (DAO) activity in serum correlates inversely with intestinal permeability of the small intestine (Luk et al., 1980; Honzawa et al., 2011). DAO is the main enzyme to catalyze the oxidation of diamines such as histamine, putrescine, and cadaverine (Shakir et al., 1977). The expression of DAO occurs predominantly in human intestinal mucosa as well as the placenta, kidney and thymus (Rangachari, 1992). However, serum DAO appears to come primarily from the small intestine (Buffoni, 1966). In the intestine, DAO is specifically located in tips of enterocyte villi, and its activity reflects the integrity and maturity of the small intestinal mucosa (Luk et al., 1980). Serum DAO activity is significantly decreased in patients with Crohn's disease and ulcerative colitis regardless of

the level of disease activity (Honzawa et al., 2011). Considering that serum DAO activity could be easily measured with Enzyme Immunoassay using a commercial kit, it might thus become a convenient method for evaluating small intestinal permeability in patients with intestinal diseases in the future.

Conclusion

Maintenance of the physical barrier in the intestine is dependent on the physical integrity of barrier components. Increased paracellular and (possibly) transcellular permeability, and epithelial cell damage will result in a gut barrier dysfunction. Subsequent translocation of PAMPs and viable bacteria are key events during liver disease. Dysfunction of the immune system might contribute to persistently elevated systemic levels of PAMPs and chronic liver disease. It is therefore important to measure and monitor intestinal permeability during liver disease. Most of the methods assessing the flow from the intestinal lumen to the blood use inert markers of different sizes that do not necessarily correlate with the uptake of larger macromolecules. And even measurement of electric resistance, which has been considered as gold standard to assess permeability for a long period of time (Li et al., 2003; Klingberg et al., 2005; Ulluwishewa et al., 2011), is not linearly correlated with permeation of small inert sugars or other molecules (Menard et al., 2010). This suggests that an ideal permeability assay will use labeled probes (such as bacteria, proteins or macromolecules). However, no universal marker provides a definitive answer on the leakiness of the intestine, and a combination of methods assessing the flow from the blood to the intestinal lumen might be useful.

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Abbreviations

LPS	lipopolysaccharide
H&E	Hematoxylin & Eosin
PAMPs	pathogen associated molecular patterns
TLR	Toll-like receptor
FITC	fluorescein isothiocyanate-conjugated
PEG	polyethylene glycols
GFP	green fluorescent protein
MLN	mesenteric lymph nodes
BDL	bile duct ligation
BSA	bovine serum albumin
A1AT	Alpha-1-Antitrypsin

FABP	Fatty acid binding proteins
DAO	Diamine oxidase

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Figure 1. Intestinal loop model in the distal small intestine
Shown is a typical photograph of the *in vivo* loop model surgery.

Figure 2. Bioluminescent imaging after injection of luciferase expressing *Escherichia coli* into a jejunal loop

A representative photograph of the jejunal loop and a corresponding bioluminescent image following injection of luciferase expressing *Escherichia coli* using the IVIS imager is shown.

Table 1

Methods to assess intestinal permeability

Methods	Assay	Advantage and disadvantage	References
Morphology analysis	H&E staining of intestinal sections	Histology is easy to perform and to interpret No functional analysis	(Szabo et al., 1985)
	Assessing intestinal tight junctions (electron microscopy, gene and protein expression)	No functional analysis	Hartmann et al., 2012; Chen et al., 2014a
Functional analysis			
Methods to assess flow from lumen to blood	Enteral administration of non-digestible markers such as sugars, radioisotopes (e.g. ⁵¹ Cr-EDTA) and polyethylene glycols (PEG)	Performed under <i>in vivo</i> conditions, but affected by factors such as gastrointestinal motility, mucosal blood flow and the distribution of the markers in the body Inert markers are being used	Sun et al., 1998; Bjarnason et al., 1984a; Bjarnason et al., 1984b; Peeters et al., 1994
	Creating isolated intestinal loops and injection of labeled bacterial products, markers or live bacteria	Serves with modifications a wide variety of purposes and are independent from intestinal motility Able to determine the site of increased leakiness Surgery required	Chen et al., 2014a Hartmann et al., 2012; Hartmann et al., 2013
Methods to assess flow from blood to lumen	Fecal albumin measurement	Non-invasive, does not require any manipulation of the animal.	Hartmann et al., 2013
	<i>In vivo</i> perfusion system	Requires normal blood albumin levels Can be used to test the effects of drugs on intestinal permeability Surgery required	Clayburgh et al., 2006; Marchiando et al., 2010b
Microbiology tests	Measurement of translocated microbial PAMPs	Direct assessment of gut-derived and translocated PAMPs in extraintestinal space Levels are dependent on the immune system	Hartmann et al., 2012; Chen et al., 2014a; Chen et al., 2014b; Cuenca et al., 2014
	Culturing translocated live bacteria	Direct assessment of gut-derived and translocated bacteria Numbers are dependent on the immune system Not able to detect non-culturable translocated bacteria	Fouts et al., 2012