New insights in intestinal ischemia-reperfusion

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New insights in intestinal ischemia-reperfusion

towards a better diagnostic work-up and therapeutic strategy

Dirk Schellekens

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New insights in intestinal ischemia-reperfusion

towards a better diagnostic work-up and therapeutic strategy

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Chapter 1

General introduction

Introduction

Intestinal ischemia is caused by blood flow that is insufficient to meet the metabolic demand of the intestinal tract. It is the result of a large variety of diseases leading to either obstruction or a diminished blood flow towards the visceral organs ¹. Injury caused by intestinal ischemia ranges from superficial damage of the mucosal layer to transmural injury of the intestinal wall. Transmural injury is characterized by perforation of the intestinal wall and results in sepsis, multiple organ failure (MOF) and ultimately death ^{2,3}. Intestinal ischemia is a severe clinical phenomenon with mortality rates up to 80% ³⁻⁵. A major factor contributing to the high mortality rate is an inaccurate or delayed diagnosis ^{1,6}. Clinical symptoms of intestinal ischemia are quite unspecific and diagnosis relies on a high index of suspicion. Despite all the advances in medical technology there are still no accurate biomarkers that allow for early diagnosis and appropriate treatment in order to minimize intestinal ischemic injury, potentially improving patient outcomes ^{7,8}.

Furthermore, although there is a growing knowledge about the pathophysiology of intestinal ischemia, there are still several issues that remain unclear ⁹.

Therefore the overall purpose of this thesis was to investigate new biomarkers in the aid of accurate diagnosis of intestinal ischemia and to provide more insight into the mechanisms responsible for intestinal tissue injury and dysfunction caused by ischemia in order to improve treatment strategies.

Anatomy and blood supply of the intestines

The intestine is the largest component of the digestive tract and is the part between the stomach and the anus. It consists of two parts; the small and the large intestine, the latter also known as the colon. The small intestine is composed of three parts, namely the duodenum, jejunum and ileum. An adult small intestine is approximately six meters in length and coils in the abdomen leading from the stomach to the colon ¹⁰. The intestinal wall is built up out of four layers. The outermost serosa is a membrane that secretes serous lubricating fluid that reduces friction from the movement of the intestines. Next, the muscularis has two layers of smooth muscle. It is responsible for the continuous peristaltic movement along the intestine. The inner circular muscle layer is thicker than the outer longitudinal layer. The submucosa is the layer of dense, irregular connective tissue supporting the mucosa and joining the mucosa to the underlying muscularis mucosae. Within the submucosa blood vessels, lymphatic vessels and nerves are situated. The innermost layer is the mucosa, containing the epithelial lining, lamina propria and muscularis mucosae ¹¹. It is a mucous membrane that absorbs water and nutrients and secretes mucin, digestive enzymes and hormones ^{12,13}. The mucosa is arranged in concentric folds, known as plicae circulares. These folds that run for one-half to two-thirds the way around the intestinal wall increase the surface area of the

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Figure 1.1 The composition of the small intestine

mucosa by five to eight times. To extend the absorptive surface even more the mucosa is equipped with villi. Villi are finger-like protrusions of the mucosa (Figure 1.1). Each villus is approximately 0.3 to 1 mm in height. The villi greatly multiplies the mucosal absorptive surface area to approximately 300 square meters¹⁴. There is no difference between the structure of the jejunum and ileum, There is however, a slight decrease in diameter, thickness of the intestinal wall and the number of mucosal folds in the lower part of the ileum.

Blood supply to the intestines comes from three major vessels: the celiac trunk, the superior mesenteric artery (SMA), and the inferior mesenteric artery (IMA). The intestines receive their blood supply based upon their embryological development. Parts of the duodenum are supplied by the celiac trunk. The distal duodenum, jejunum, ileum, cecum, ascending colon, and two-thirds of the transverse colon receive their blood supply from the SMA. The distal one-third transverse colon, descending and sigmoid colon, and rectum are supplied by the IMA. There is an abundant supply of collateral vessels and thereby a significant territorial overlap of blood flow that protect the intestines from transient periods of diminished perfusion. The major collateral vasculature of the gastrointestinal tract protects the bowel from massive ischemia. Normally, when the collateral vessels are sufficiently patent and there is occlusion of a mesenteric vessel, they will provide the needed blood supply. The main anastomotic regions include the pancreaticoduodenal arteries (celiac trunk with the SMA), the marginal artery of Drummond (SMA and IMA), and the arc of Rioloan (SMA and IMA). If the celiac artery is occluded, then the SMA can provide collateral flow via the pancreaticoduodenal arteries, and the same is true in reverse should the SMA occlude. Should the IMA occlude, then collateral flow via the arc of Rioloan and marginal artery can provide anterograde flow (Figure 1.2). However, the extent of the occlusion and its

Figure 1.2 Intestinal blood supply

The arterial blood supply to the small intestine and colon from the celiac artery, superior mesenteric artery (SMA), inferior mesenteric artery (IMA) and their collateral vessels.

location will determine whether collateral flow is successful or intestinal ischemia will develop.

The venous circulation parallels the arterial architecture. The inferior mesenteric vein (IMV) facilitates venous return from the IMA distribution. The superior mesenteric vein (SMV) facilitates blood return from the SMA region. Together they join the splenic vein and form the portal vein that then drains into the liver ^{10,15}.

Intestinal ischemia-reperfusion

The splanchnic circulation receives between 25 and 35 % of the cardiac output, depending upon whether the person is in fasting or feeding state ¹⁶. Two-thirds of this bloodflow is directed to the mucosal and submucosal layers of the intestines to facilitate nutrient exchange ^{1,17}. The splanchnic blood flow is regulated by the intrinsic (metabolic and myogenic) and the extrinsic (neural and humoral) system. The intrinsic metabolic regulatory system reacts on an imbalance between oxygen demand and supply by secreting local metabolites (adenosine, carbon dioxide etc.) resulting in vasodilatation and hyperemia. The myogenic mechanism compensates for a decrease in perfusion pressure by a direct arteriolar muscle relaxation, resulting in a preservation of the mesenteric blood supply. Neuronal and humoral mechanisms can also increase or decrease the blood flow. These consist of the sympathetic nervous system, the renine-

angiotensine system (RAAS) and numerous other endogenous or exogenous humoral factors ².

Ischemic injury to the intestinal tract develops when there is insufficient supply of oxygen and nutrients required for the cell's metabolism. The intestines are able to compensate the diminished oxygen supply by opening the collateral circulation and increasing the oxygen extraction, but these mechanisms are limited. The severity of ischemia depends on the quality of the systemic perfusion and the collateral circulation, the number and the type of splanchnic vessels that are affected, the degree of occlusion and the duration of the ischemic insult ¹⁸. The injury to the intestinal tract following ischemia is a result of hypoxia as well as reperfusion injury. The damage induced by ischemia starts at the tips of the villi and progress to transmural infarction, depending on the duration and severity of the ischemic period ¹⁹. The severity of the injury can range from reversible mucosal infarction, to mural infarction of the mucosa and submucosa, to transmural injury, involving all intestinal layers including the muscular wall causing perforation, peritonitis, sepsis and death ^{2,20}. Hypoxia causes damage to the villi of the mucosa within one hour, whereas transmural infarction occurs within eight to 16 hours ¹⁹. The enterocytes at the tips of the villi are more susceptible to the effect of ischemia than cells located at the bottom of the crypts. This is due to the location at the end of the distribution of the central arteriole which leads to a lower oxygen tension compared to the crypts ²¹.

Reperfusion injury occurs when oxygen-enriched blood re-enters the ischemic intestinal tract. Reperfusion begins after dissolution of an embolus or thrombus, after revascularization or adequate resuscitation of the blood flow. Paradoxically, reperfusion injury causes the most damage, initiated through the production of reactive oxygen species (ROS), activation of the complement system and increased influx of leukocytes ⁹. Ischemia-reperfusion (IR) injury disrupts the intestinal barrier function that results in an increased intestinal permeability. The latter causes bacteria and other luminal content entering the systemic and portal circulation ^{22,23}. Bacterial translocation together with leukocyte activation can result in the systemic inflammatory response syndrome (SIRS) and sepsis. Sepsis ultimately results in multiple organ failure (MOF) as remote organ injury occurs ²⁴.

Types of intestinal ischemia

Intestinal ischemia can be of acute (90%) or chronic type. Acute mesenteric ischemia (AMI) refers to the sudden onset of intestinal hypoperfusion. There are four specific types of acute mesenteric ischemia based on different causes. The most frequent cause of AMI are arterial emboli with an incidence of 40-50% ¹⁻³. These emboli originate most frequently from the left atrium, left ventricle or cardiac valves. Sometimes a mesenteric artery embolus can form during angiographic procedures of the coronary or cerebral circulation. Arterial emboli most often occlude the SMA; the IMA is rarely affected. Acute mesenteric thrombosis occurs in 25-30% of the AMI cases. Severe atherosclerosis,

Figure 1.3 The different types of intestinal ischemia and their etiological background

especially at the origin of the mesenteric artery, is the underlying cause of acute mesenteric thrombi in most of the patients. In addition, angiographic procedures, surgical accidents, hypercoagulability and oral contraceptives can cause arterial thrombi¹⁻³. In 5-15% of cases of mesenteric ischemia the patients have a venous thrombosis. Risk factors for the development of venous thrombi are hypercoagulability, right-sided heart failure, malignancy in the portal region, cirrhosis, abdominal trauma, recent abdominal surgery or infection, pancreatitis, splenectomy, and portal hypertension. Nonocclusive mesenteric ischemia (NOMI) accounts for approximately 20% of the patients with AMI. It is the result severe hypotension leading to a diminished mesenteric blood flow caused by cardiac failure, hypovolemic and septic shock, neonatal necrotizing enterocolitis, major surgery or vasoconstrictive drugs^{2,25-30}.

Chronic mesenteric ischemia refers to episodic or constant intestinal hypoperfusion. More than 90% of cases of chronic mesenteric ischemia is related to progressive mesenteric atherosclerotic disease (Figure 1.3)³¹. This thesis will focus on improving the early diagnosis and to study the pathophysiological mechanisms of AMI.

Clinical presentation of AMI

Symptoms of AMI are nonspecific, ranging from sudden severe periumbilical abdominal pain to nausea and vomiting. A classic finding for AMI includes abdominal pain out of proportion to physical examination. The symptoms and signs are different depending on the underlying cause^{1,2}. Patients with an SMA embolism or thrombosis have an acute

onset of symptoms and their clinical situation can change very fast. The presentation of mesenteric vein thrombosis is in most of the cases insidious. They only complain about nonspecific abdominal pain associated with anorexia and diarrhea, 1-2 weeks after the onset ^{7,8}. Nonocclusive mesenteric ischemia has also a more gradual onset. The clinical presentation is often dominated by the precipitating disorders (eg. hypotension, cardiac failure, hypovolemia, etc.) ^{1,2}. Chronic mesenteric ischemia typically presents itself with postprandial pain (abdominal angina), weight loss as a result from fear of eating and unexplained diarrhea ^{2,7,8}.

Diagnosis of AMI

Early diagnosis and treatment of AMI is critical to prevent progression of intestinal IR to irreversible transmural infarction causing leakage of intestinal content into the abdominal cavity leading to peritonitis, an overwhelming SIRS response and eventually death ^{22,23,26}. Fast revascularization when the ischemic damage is potentially reversible is most beneficial to improve patient's outcome ^{7,8,32}. Early diagnosis however is difficult because of the non-specific presentation and paucity in accurate biomarkers ³³.

Diagnosis of intestinal ischemia nowadays relies on a high index of clinical suspicion, particularly for patients with known risk factors (eg. older than 60 years, atrial fibrillation, myocardial heart failure, congestive heart failure, arterial emboli, postprandial abdominal pain and weight loss). Several guidelines have been developed for the diagnosis and treatment of AMI ^{3,7,8}. Patients suspected of having AMI should undergo an intravenous contrast enhanced CT scan (CTA) with a slice thickness of 1 mm following a triphasic protocol (without contrast, arterial and portal venous phases). This is the most sensitive and specific diagnostic tool, available around the clock ^{7,8}. The presence of intestinal wall or portomesenteric gas on CTA is a sign of severe transmural infarction, but this sign is not specific for either infarction or ischemia. CT scans are also most suitable for patients with mesenteric venous thrombosis ³⁴.

Next, routinely used laboratory markers lack diagnostic accuracy to identify AMI. Some laboratory abnormalities that can appear with AMI are elevated white blood cell count, hematocrite and metabolic acidosis with a high anion gap and lactate concentrations. None of these tests are sufficiently sensitive or specific to diagnose AMI in the acute setting ^{7,8,35}. However, D-dimer showed a high sensitivity of > 95%, making it a useful marker to exclude AMI in patients with acute abdominal complaints. But many other conditions are associated with high D-dimer levels and it does not differentiate between patients with reversible and irreversible intestinal ischemia ³⁶.

New biomarkers for AMI

A perfect biomarker for AMI needs to be specific for AMI and highly sensitive. The biomarker that will be measured needs to be present in sufficiently high concentrations in the intestinal wall. Thereby it must be measurable in venous blood samples, so the

structural integrity of the protein must be kept after passage through the portal circulation and the liver ^{37,38}.

Proposed biomarkers for AMI that already have been investigated are D- lactate, I-FABP, α -glutathione S-transferase and creatine kinase. These four markers all originate from different sites in the intestinal wall, respectively the lumen, the epithelium, the small arteries and veins and the muscular layer. Despite the many investigations conducted to date, no clinically useful biomarker has yet entered routine clinical practice ^{17,35}.

I-FABP is probably the most promising biomarker for AMI today. As ischemia starts from the mucosa and progresses to the serosa, a mucosa-derived marker would be most useful for early diagnosis ³⁵. I-FABP is a small (14-kD) cytosolic protein solely present in mature enterocytes at the tip of the villus, located in the mucosa of the intestinal ³⁹. Release of this biomarker in plasma indicates injury to the enterocytes membrane (Figure 1.4). It can be used in the early phase to diagnose ischemia-reperfusion injury of the intestines ⁴⁰⁻⁴⁴. I-FABP however, only detects injury of the mucosal layer of the intestinal wall. Mucosal injury is mostly still reversible in contrast with transmural infarction. Transmural infarction leads to perforation of the intestinal wall which can cause peritonitis, sepsis and in the worst case MOF (Figure 1.5). Most of the patients with intestinal ischemia can be treated conservatively by restoring the intestinal blood flow,

Figure 1.4 Leakage of cytosolic proteins from damaged enterocytes during intestinal ischemia-reperfusion

(top), cytosolic proteins like I-FABP (purple) are abundantly present in mature enterocytes. **(bottom)**, Intestinal ischemia-reperfusion leads to epithelial membrane integrity loss causing leakage of I-FABP into the circulation, where it can be measured using an I-FABP ELISA.

but patients with transmural infarction need to undergo surgery^{7,8,45}. Therefore, transmural infarction needs to be detected as soon as possible because mortality can increase rapidly.

Figure 1.5 Stages of intestinal ischemia

The severity of intestinal ischemic injury ranges from *mucosal infarction* when the intestinal damage extends no deeper than the muscularis mucosae, sparing the muscular wall, to *transmural infarction* of the intestine, involving all intestinal wall layers leading to perforation of the intestinal wall.

This leads to the following questions:

1. What is the diagnostic value of the currently known circulating biomarkers to detect acute intestinal ischemia in man?
2. Can we provide more insight into the clinical usefulness of circulating I-FABP as early diagnostic marker to detect intestinal ischemia?
3. Are proteins, derived from the intestinal wall, useful as biomarker to detect transmural infarction, selecting patients who should undergo prompt surgical intervention?
4. Can we develop and validate a new reliable sandwich enzyme-linked immunosorbent assay (ELISA) for quantification of potential new biomarkers to detect transmural intestinal infarction?

Histology and intestinal barrier function

The intestine's primary function is the digestion and uptake of nutrients, water and electrolytes ^{12,13}. To this end, the intestinal mucosa is covered by a single layer of columnar epithelial cells. This epithelial lining is characterized by the regularly distributed finger-like villi, with at the base of these villi depressions known as intestinal crypts, or the crypts of Lieberkuhn ¹¹. The epithelial lining is composed of four different type of cells, all originated from stem cells in the crypt ⁴⁶. Deep within the crypts, epithelial stem cells are abundant, which differentiate into Paneth cells, absorptive enterocytes, goblet cells and enteroendocrine cells (Figure 1.6). While Paneth cells remain in the crypts, goblet cells, enterocytes and enteroendocrine cells migrate to the tip of the villi, where they undergo apoptosis at the end of their lifecycle and shed into the intestinal lumen ⁴⁷. This mechanism allows renewal of the epithelial lining every 3-6 days. Enterocytes have apical microvilli, also known as the brush border, which help with de digestion and absorption function of the enterocytes. The enterocytes are interspersed with mucin secreting goblets cells and enteroendocrine cells producing different kinds of digestive hormones. At the lower portion of the crypts the Paneth cells secrete protective antimicrobial proteins like lysozyme and defensins from eosinophilic granules ⁴⁸. These proteins are released into the crypt lumen to prevent microbial invasion of the epithelial

Figure 1.6 Composition of the small intestinal epithelial lining

lining⁴⁹. Next, Paneth cells constitute the niche for stem cells in the intestinal crypts by producing essential growth factors, vital for appropriate stem cell function⁵⁰.

The lamina propria of the individual villus consists mainly of loose collagenous tissue containing a small central arteriole, branches at the tip of the villus to form the capillaries, in turn, empty into a collecting venule that runs to the bottom of the villus. Together with central lymphatics these help transport nutrients absorbed by the enterocytes. The small intestinal lamina propria also contains low numbers of immune cells, including lymphocytes and white blood cells.

The intake of nutrition exposes the intestinal epithelium to a wide variety of antigens from food and microbiota, making it the largest site of exposure to the outside environment in the human body. The secondary function of the intestine is therefore to monitoring and sealing the host interior from potentially harmful intruders^{12,13}. This function of the gastrointestinal tract is referred to as intestinal barrier function. The intestinal barrier can be subdivided into a physical and an immunological part^{51,52}. The physical intestinal barrier consists of the mucus layer and the epithelial barrier. The mucus layer is the first line of defense, preventing direct contact of bacteria and toxins with the epithelial cells. The main component of the mucus layer are highly glycosylated mucin proteins that form a gel-like structure overlying the epithelial cells. Immune regulators like IgA molecules, are released in the mucus gel to reinforce the mucus layer⁵³. Next, the intestinal barrier consists of a single but cohesive layer of epithelial cells covered with mucus stretching from the lower esophageal sphincter to the anus⁵⁴. The plasma membrane of the cells prevent translocation of micro-organisms and antigens to the underlying lamina propria and systemic circulation. The epithelial cells are firmly connected to another by junctional complexes located at the lateral membrane⁵⁵. Transport of important molecules in-between the epithelial cells is regulated by these junctional complexes. They serve an important role in the intestinal barrier function as a selective fence sealing the paracellular pathway⁵⁶. The three most important complexes are tight junctions (TJs), adherens junctions (AJs) and desmosomes. TJs are the most apical complexes that are built up by the transmembrane sealing proteins claudins and occluding⁵⁷. These proteins interact with cytoplasmic proteins, including zonula-occludin (ZO)⁵⁸. ZO proteins act as an anchor linking the transmembrane proteins to the actin cytoskeleton. Together with the AJs, the TJs are the most important complexes which regulate cell-cell adhesion, paracellular permeability and cell polarity^{56,58}. AJs are found beneath the TJs and are required for their assembly. Together with desmosomes, AJs provide strong adhesive bonds to maintain the epithelial barrier integrity (Figure 1.7)⁵⁶. The physical intestinal barrier is supported by the immunological intestinal barrier. Intestinal cells responsible for the immunological part of the intestinal barrier are enterocytes and Paneth cells¹². Enterocytes act as immune sensors of microbial pathogens and commensal organisms. Recognition of hazardous components is

Figure 1.7 Enterocytes with different types of intercellular contacts being essential for maintenance of the intestinal barrier function

Tight junctions (TJs) and the subjacent adherens junctions (AJs) act as a fence, regulating the passive diffusion of solutes across the paracellular pathway. TJs are composed of transmembrane proteins such as occludin and members of the claudin family, which are anchored to the cell cytoskeleton by zonula occludens proteins (ZO-1, ZO-2 and ZO-3). AJs are important for connecting neighbouring cells and consist, mainly, of the transmembrane protein E-cadherin.

achieved by Pattern-Recognition Receptors (PRRs). PRRs consist of four major type of receptors including Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) ^{59,60}. PRRs response to pathogen-associated molecular patterns (PAMPs) or Damage-Associated molecular patterns (DAMPs). Damage-associated molecular patterns (DAMPs) originate from nuclear or cytosolic proteins, and are released as a consequence of cell or tissue injury ^{54,59}. In contrast, PAMP's are expressed on both commensal and pathogenic microorganisms and include bacterial lipopolysaccharide (LPS), lipoprotein and peptidoglycans ¹². PRRs activate intracellular signaling pathways, resulting in upregulation of inflammatory genes and stimulation of mechanisms involved in the NF- κ B associated inflammatory responses ⁶¹. The epithelial lining also harbors intraepithelial lymphocytes. They are components of gut associated lymphoid tissue (GALT) and produce large amounts of secretory immunoglobulin A via T cell or specialized rapid "natural" antibody producing B cell dependent pathways ⁶². Detection of pathogens by PRRs also leads to the activation of the immune cells present in the lamina propria required to contain the infection ⁶². The lamina propria accommodates large amount of immune cells of both the innate immune system (macrophages, dendritic cells, mast cells) and the adaptive

immune system including T regulatory cells, B cells, TCD4+ cells and plasma cells. The latter reflecting the central role of the intestine in immunity and protection against pathogens ⁶³.

Next, the Paneth cells abide in the crypts of the small intestine between the stem cells reinforce the immunological barrier. They are specialized epithelial cells that secrete antimicrobial peptides like defensins and lysozyme ⁴⁸. These antimicrobial peptides maintain a sterile environment within the crypts, which is important to protect the intestinal stem cells ^{46,49}. Damage to stem cells would have severe consequences for the maintenance of the normal gut epithelium.

Role of IR in intestinal barrier loss

Over the past decades it has become evident that dysfunction of the intestinal barrier has a significant impact on the health of an individual. It has become apparent that disruption of either epithelial cells membrane or tight junctions are playing a key role in the development, perpetuation and/or progression of a variety of diseases including inflammatory bowel disease (IBD), celiac disease, necrotizing enterocolitis (NEC) and intestinal ischemia ^{54,59,63}. An impaired intestinal barrier function in patients with decreased intestinal perfusion following major surgery, trauma or (hemorrhagic) shock is associated with the development of sepsis and multiple organ failure (MOF) ^{22,29,64}.

Evaluation of intestinal pathology in patients of all age-groups remains to be a challenge to both clinicians and scientists. This is mainly attributable to the non-specific clinical presentation of patients with abdominal complaints and a paucity in clinically useful tests to detect intestinal barrier loss. Therefore, a major delay in diagnosis is often present in patients with e.g. NEC, chemotherapy-induced mucositis, acute mesenteric ischemia and celiac disease ^{2,54,65}. A diagnostic delay results in postponed correct treatment, which is accompanied by higher morbidity and mortality rates. In line with these diagnostic concerns, the follow-up of numerous intestinal diseases is hampered by the absence of non-invasive, rapid diagnostic means to assess intestinal damage for evaluation of the effects of treatment on the recovery of the disorder ⁶³. The current standard technique for assessing intestinal status is endoscopy with bowel biopsy, which is often inadequate as it is invasive, sometimes requires sedation, is expensive and only assesses the function of the biopsied fraction. In conclusion, there is a need for specific sensitive biomarkers which can evaluate intestinal barrier function ⁶⁶.

Improved insight in IR-induced intestinal barrier loss

Given the importance of IR injury as underlying pathology in a variety of diseases, it is crucial to understand the molecular mechanisms leading to intestinal barrier loss and inflammation. However, most of the current knowledge on these mechanisms are obtained from animal models. This let our research group to develop a human experimental intestinal IR model using patients undergoing a pancreatico-

duodenectomy. In these patients a variable length of healthy jejunum is routinely resected in continuity with the head of the pancreas and duodenum as part of the surgical procedure. The terminal 6 cm of this jejunal segment is isolated using a linear cutting stapler and subjected to various periods of warm ischemia by placing two atraumatic vascular clamps across the mesentery (Figure 1.8). Removal of the clamps allow for reperfusion as confirmed by regaining of normal pink color and restoration of gut motility. A similar model was developed to study the pathophysiology of IR in the colon, including patients undergoing low-anterior-resection or abdominoperineal resection for colorectal cancer. During surgery, the colonic segment that had to be removed for surgical reasons, was identified and the proximal six cm of this segment, located just distal from the initial site of surgical transection, was carefully isolated similarly to the small intestine IR procedure and exposed to ischemia by placing two atraumatic vascular clamps across the mesenteric arteriole and venule (Figure 1.9). These models allow for the collection of both plasma and tissue during the experimental protocol at set time points, yielding reproducible and consistent results. In short, tissue can be sampled at any required time point using the linear cutting stapler. Blood is sampled at different time points by direct puncture of the venule draining the isolated intestinal segment. Arterial blood is sampled from the radial artery line, present in all patients as part of routine intraoperative monitoring. This allowed assessing concentration gradients in plasma across the isolated intestinal segment. Meanwhile,

Figure 1.8 Schematic illustration of the experimental human small intestinal IR model

The model takes advantage of the fact that during pancreaticoduodenectomy, a variable segment of the jejunum is routinely resected in continuity with the gallbladder, the head of the pancreas and duodenum (depicted in grey) as part of the surgical procedure. The terminal six cm of this jejunal segment (depicted in pink) was isolated by staple transection from the remaining bowel(dotted lines) and mesentery. Next, the isolated segment is subjected to ischemia by placing two atraumatic vascular clamps across the mesenteric arteriole and venule. Meanwhile, the surgery proceeded as planned.

Adapted from "Life and death at the mucosal-luminal interface: a new perspective on human intestinal ischemia-reperfusion", Joep Grootjans, 2012, p.14

surgery proceeds as planned. At the end of the experiment, 2 cm of intestine that remained untreated during surgery was resected and served as internal control ^{67,68}.

Figure 1.9 Schematic illustration of the experimental human colon IR model

These models have allowed a better understanding of the pathophysiological mechanisms underlying the development of intestinal injury and inflammation during human intestinal IR. First, a unique, endogenous clearing mechanism for injured enterocytes was revealed upon short periods (30 minutes) of ischemia with rapid detachment of damaged apoptotic enterocytes into the lumen ^{69,70}. This process is quickly followed by repair of the intestinal continuity during reperfusion by 'purse-string' contraction. This results in a restored epithelial lining ⁷⁰, which is hypothesized to be a key factor in the prevention of the development of inflammation. However, prolonged periods (>45 minutes) of ischemia followed by reperfusion leads to irreversible tissue damage and initiation of inflammation ²³. This was accompanied by increasingly evident endoplasmatic reticulum stress in Paneth cells, resulting in apoptotic Paneth cells, which are shed into the crypt lumen, lowering their numbers. Paneth cell loss resulted in diminished gut barrier function and subsequent bacterial translocation and systemic inflammation ⁴⁹. In conclusion, the human in vivo models for intestinal IR have given more insight in several physical (mucus-layer, epithelial lining) and immunological (Paneth cells) intestinal barriers response upon ischemic damage. However, these observations raised several new questions concerning the way the intestinal barrier is restored during IR. One important remaining question is whether functional barrier restoration is achieved during reperfusion. This is in particular important as few human experimental studies exist that directly correlate barrier function to histological appearance. Also, the methods to measure intestinal barrier permeability and enterocytes function is argued ⁷¹. So we set out to study the relation between

histological and functional barrier recovery using a human experimental small intestinal IR model.

Next, although the consequences of IR in patients participating in the previous studies were consistent, some patients had considerably less intestinal IR-induced damage than others. This leads us to investigate whether such differences in disease susceptibility are sex-related. This is important since recognition of potential sex differences is expected to be beneficial for treating both males and females with personalized novel therapeutic approaches to reduce IR-associated morbidity and mortality.

The observed distinct responses of villus and crypt epithelium to mucosal injury and inflammation led our group to investigate differential gene expression patterns in this model. Data showed that cyclooxygenase-2 (COX-2) gene expression was significantly upregulated during reperfusion. As COX-2 plays a key function in the human wound healing, several animal studies suggest an important role for COX-2 in intestinal wound healing^{72,73}. It is also known from clinical studies that inhibition of COX-2 with the use of non-steroidal anti-inflammatory drugs increases the risk of developing anastomotic leakage^{74,75}. However, controversy on whether COX-2 is beneficial and hence protective or, detrimental to the mucosa during intestinal damage remains^{72,73,76}. COX-2 is responsible for the conversion of arachidonic acid into prostaglandins, of which PGE2 is responsible for mucosal restitution in response to epithelial injury. It is demonstrated that COX-2 deficiency led to major intestinal barrier dysfunction, increased bacterial translocation and severe inflammation^{73,76}. Other reports show that elevated COX-2 expression leads to an increase of IR-induced intestinal damage⁷².

Stimulation or inhibition of COX-2 could be an interesting new therapeutic target in the treatment of IR-induced intestinal tissue damage, so the underlying molecular mechanisms should therefore be investigated.

Furthermore, better knowledge on danger signals from the lumen causing the intestinal damage and inflammation ultimately leading to transition into systemic inflammation is needed. These danger signals were long thought to consist of bacteria or their products. However, interventions against bacteria that are aimed at reducing mortality in patients with sepsis have been unsuccessful in clinical trials⁷⁷. Recent rat studies undergoing intestinal IR indicate that pancreatic enzymes play a major role in acute inflammatory processes resulting from intestinal ischemia, hemorrhagic and endotoxic shock⁷⁸⁻⁸⁰. The pathognomonic role of proteases was discovered by adding protease-inhibitors intraluminally in rats, showing abrogation of the usual sequelae of intestinal IR, inflammation and shock⁸¹. These results gave rise to the 'autodigestion' hypothesis suggesting that powerful proteases leak across the intestinal mucosal barrier, initiating self-digestion of the intestinal wall causing severe inflammation, leading to SIRS with MOF⁸². Therefore the role of pancreatic digestive enzymes need to be studied in the pathogenesis of human intestinal IR. Elucidation of the mechanisms involved will

potentially lead to new therapeutic opportunities; especially new therapies directed at the inhibition of pancreatic enzymes.

This all leads to the following pathophysiological questions:

1. What is the relation between the morphological and functional restoration of the intestinal barrier during IR?
2. Is there a sex-difference in the response to intestinal IR in man and what are the possible underlying mechanisms?
3. What is the role of COX-2 in the restoration of intestinal damage?
4. Does a relationship exist between pancreatic digestive enzymes and the increased inflammatory response during human intestinal IR?

Aims of the thesis

In this thesis I set out to improve our knowledge on A. potential (new) biomarkers to detect intestinal IR at an early stage and B. to provide better insight into the pathophysiology of intestinal IR. On the basis of the questions brought forward in the previous paragraphs, the following aims were subject of this thesis:

Part A: potential new biomarkers for the detection of intestinal IR

- 1) To study the safety of our two human in vivo intestinal IR models
- 2) To systemically review the literature regarding human plasma biomarkers for acute mesenteric ischemia reported during the last ten years.
- 3) To investigate the relation between plasma marker for intestinal mucosal damage (I-FABP levels) and the severity of ischemic mucosal injury and define the clinical usefulness of systemic I-FABP following ischemia.
- 4) To discover a new marker for transmural intestinal ischemic damage by developing a new quantitative ELISA for smooth muscle protein of 22 kDa (SM22) and to evaluate the diagnostic potential of SM22 as plasma biomarker for the detection of transmural intestinal ischemia.

Part B: pathophysiology of human experimental IR

- 5) To study the relation between histological and functional intestinal epithelial barrier recovery during human small intestinal IR.
- 6) To find out whether there is a sex-difference in response to intestinal ischemic hit and to investigate the mechanisms involved in the differences in human intestinal mucosal responses to IR between males and females
- 7) To study the role of Cyclooxygenase2 (COX-2) during intestinal epithelial damage and recovery.
- 8) To investigate the role of pancreatic digestive enzymes in intestinal inflammation associated with human intestinal IR

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Chapter 2

Evaluating the safety and complications of two human experimental intestinal ischemia reperfusion models: a retrospective observational study

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Submitted

Abstract

Background: We developed a jejunal and colonic experimental human ischemia-reperfusion (IR) model to study pathophysiological mechanisms of human intestinal IR and potential new biomarkers for intestinal ischemia. Our objective was to evaluate the safety of these IR models by comparing patients undergoing surgery with and without experimental in vivo intestinal IR

Methods: A retrospective study was performed to evaluate the safety of the two IR models. In 10 patients undergoing a pancreaticoduodenectomy and 10 patients undergoing colorectal surgery, 6 cm of jejunum or colon was isolated and exposed to ischemia followed by various reperfusion periods. The complication rates of patients participating in the IR studies were compared as primary outcome parameter to non-participating patients undergoing the same type of surgery. Patients were matched for age, sex, comorbidities and tumor characteristics. Postoperative complications were recorded based on the Clavien-Dindo classification system. Secondary outcome parameters were operative time, blood loss, 90-day mortality and length of hospital stay.

Results: Following pancreatic surgery, 63% of the patients suffered from one or more postoperative complication. There was no significant difference in incidence of complications between patients undergoing pancreatic surgery with or without experimental in vivo jejunal IR (7 of 10 jejunal IR patients (70%) vs 12 of 20 No IR patients (60%), $P = 0.7$). Operative time, amount of blood loss, length of hospital stay or mortality were equal in both groups. 60% of patients undergoing colorectal surgery experienced postoperative complications of any Clavien-Dindo grade. Complication rate was similar in patients undergoing experimental in vivo colonic IR and patients without IR (5 of 10 colonic IR patients (50%) vs 13 of 20 No IR patients (65%), $P = 0.46$). No significant differences were observed concerning operative time, amount of blood loss, length of hospital stay or mortality.

Conclusion: This study showed that these human experimental intestinal IR models are safe and feasible in patients undergoing pancreatic or colorectal surgery.

Introduction

Intestinal ischemia reperfusion (IR) is a frequent occurring phenomenon following multiple clinical situations. It is the result of thromboembolic occlusion of the mesenteric arterial blood supply causing acute mesenteric ischemia. More often intestinal IR is observed as part of intestinal hypoperfusion in patients suffering from shock, trauma, sepsis or major surgery¹⁻⁴. These severe stress situations lead to a significant reduction of mesenteric blood flow which preserves central hemodynamic stability, but results frequently in nonocclusive mesenteric ischemia. Paradoxically, the subsequent return of oxygenated blood during reperfusion aggravates ischemia induced tissue damage^{3,5}. Intestinal IR is characterized by a decrease of gut barrier function. Dysfunction of the gut barrier has been implicated as a major contributor to the systemic inflammatory responses following intestinal IR, which can evolve to multiple organ failure and death^{6,7}. The latter accounts for the high morbidity and mortality rates of 60-80% associated with intestinal IR^{1,8}.

It is intriguing that the morbidity and mortality rates of intestinal IR did not improve over the last decades despite improvements in surgery, interventional radiology and intensive care medicine⁸. This is partly due to the lack of insight in the pathophysiological processes during intestinal IR and consequently the shortcoming in preventive and/or therapeutic options. Next, these grave outcomes are closely linked to a delay in diagnosis. This is mainly attributable to the nonspecific clinical presentation in combination with a paucity of early, non-invasive diagnostic markers for intestinal ischemia⁹⁻¹¹. Although there is increasing knowledge about mechanisms involved in intestinal tissue injury and dysfunction caused by IR, many questions remain. Animal models have been indispensable to obtain more insight into the mechanisms of intestinal IR. However, due to differences between various animals and models most results cannot be translated to the clinical setting¹². For this purpose our group developed a in vivo human model for intestinal IR, in which ischemia is applied to either the small intestine or the colon of included patients undergoing surgery, followed by a period of reperfusion^{13,14}. This model allowed our research group to investigate small intestinal and colonic IR injury in more detail, resulting in better insight in the mechanisms of human intestinal IR injury. Furthermore, this model made it possible to evaluate new biomarkers to facilitate the earlier diagnosis of acute intestinal ischemia¹⁵.

In our experience, the models seemed safe and harmless for the individual participating patients with no serious adverse events reported. However, objective evidence is needed regarding the long-term post-operative outcomes and complication rates of these patients as a group, compared to a group of control patients undergoing the same type

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of surgery, performed by the same surgeons in the same hospital without being exposed to IR-protocol.

In an attempt to confirm the safety of our model, this study aimed to compare the post-operative outcomes and complication rates between the IR-groups and a group of matched control patients.

Materials and Methods

Ethics

The study was approved by the Medical Ethical Committee of Maastricht University Medical Center and was conducted according to the revised version of the Declaration of Helsinki (October 2013, Fortaleza). Written informed consent of all patients participating in the IR studies was obtained. The responsible ethics committee granted an exemption from requiring informed consent for the retrospective gathering and analysis of medical data from control patients, who did not participate in the IR studies.

Experimental IR procedures and control patients

The studies were carried out in the Maastricht University Medical Center (MUMC) in Maastricht, the Netherlands. This is a high volume, tertiary referral center for Hepato-Pancreatico-Biliary (HPB) and colorectal surgery with ⁴⁵⁻⁶⁵ pancreatic resections per year and 90-100 colorectal procedures.

Jejunal IR

To investigate human small intestinal IR, ten patients undergoing a pancreaticoduodenectomy for benign or malignant pancreatic head tumors were included at the Maastricht University Medical Center from December 2008 until August 2010.

Six cm of healthy small intestine from these patients was exposed to 60 minutes of ischemia (60I), followed by 30 and 120 minutes of reperfusion according to the human jejunal IR model as described elsewhere ¹³. In short, during pancreaticoduodenectomy, a variable segment of the jejunum is routinely resected in continuity with the head of the pancreas and duodenum as part of the surgical procedure. The terminal six cm of this jejunal segment was isolated by staple transection from the remaining bowel and mesentery. All collateral blood vessels were clamped and ligated, leaving only one central mesenteric supplying arteriole and draining venule. Next, the isolated segment was subjected to 60I by placing two atraumatic vascular clamps across the mesenteric arteriole and venule. Meanwhile, the surgery proceeded as planned. After the period of ischemia, one third (two cm) of the isolated ischemic jejunum was resected using a linear cutting stapler (GIA; Medtronic, Eindhoven, the Netherlands). Next, clamps were removed to allow reperfusion, as confirmed by regaining of normal pink color and restoration of gut motility. Another segment of the isolated jejunum (two cm) was resected similarly after 30 minutes of reperfusion (30R). The last part (two cm) was resected after 120 minutes of reperfusion (120R). Simultaneously, two cm of the jejunum, which remained untreated during surgery, was resected, serving as the internal control tissue. This segment underwent similar surgical handling as the isolated part of the

jejunum but was not exposed to IR. Arterial blood was sampled from the radial artery before ischemia, immediately after ischemia, and at 30 and 120 minutes after start of reperfusion. Simultaneous with each respective arterial blood sample, blood was drawn from the venule draining the isolated jejunal segment to assess concentration gradients across the isolated jejunal segment. All tissue samples were immediately formalin-fixed for immunohistochemical analysis.

Colon IR

For the experimental colon IR protocol, ten patients undergoing low-anterior-resection or abdominoperineal resection for colorectal cancer were included from March 2009 until October 2010 at the Maastricht University Medical Center. In these patients, a piece of healthy colon was subjected to 60 minutes of ischemia (60I), followed by 30 and 60 minutes of reperfusion according to the human colon IR model previously described ¹⁴. During surgery, the distal colonic segment that had to be removed for surgical reasons, was identified and the proximal six cm of this segment, located just distal from the initial site of surgical transection, was carefully isolated using a cutting stapler similarly to the small intestine IR procedure. All collateral blood vessels were ligated and the isolated segment was exposed to 60 minutes of ischemia (60I) by placing two atraumatic vascular clamps across the mesenteric arteriole and venule. During IR, surgery was continued in accordance with the standard operating procedures for a low-anterior resection or abdominoperineal resection. After 60I and tissue collection, reperfusion was allowed by removal of the clamps, and tissue was again collected at 30 minutes (60I30R, short reperfusion) and 60 minutes of reperfusion (60I60R, prolonged reperfusion). At the end of the experimental protocol, a small part of colonic tissue located just proximal from the site of the initial transection, not exposed to IR, was resected and served as internal control tissue. Because of differences in duration of surgery, we were only able to reach 60R in the colonic IR model, whereas in the small intestinal IR model 120R could be achieved. For both the jejunal IR and the colon IR study, patients with other underlying intestinal diseases were excluded.

Control patients

To evaluate the safety of the IR models, we extracted data from the medical charts of 40 patients (n = 20 as controls for the jejunal IR model and n = 20 as controls for the colonic IR model), who underwent the same type of surgery in the same hospital over the same time period as the IR-patients, but were not exposed to the IR protocols. For every patient subjected to the IR-model, two control patients were selected. These patients were matched for age, sex, tumor characteristics/classification and operation period.

Data collection and definitions of outcome

Health record files of all the patients (study group and control group) were studied to obtain clinical parameters regarding sex, age, diagnosis, medical history, medication use, date, type of surgery, pre-operative status, intra-operative variables, post-operative course, tumor characteristics, complications, length of hospital stay, reinterventions, re-admissions and death. Readmission was defined as any additional hospitalization, excluding admission to a subacute care or rehabilitation facility within 30 days^{16,17}. All procedures were performed by experienced surgeons who had completed training in pancreatic and colorectal surgery. The general health condition of the patient was appraised using the Charlson comorbidity index¹⁸. All postoperative complications were assessed using the Clavien-Dindo classification system¹⁹. Data on the postoperative course were collected up to the 90th postoperative day.

The primary endpoint for this study was 90-day Clavien-Dindo classification of surgical complications. Secondary endpoints were duration of surgery, intraoperative blood loss or iatrogenic injuries, infections and complications in wound healing, cardiovascular complications, organ failure, neurologic complications, gastro-intestinal complications, length of postoperative hospital stay, readmission within 30 days after discharge and death within 90 days postoperative. Pancreatic fistula, delayed gastric emptying (DGE) and postpancreatectomy hemorrhage were defined according to the International Study Group of Pancreatic Surgery definitions²⁰⁻²².

Statistics

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS, Chicago, IL), version 23. Continuous variables are presented as median values with interquartile ranges (IQRs), and categorical variables as frequencies, number of patients and percentages. The primary endpoint for this study, the 90-day Clavien-Dindo classification of surgical complications, was compared using the χ^2 -test. Other secondary outcomes were similarly compared between the two groups for categorical variables using the χ^2 -test or by the two-sided Fisher exact test as appropriate. For continuous variables, the student's t-test and the nonparametric Mann-Whitney U test were used. A *P* value of 0.05 was considered statistically significant.

Results

Patient's characteristics

In the jejunal IR group, there were eight male (80%) and two female (20%) patients with a median age of 67 years (range 48–79 years). Eight (80%) patients had malignant disease and the remaining two (20%) patients had chronic pancreatitis.

To test whether the post-operative outcome or incidence and severity of complications were different in the experimental IR group, we analyzed a matched control group of 20 patients. There were 16 (80%) male and four (20%) female patients with a median age of 65 years (range 45–79 years). When comparing both groups no significant differences were found concerning comorbidities, tumor location, preoperative therapies, intoxications, or ASA classification. Furthermore, groups did not differ with respect to the frequency of positive resection margins. In both the experimental jejunal IR group and No IR group, positive margins (R1) was recorded in one patient. Patient's characteristics, comorbidities and postoperative diagnoses are presented in Table 2.1.

Eight male (80%) and two female (20%) patients with a median age of 60 years (range 45-66 years) were included in the colonic IR group. To evaluate the safety of the experimental colonic IR protocol, these patients were compared with 20 control patients included in the same time period. This group consists of 16 male (80%) and four female (20%) patients with a median age of 65 years (range 48-79 years). All patients suffered from malignant disease. Low-anterior-resection (n = 26) was the most performed surgical technique compared with the abdominoperineal resection (n=4). No significant differences were found concerning co morbidities, AJCC cancer stage, preoperative therapies, intoxications, or ASA classification of patients undergoing colorectal surgery with and without experimental in vivo colonic IR. All patients underwent an open colorectal procedure. No positive resection margins (R1) were recorded in both the experimental in vivo colonic IR and the No IR group. Patient characteristics, co morbidities and postoperative diagnoses are presented in Table 2.2.

Table 2.1 Demographics and comorbid conditions of patients undergoing pancreatic surgery with and without experimental in vivo jejunal IR

N (%) or median (IQR)	All (30)	Jejunal IR (10)	No IR (20)	P-value
Sex				1.0*
Male	24 (80)	8 (80)	16 (80)	
Female	6 (20)	2 (20)	4 (20)	
Age (range)	67 (45-79)	67 (48-79)	65 (45-79)	0.83**
Charlson index score				1.0*
0-5	6 (20)	2 (20)	4 (20)	
5-10	24 (80)	8 (80)	16 (80)	
>10	0 (0)	0 (0)	0 (0)	
Tumor location				0.3*
Pancreatic head	12 (40)	2 (20)	10 (50)	
Papil of Vater	8 (26.7)	4 (40)	4 (20)	
Extrahepatic biliary ducts	3 (10)	2 (20)	1 (5)	
Duodenum	1 (3.3)	0 (0)	1 (5)	
Benign / pancreatitis	6 (20)	2 (20)	4 (20)	
Coronary artery disease	4 (13.3) ^a	1 (10)	3 (15.8) ^a	1.0
Hypertension	10 (35.7) ^a	4 (40)	6 (33.3) ^a	1.0
Peripheral vascular disease	1 (3.4) ^a	1 (10)	0 (0) ^a	0.35
COPD	0 (0) ^a	0 (0)	0 (0) ^a	1.0
Diabetes mellitus	7 (24.1) ^a	2 (20)	5 (26.3) ^a	1.0
Preoperative pancreatitis	4 (14.8) ^a	0 (0)	4 (23.5) ^a	0.26
Liver insufficiency	0 (0) ^a	0 (0)	0 (0) ^a	1.0
Preoperative jaundice	12 (85.7) ^a	3 (33.3) ^a	9 (52.9) ^a	0.43
Renal insufficiency	0 (0) ^a	0 (0)	0 (0) ^a	1.0
Previous abdominal surgery	9 (32.1) ^a	4 (44.4) ^a	5 (26.3)	0.4
Preoperative radiotherapy	0 (0)	0 (0)	0 (0)	1.0
Preoperative chemotherapy	0 (0)	0 (0)	0 (0)	1.0
Tobacco use ^b				1.0
Ever	14 (66.7)	6 (66.7)	8 (66.7)	
Never	7 (33.3)	3 (33.3)	4 (33.3)	
Alcohol use ^b				0.63*
Current	14 (66.7)	7 (77.8)	7 (58.3)	
Abusus	4 (19.0)	1 (11.1)	3 (25)	
Never	3 (14.3)	1 (11.1)	2 (16.7)	
Body mass index (kg/m ²)	23 (22-25)	25 (23-29)	22 (21-24)	0.19**
ASA ^a				0.41*
1	1 (3.4)	0 (0)	1 (5.3)	
2	22 (75.9)	9 (90)	13 (68.4)	
3	6 (20.7)	1 (10)	5 (26.3)	
4	(0)	0 (0)	0 (0)	
Surgical margin status				1.0
R0	28 (93.3)	9 (90)	19 (95%)	
R1	2 (6.7)	1 (10)	1 (5)	

* χ^2 test. ** Mann Whitney u test. Others: Fisher exact test. IQR= interquartile range.

COPD = Chronic obstructive pulmonary disease. ASA = American Society of Anesthesiologists. 1: normal healthy patient. 2: Patient with mild systemic disease. 4: patient with severe, life-threatening systemic disease

^a1-3 missing values

^b4-9 missing values

Table 2.2 Patient demographics and comorbid conditions of patients undergoing colorectal surgery with and without experimental in vivo colonic IR

N (%) or median (IQR)	All (30)	Colonic IR (10)	No IR (20)	P-value
Sex				1.0*
Male	24 (80)	8 (80)	16 (80)	
Female	6 (20)	2 (20)	4 (20)	
Age (range)	63 (45-79)	60 (45-66)	65 (48-79)	0.29**
Charlson index score				0.21*
0-5	9 (30)	4 (40)	5 (25)	
5-10	20 (67)	5 (50)	15 (75)	
>10	1 (3)	1 (10)	0 (0)	
AJCC stage				1.0*
1	3 (10)	1 (10)	2 (10)	
2	12 (40)	4 (40)	8 (40)	
3	15 (50)	5 (50)	10 (50)	
4	0 (0)	0 (0)	0 (0)	
Coronary artery disease	3 (10)	1 (10)	2 (10)	1.0
Hypertension	11 (37)	3 (30)	8 (40)	0.71
Peripheral vascular disease	1 (3)	0 (0)	1 (5)	1.0
COPD	3 (10)	0 (0)	3 (15)	0.53
Diabetes mellitus	6 (20)	2 (20)	4 (20)	1.0
Liver insufficiency	4 (13)	1 (10)	3 (15)	1.0
Renal insufficiency	1 (3)	0 (0)	1 (5)	1.0
Previous abdominal surgery	11 (37)	6 (60)	5 (25)	0.1
Preoperative radiotherapy	29 (100)	10 (100)	19 (100)	1.0
Preoperative chemotherapy	25 (86)	8 (80)	17 (89)	0.6*
Tobacco use ^b				1.0
Ever	12 (46.1)	3 (50)	9 (45)	
Never	14 (53.8)	3 (50)	11 (55)	
Alcohol use				0.8*
Current	12 (40)	3 (60)	9 (45)	
Abusus	6 (20)	1 (20)	5 (25)	
Never	7 (23)	1 (20)	6 (30)	
Body mass index (kg/m ²)	27 (22-29)	27 (24-29)	26 (22-29)	0.66**
ASA				0.5*
1	8 (27)	3 (30)	5 (25)	
2	18 (60)	7 (70)	11 (55)	
3	3 (10)	0 (0)	3 (15)	
4	1 (3)	0 (0)	1 (5)	
Surgical margin status				1.0
R0	30 (100)	10 (100)	20 (100)	
R1	0 (0)	0 (0)	0 (0)	

* χ^2 test. ** Mann Whitney u test. Others: Fisher exact test. IQR= interquartile range.

COPD = Chronic obstructive pulmonary disease. ASA = American Society of Anesthesiologists. 1: normal healthy patient. 2: Patient with mild systemic disease. 4: patient with severe, life-threatening systemic disease

^b 4-9 missing values

The incidence and severity of postoperative complications with or without in vivo jejunal IR

The overall postoperative complication rate using the Clavien-Dindo classification following pylorus-preserving pancreaticoduodenectomy in the total population of 30 patients was 63.3% (19 out of 30 patients). 48 adverse events occurred in these 19 patients. The frequency and severity of postoperative complications were equivalent for patients undergoing pancreatic surgery with and without experimental in vivo jejunal IR (7 out of 10 jejunal IR patients (70%) vs 12 out of 20 No IR patients (60%), $P = 0.23$). As shown in Table 2.3, grade 1 complications were not statistically different for both groups (50% after jejunal IR; 20% after no IR). Also, no significant differences were found for grade 2 (40% vs 45%), grade 3a (50% vs 40%) and grade 3b (20% vs 20%). The incidence of grade 4a (30% vs 5%) and 4b (0% vs 10%) complications were found not to be significantly different between the jejunal IR group and the No IR group. The grade 5 complication rate (mortality) was 5% in the no IR group (one patient) compared to 0% in the jejunal IR group. The cause of death was sepsis with multiple organ failure following anastomotic leakage.

In both the jejunal IR and No IR groups anastomotic leakage, infection-related complications, gastrointestinal and vascular adverse events represented the five most common postoperative complications. These complication rates were similar after jejunal IR and no IR, except the rates of DGE. These rates were significantly higher after jejunal IR compared to no IR ($P = 0.03$).

The duration of hospitalization was not affected by inclusion of patients in the jejunal IR protocol or not. The median hospital stay in patients undergoing pancreatic surgery with experimental in vivo jejunal IR was 18 days (IQR 9 to 38 days) and the length of stay for patients without the experimental in vivo jejunal IR was 19 days (IQR 11 to 29 days; $P = 0.86$).

Table 2.3 Morbidity and mortality of patients undergoing pancreatic surgery with and without experimental in vivo jejunal IR

N (%) or median (IQR)	All (30)	Jejunal IR (10)	No IR (20)	P-value
<i>Primary endpoint</i>				
Patients with complications	19 (63.3)	7 (70)	12 (60)	0.7
Total complications	48	19	29	
Clavien Dindo score				0.23*
1	9 (30)	5 (50)	4 (20)	
2	13 (43.3)	4 (40)	9 (45)	
3a	13 (43.3)	5 (50)	8 (40)	
3b	6 (20)	2 (20)	4 (20)	
4a	4 (13.3)	3 (30)	1 (5)	
4b	2 (6.7)	0 (0)	2 (10)	
5	1 (3)	0 (0)	1 (5)	
<i>Secondary endpoints</i>				
Infection and wound healing				
Anastomotic leakage	9 (30)	3 (30)	6 (30)	1.0
Intestinal necrosis	0 (0)	0 (0)	0 (0)	1.0*
Pneumonia	3 (10)	3 (30)	0 (0)	0.10
Wound infection	8 (26.7)	4 (40)	4 (20)	1.0
Sepsis	6 (20)	2 (20)	4 (20)	1.0
Wound dehiscence	0 (0)	0 (0)	0 (0)	1.0
Intra-abdominal abscess	7 (23.3)	1 (10)	6 (30)	0.37
Urinary tract infection	3 (10)	1 (10)	2 (10)	1.0
Cardiovascular complications				
Central venous catheter infection	0 (0)	0 (0)	0 (0)	1.0
Intra-abdominal hemorrhage	3 (10)	1 (10)	2 (10)	1.0
Thromboembolic event	0 (0)	0 (0)	0 (0)	1.0
Arrhythmia	1 (3)	0 (0)	1 (5)	1.0
Myocardial infarction	0 (0)	0 (0)	0 (0)	1.0
Cerebral vascular accident	1 (3)	1 (10)	0 (0)	1.0
Organ failure				
Respiratory failure	1 (3)	0 (0)	1 (5)	1.0
Hepatic failure	0 (0)	0 (0)	0 (0)	1.0
Renal failure	1 (3)	0 (0)	1 (5)	1.0
Gastro-intestinal complications				
Ileus	3 (10)	2 (20)	1 (5)	0.25
DGE	5 (16.7)	4 (40)	1 (5)	0.03
Fistulas	0 (0)	0 (0)	0 (0)	1.0
Length of hospital stay	19 (10-31)	18 (9-38)	19 (11-29)	0.86**
Hospital readmission <30 days	5 (16.7)	2 (20)	3 (15)	0.58
Reoperation	6 (20)	2 (20)	4 (20)	1.0
Death < 90 days	1 (10)	0 (0)	1 (5)	1.0

* X² test. ** Mann Whitney u test. Others: Fisher exact test. IQR= interquartile range.

DGE indicates delayed gastric emptying

The incidence and severity of postoperative complications with or without in vivo colonic IR

Table 2.4 provides a summary of all postoperative complications observed in both groups in terms of type and severity according to the Clavien-Dindo classification. 18 out of the 30 patients (60%) experienced a complicated postoperative course after colorectal surgery. Overall 33 complications were found in these 18 patients. When comparing both groups, five patients (50%) in the colonic IR group and 13 patients (65%) in the No IR group had one or more postoperative complications. The difference in the incidence of complications was not statistically significant ($P = 0.44$).

Patients enrolled in the experimental colonic IR group showed no increase in complication rate in Clavien-Dindo grade 1 (30% vs 30%), 2 (10% vs 30%) and 3 (3a 10% vs 20% and 3b 20% vs 25% respectively). Grade 4a and 4b were exclusive to the no IR patient cohort. No patients died (grade 5) within a 90 days postoperative period in neither of the two groups.

When comparing the incidence of complications by diagnosis, the most common complications were anastomotic leakage, infection-related complications, and gastrointestinal adverse events.

Interestingly, although the rates of all complications were similar after colonic IR or No IR, anastomotic leakage only occurred in the No IR group but failed to reach statistical significance ($P = 0.27$).

Hospitalization period was similar for both groups: patients in the colonic IR group had a median hospital stay of 13 days (IQR 8 to 16 days) compared with a median hospital stay of 12 days (IQR 9 to 15 days) in patients in the No IR group ($P = 0.79$).

Table 2.4 Morbidity and mortality of patients undergoing colorectal surgery with and without experimental in vivo colonic IR

N (%) or median (IQR)	All (30)	Colonic IR (10)	No IR (20)	P-value
<i>Primary endpoint</i>				
Patients with complications	18 (60)	5 (50)	13 (65)	0.46
Total complications	33	7	26	
Clavien Dindo score				0.44*
1	9 (30)	3 (30)	6 (30)	
2	10 (33.3)	1 (10)	9 (30)	
3a	5 (16.7)	1 (10)	4 (20)	
3b	7 (23.3)	2 (20)	5 (25)	
4a	1 (3)	0 (0)	1 (5)	
4b	1 (0)	0 (0)	1 (5)	
5	0 (0)	0 (0)	0 (0)	
<i>Secondary endpoints</i>				
Infection and wound healing				
Anastomotic leakage	4 (13.3)	0 (0)	4 (20)	0.27
Intestinal necrosis	1 (3)	0 (0)	1 (5)	1.0
Pneumonia	3 (10)	0 (0)	3 (15)	0.53
Wound infection	6 (20)	2 (20)	4 (20)	1.0
Sepsis	1 (3)	0 (0)	1 (5)	1.0
Wound dehiscence	2 (7)	1 (10)	1 (5)	1.0
Intra-abdominal abscess	7 (23)	2 (20)	5 (25)	1.0
Urinary tract infection	6 (20)	1 (10)	5 (25)	0.64
Cardiovascular complications				
Central venous catheter infection	0 (0)	0 (0)	0 (0)	1.0
Intra-abdominal complications				
Intra-abdominal hemorrhage	0 (0)	0 (0)	0 (0)	1.0
Thromboembolic event	0 (0)	0 (0)	0 (0)	1.0
Arrhythmia	0 (0)	0 (0)	0 (0)	1.0
Myocardial infarction	0 (0)	0 (0)	0 (0)	1.0
Cerebral vascular accident	0 (0)	0 (0)	0 (0)	1.0
Organ failure				
Respiratory failure	1 (3)	0 (0)	1 (5)	1.0
Hepatic failure	0 (0)	0 (0)	0 (0)	1.0
Renal failure	0 (0)	0 (0)	0 (0)	1.0
Gastro-intestinal complications				
Ileus	5 (13)	1 (10)	4 (20)	0.64
DGE	0 (0)	0 (0)	0 (0)	1.0
Fistulas	0 (0)	0 (0)	0 (0)	1.0
Length of hospital stay	12 (9-16)	13 (8-16)	12 (9-15)	0.79**
Hospital readmission <30 days	4 (13)	2 (20)	2 (10)	0.59
Reoperation	4 (13)	0 (0)	4 (20)	0.27
Death < 90 days	0 (0)	0 (0)	0 (0)	1.0

* X² test. ** Mann Whitney U test. Others: Fisher exact test. IQR= interquartile range.

DGE indicates delayed gastric emptying

Intraoperative complications with or without in vivo jejunal IR

Possible intraoperative complications associated with the experimental IR protocol were obtained from the patient's health records and operative reports. Factors evaluated in the analyses included operative time, estimated blood loss and intraoperative hemorrhage or iatrogenic injuries.

The duration of surgery in patients subjected to the jejunal IR protocol was not significantly prolonged compared to the No IR group (288 min. IQR 228 to 395 min. vs 353 min. IQR 227 to 398 min., $P = 0.49$, Table 2.5A). Next, the jejunal IR model did not lead to significantly more blood loss ($P = 0.83$), intraoperative hemorrhage ($P=0.54$) or iatrogenic injuries.

Intraoperative complications with or without in vivo colonic IR

Table 2.5B gives a detailed overview of the surgical and peri-operative outcomes in patients undergoing colorectal surgery with and without experimental colonic IR. There were no differences between the colonic IR group and No IR group regarding duration of surgery (274 min. (IQR 251 to 329 min.) vs 228 min. (IQR 182 to 306 min.), $P=0.08$), total blood loss (500 mL (IQR 0 to 2049 mL) vs 600 mL (IQR 100 to 1758 mL), $P=0.77$), intraoperative hemorrhage (20% vs 15%, $P=1.0$) or iatrogenic injuries (10% vs 5%, $P=1.0$).

Table 2.5A Operative data of patients undergoing pancreatic surgery with and without experimental in vivo jejunal IR

N (%) or median (IQR)	All (30)	Jejunal IR (10)	No IR (20)	P-value
Duration of surgery (min)	317 (228-395) ^a	288 (239-380) ^a	353 (227-398)	0.49**
Vascular resection	4 (13)	1 (10)	3 (15)	1.0
Blood loss (mL)	550 (150-775) ^a	600 (200-2000) ^a	500 (0-700) ^a	0.83**
Intraoperative complications				
Hemorrhage	2 (7)	0 (0)	2 (10)	0.54
Iatrogenic injuries	0 (0)	0 (0)	0 (0)	1.0

* χ^2 test. ** Mann Whitney u test. Others: Fisher exact test. IQR= interquartile range.

^a1-3 missing values

Table 2.5B of patients undergoing colorectal surgery with and without experimental in vivo colonic IR

N (%) or median (IQR)	All (30)	Colonic IR (10)	No IR (20)	P-value
Duration of surgery (min)	251 (199-314) ^a	274 (251-329) ^a	228 (182-306) ^a	0.08**
Vascular resection	0 (0)	0 (0)	0 (0)	1.0
Blood loss (mL)	600 (100-1758) ^a	500 (0-2049) ^a	600 (100-1758) ^a	0.77**
Intraoperative complications				
Hemorrhage	5 (17)	2 (20)	3 (15)	1.0
Iatrogenic injuries	1 (3)	1 (10)	1 (5)	1.0

* χ^2 test. ** Mann Whitney u test. Others: Fisher exact test. IQR= interquartile range.

^a1-3 missing values

Discussion

In this study we describe the safety of human experimental models to study IR of the small intestine or colon. Intestinal ischemia is a life threatening, frequently observed event associated with persistently high mortality and morbidity rates ³. To improve patient outcome it is important to provide better insight in the pathophysiological mechanisms of human intestinal IR and to evaluate potential modalities for the early diagnosis of intestinal ischemia ^{1,4,9}.

Using both models we were able to elucidate several key processes involved in the pathophysiology of human intestinal IR. We found that the human intestine was capable to rapidly restore the epithelial lining after short periods of ischemia (≤ 30 minutes) followed by reperfusion using a “zipper-like” constriction through active contraction of non-muscle myosin fibers at the basal side of epithelial cells ^{23,24}. Prolonged periods of ischemia (≥ 45 minutes) led to permanent physical intestinal barrier loss and the occurrence of increased systemic inflammation ²⁵. The latter was linked to apoptosis of Paneth cells (immunological barrier integrity loss) caused by IR induced endoplasmic reticulum (ER) stress leading to increased bacterial translocation ²⁶. The results for colonic IR revealed that the human colon is more resistant to IR-induced damage than the human jejunum leading only to breaches in the mucus layer ²⁷. Interestingly, this was followed by rapid goblet cell compound exocytosis restoring the mucus layer and flushing out bacteria from the crypts ¹⁴.

The human intestinal IR models also let us evaluate several potential plasma biomarkers for intestinal ischemia, including I-FABP and SM22 in the search of new diagnostic tools for the detection of intestinal ischemia ^{15,28}.

During the inclusion-period no serious adverse events were reported in our patients undergoing the experimental IR protocol. However, although adverse events were accurately tracked and reported back to the medical ethical committee, no structural evaluation was yet provided of complications following our human in vivo intestinal IR. Moreover, we wanted to evaluate whether patients with IR were non-inferior compared with no IR patients regarding peri-operative complication rates. As 60 minutes of ischemia is associated with the most morphological/histological intestinal damage and increased systemic inflammation, included patients subjected to these periods were likely to have the biggest risk of complications. It is therefore that we focused on this subgroup for our safety and complication analysis.

First our cohorts of patient with jejunal or colonic in vivo intestinal IR were well matched with respect to age, sex, distribution, tumor characteristics and Charlson comorbidity index when compared to their control-groups without in vivo IR.

We observed a similar percentage of complications after jejunal in vivo IR compared to No IR regarding all complications classified by the Clavien-Dindo system.

Pancreaticoduodenectomies are complex abdominal operations with high risk of morbidity and mortality. In our study, this rate is 63.3%, which is in agreement with the current literature ^{29,30}. When analyzing the complications by diagnosis, a significantly higher rate of 40% of the patients suffering from DGE was found in the jejunal IR group. Such rates of DGE after pancreaticoduodenectomies, however, have been reported by Glowka et al. with incidences as high as 61% ³¹. Mortality was 5% in our population. This is also in concordance with rates reported in literature revealing that high-volume centers significantly improve survival rates ³². Operative time and blood loss were not different between our patients and previously reported data ^{33,34}.

The overall complications rates in our patients undergoing colorectal surgery with and without experimental in vivo colonic IR did not differ significantly between the IR and no IR group but are higher than recently published rates. These show an overall complication rate of 20% up to 51% ³⁵⁻³⁷. A potential reason for this discrepancy seems to be the meticulous documentation of all complications according to the Clavien-Dindo classification system in our analysis. In both groups most patients with a complicated postoperative course experienced only minor complications. The incidence of anastomotic leakage in our cohort is 13.3% (4/30 patients). This observed anastomotic leakage rate is similar in comparison to recent literature, reporting rates of 13.4% to 20.0% after a follow-up of more than 30 days ³⁸. The recorded amount of blood loss, other intraoperative complications and length of hospital stay parallels results of the open surgery groups from the recent COLOR and CLASICC trials ^{35,36}.

Finally, the positive surgical margin rate in both the in vivo jejunal IR group and colonic IR group did not differ when compared to their own control groups, reflecting oncologically adequate and save surgery despite being subjected to the experimental IR-model.

Our study has several limitations. These include its low sample number. Next, it is retrospective in design, indicating that postoperative complications might have been missed. Furthermore, comparability with other studies using the same Clavien-Dindo grading system for severity of postoperative complication can still be restricted since inter-observer reproducibility of the classification system might be biased. However, this study was not designed to test whether our postoperative complication rates were superior or inferior to previously reported complication rates in the literature.

In conclusion, our findings show that it is safe to study in vivo human intestinal IR by applying intestinal ischemia to six cm of isolated jejunum or colon during surgery.

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A microscopic image of intestinal tissue, showing the characteristic villi and crypts, is positioned in the top-left corner of the page. The image is in grayscale and has a slightly faded, artistic appearance.

Chapter 3

Serological markers for human intestinal ischemia: a systematic review

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Abstract

Early and accurate diagnosis of intestinal ischemia is important in order to provide rapid and correct treatment and reduce morbidity and mortality rates. Clinical signs and symptoms are often unspecific. This systemic review sums up literature regarding human plasma biomarkers for acute mesenteric ischemia reported during the last ten years. Classic, general markers, including lactate, white cell count, base excess, show poor diagnostic accuracy for intestinal ischemia. Preliminary results for ischemia-modified albumin are promising, which is also true for the inflammatory marker procalcitonin. Best diagnostic accuracy is described for D-dimer, α -Glutathione S-transferase (α -GST) and Intestinal fatty acid binding protein (I-FABP), reflecting coagulation activity and mucosal damage respectively. Future studies should be directed at phase four questions (Do patients who undergo the diagnostic test fare better (in their ultimate health outcomes) than similar patients who do not?) for these markers and the identification of additional, novel plasma biomarkers signaling various types and stages of intestinal ischemia.

Introduction

Acute mesenteric ischemia is a vascular and gastrointestinal surgical emergency that results from either arterial thromboembolic occlusion, venous thrombotic occlusion or non-occlusive causes due to mesenteric hypoperfusion in patients with low cardiac output and/or sepsis. Intestinal ischemia may also be caused by bowel strangulation due to intra-abdominal adhesions or incarcerated hernias or due to necrotizing enterocolitis ^{1,2}. Histologically, ischemic damage starts from the mucosa and extends towards the serosa. Intestinal mucosal ischemic injury is often reversible, but transmural injury often results in inflammation, necrosis, sepsis and multiple organ failure ^{1,3}.

Mortality rates of intestinal ischemia have remained unchanged the last decades, ranging from 60-80%, despite advances in imaging and treatment ^{1,2}. A critical factor contributing to the high mortality rates of intestinal ischemia is the delay in diagnosis and treatment ¹. Symptoms of intestinal ischemia are quite unspecific and diagnosis relies on a high index of suspicion. Furthermore, there is a lack of specific markers to detect intestinal ischemia ^{1,4-6}. All patients suspected to have intestinal ischemia should undergo CT-scanning of the abdomen with intravenous contrast to improve the chance of survival, as CT with intravenous contrast enhancement is the most sensitive and specific diagnostic tool available today ^{1,2}. Mesenteric arterial revascularization is done preferably before any bowel surgery. This should especially be performed when there is no sign of transmural infarction and the ischemia is potentially reversible ^{1,2}.

Therefore, early diagnosis using routine laboratory tests is of great need. An ideal plasma biomarker would be tissue specific, metabolic stable from intestine to peripheral blood, reflect actual damage and be specific for ischemia and have high sensitivity so it can rule out the disease. The aim of this study was to systemically review the literature regarding human plasma biomarkers for acute mesenteric ischemia reported during the last ten years.

Methods

Search strategy

A literature search was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement ⁷. In an attempt to identify all studies that evaluate serological markers for human intestinal ischemia three methods were used to retrieve information for this review. First, the on-line databases PubMed, MEDLINE and EMBASE for the years 2006 to 2016 were searched. This time frame was chosen as previous good quality reviews concerning plasma biomarkers for mesenteric ischemia already evaluated the literature before 2006. The search was conducted using the following terms: (((("mesenteric ischemia" [MeSH Terms] OR ("mesenteric" [All Fields] AND "ischemia" [All Fields]) OR "mesenteric ischemia" [All Fields]) OR (("intestines"[MeSH Terms] OR "intestines" [All Fields] OR "intestinal" [All Fields]) AND ("ischaemia" [All Fields] OR "ischemia" [MeSH Terms] OR "ischemia"[All Fields])))) AND ("diagnosis"[Subheading] OR "diagnosis"[All Fields] OR "diagnosis" [MeSH Terms])) AND ("biomarkers" [MeSH Terms] OR "biomarkers"[All Fields] OR "marker"[All Fields])). The second method was to examine the references of the articles found by the electronic searches methods for additional citations. The last method was to search unpublished material relevant to this topic using the Clinical Trials Search and ClinicalTrials.gov databases.

Study selection

With the use of the above-mentioned retrieval method, all English-language studies describing human subjects and published in the last 10 years were considered for this review.

One author performed searches. Any duplicates were removed. Two authors selected resulting articles independently. A first selection was made by screening the titles and abstracts of all articles. Next, full articles were read to make a final selection. Where there was no consensus, the article was discussed to make a final decision about inclusion.

Figure 3.1 A PRISMA flow diagram for selection of studies in this systematic review

Results

Search

We identified a total of 102 reports that were published between 2005 and 2016. After the initial screening, 38 candidate studies were identified. A total of 23 studies were included in the final review. Figure 3.1 shows the selection process. All included studies were about human patients suffering from acute mesenteric ischemia. Eleven investigated the already available and classical biomarkers like L-lactate, LDH and pH, two studies investigated D-lactate, four articles were about D-dimer, two investigated procalcitonin. Ischemia-modified albumin was studied by two studies, one investigated endothelial factors, one was about a-Glutathione S-transferase and four studies investigated intestinal fatty acid binding protein (Figure 3.2 and Table 3.1). Part of the studies identified was already analyzed in recent reviews or meta-analysis and are therefore not separately referenced to in this review.

Study results

Classical, general markers

L-lactate is the serum marker, which clinicians traditionally rely on if a patient presents with suspicion of intestinal ischemia. However, revision of lactate metabolism shows that many factors may lead to increased serum lactate levels and that measuring L-lactate is merely an unspecific marker of tissue hypoperfusion and does not reflect early, specific intestinal ischemia ⁸. Van der Voort et al. studied L-lactate recently showing no difference in blood levels between patients with proven ischemia and those without ischemia in 120 patients admitted to the ICU with suspected intestinal ischemia ⁹. Matsumoto et al. also reported on the diagnostic accuracy of circulating L-lactate in patients with suspected intestinal ischemia; they showed an AUC of 0.72 (95% CI 0.58-0.86) for L-lactate in 86 patients with proven intestinal ischemia out of 208 patients admitted to their emergency unit in 2009 and 2010 ¹⁰. Studer et al. showed that nor peak nor serial measurements of lactate and pH were correlated with the length of necrotic bowel ¹¹. Van der Voort showed same blood values of Lactate dehydrogenase (LDH) and Creatinine kinase (CK), both markers for muscular damage, in patients with and without intestinal ischemia ⁹. Matsumoto showed AUCs of 0.78 (95% CI 0.68-0.88) for LDH comparing patients with and without intestinal ischemia at their emergency department ¹⁰. Block et al. included 71 patients with suspected intestinal ischemia at the department of surgery in their Swedish hospital. The ten patients with proven intestinal ischemia had higher plasma levels of LDH than the patients without ischemia, however this difference was not clinical relevant since the diagnostic accuracy was low with an AUC of 0.46 (95% CI 0.34-0.58) and sensitivity of 0.70 (95% CI 0.42-0.98) and specificity of 0.42 (95% CI 0.29-0.55) ¹². Thuijls et al. studied 46 patients with suspected intestinal

Figure 3.2 The origin of proposed biomarkers ideally reflecting the different types and stage of intestinal ischemia

1, (straight arrow) in the lumen. 2, (short dotted arrow) markers for intestinal mucosal damage. 3, (thin arrow) markers for intravascular coagulation and systemic inflammation. 4, (dotted arrow) markers for transmural damage.

ischemia of whom 22 had proven ischemia and they found that the classical plasma biomarkers (lactate, leukocyte count and base excess) lacked diagnostic accuracy for intestinal ischemia since there was no difference in its blood values between patients with intestinal ischemia and those without¹³. This was in line with the data of the review of Evennett et al. in 2009¹⁴. They reported a sensitivity ranging between 1.00 and 0.33 and specificity between 0.74 and 0.53 for L-lactate. Pooled analysis for leukocyte count revealed a sensitivity of 0.80 (95% CI 0.66-0.91) and specificity of 0.50 (95% CI 0.31-0.69). Pooled analyses for base excess showed a sensitivity of 0.74 (95% CI 0.58-0.86) and specificity of 0.42 (95% CI 0.20-0.67). Matsumoto et al. reported an AUC for leukocyte count of 0.54 (95% CI 0.39-0.70) in their study of patients with and without intestinal ischemia¹⁰.

Bacterial product

D-lactate, the stereoisomer of L-lactate, is strictly produced by bacterial fermentation in the gastrointestinal tract. The presence of D-lactate in the circulation can therefore be regarded as proof of decreased mucosal integrity caused by intestinal ischemia. Van der Voort et al. studied 120 patients admitted to the ICU with suspected ischemia of

whom the 23 patients with proven ischemia had no significant difference D-lactate plasma value than patients without ischemia ⁹. Block et al. also studied plasma values of D-lactate in 2008 and they also found a low diagnostic accuracy for discriminating patients with intestinal ischemia from those without with an AUC of 0.32 (95% CI 0.22-0.43), sensitivity of 0.90 (95% CI 0.71-1.0) and specificity of 0.23 (0.12-0.34) ¹².

Coagulation

D-dimer is a fibrinolytic marker increasing and accumulating after local activation of intravascular coagulation with a half-life of approximately 6 hours. Cudnik et al. evaluated in 2013 pooled data from five studies showing D-dimer as a promising marker for intestinal ischemia. They reported a pooled sensitivity of 0.96 (95% CI 0.89-0.99) and specificity of 0.40 (95% CI 0.33-0.47) ⁶. Yang et al. included 50 consecutive patients with proven acute mesenteric venous thrombosis admitted to their intestinal stroke center between May 2010 and June 2014 and showed a good correlation with multiple organ failure, death within 30 days and short-bowel ¹⁵.

Matsumoto et al. included 208 patients suspected of intestinal ischemia at the emergency department, of whom 86 had proven ischemia. They showed that D-dimer had an AUC of 0.74 (95% CI 0.63-0.85) ¹⁰.

Yang et al. studied 274 patients undergoing surgery because of acute intestinal obstruction of whom 17 had reversible ischemia, 99 developed bowel necrosis and 158 showed no signs of ischemia. D-dimer levels had an AUC of 0.64 (95% CI 0.55-0.73) in discriminating patients with bowel necrosis from other patients operated because of bowel obstruction. An AUC of 0.77 (95% CI 0.61-0.93) was reported to distinguish the patients with reversible ischemia from those with bowel necrosis. They showed a weak correlation between D-dimer levels and length of necrotic bowel ¹⁶.

Inflammatory markers

Procalcitonin (PCT) is a 13kDa precursor of calcitonin and in pathologic conditions it is thought to be released by liver parenchyma upon stimulation by bacterial endotoxins, TNF α and IL-6. Its half-life is 24 hours. Cosse et al reviewed the articles related to diagnosis of intestinal ischemia by PCT between 1993 and 2014 and found five clinical studies with a total of 659 patients showing an AUC between 0.77 and 0.92 with a sensitivity of 0.72-1.00 and specificity of 0.68-0.91 discriminating patients with and without intestinal ischemia ¹⁷. Furthermore, in 2015 Cosse et al. reported that PCT possesses predictive values as marker for intestinal necrotic damage, the degree of extension of tissue damage and mortality in 128 patients with intestinal ischemia ¹⁸.

Marker for ischemia (IMA)

Human serum albumin has a binding site at the N-terminus for metal ions, such as cobalt. Ischemia is hypothesized to lead to alterations in this binding site making it incapable of binding metal ions, which is called ischemia-modified albumin (IMA). In 2008, two preliminary studies showed that patients with intestinal ischemia had significant higher serum IMA levels than patients without intestinal ischemia or healthy individuals ^{19,20}.

Endothelial factors

Endothelin-1 (ET-1) has been postulated as one of the endothelial factors that most contribute to organ damage associated with ischemia-reperfusion (IR) in different organs. In line, Groesdonk et al. studied 156 patients undergoing cardiac surgery with extracorporeal circulation of whom 78 developed non-occlusive mesenteric ischemia, assessed by arterial angiography. Preoperative and postoperative (day 1) ET-1 serum levels were higher in patients with NOMI compared with their matched controls with an AUC of 0.67 (95% CI 0.58-0.75) and 0.77 (95% CI 0.69-0.84) respectively ²¹. Increased ET-1 serum levels could reflect pre- and postoperative vasoconstriction.

Epithelial damage markers

Intestinal fatty acid binding protein (I-FABP) is a well-established plasma marker for early enterocyte cell death. The small (14–15 kDa) cytosolic I-FABP is specifically present in mature enterocytes of small and large intestine and is released as soon as cell membrane integrity is compromised. I-FABP is present in very small amounts in the plasma of healthy individuals, probably representing the normal turnover of enterocytes, but levels rise rapidly after episodes of acute intestinal ischemia and inflammation, including NEC. Because of its low molecular weight, I-FABP present in the systemic circulation passes through the glomerular filter (fractional renal excretion 28%; half life time 11 minutes) and can readily be detected in the plasma. A recent meta-analysis on the accuracy of circulating I-FABP for the diagnosis of human acute intestinal ischemia selected nine studies, collectively including 1246 patients, showing that the area under the summary receiver-operating curve (AUC of SROC) is 0.86 (95% CI: 0.83-0.89) with pooled sensitivity of 0.80 (95% CI: 0.72-0.86) and specificity of 0.85 (95% CI; 0.73-0.93) ²². In addition, Schellekens et al. studied the relation between plasma I-FABP and morphologic epithelial intestinal damage in their human translational ischemia-reperfusion (IR) model and showed a relation between the duration of ischemia and the increase of plasma I-FABP levels, which even is helpful to discriminate mild, reversible jejunal ischemia from more extensive, irreversible ischemia with an AUC of 0.97 (95% CI 0.89-1.03) ³.

α -Glutathione S-transferase (α -GST) is a detoxifying enzyme, which is highly active in intestinal mucosa and liver. It conjugates glutathione in response to cellular damage.

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Cudnik et al. already reviewed the diagnostic accuracy of this biomarker in the diagnosis of intestinal ischemia in 2013 and since then no new articles were found using a-GST. They showed pooled data of three studies revealing that a-GST has a pooled sensitivity of 0.68 (95% CI 0.55-0.80) and specificity of 0.85 (95% CI 0.76-0.92) ⁶.

Table 3.1 overview of reported serological markers for human intestinal ischemia

Marker	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	Remarks-Limitations
L-lactate	- 0.33-1.00	- 0.53-0.74	0.72 (0.58-0.86) [10]- [14]	Other studies show no differences between patients with and without intestinal ischemia [9, 13]. ESTES guideline also recommends not to rely on L-lactate [4].
LDH	- 0.70 (0.42-0.98)	- 0.42 (0.29-0.55)	0.78 (0.68-0.88) [10] 0.46 (0.34-0.58) [12]	Other study shows no differences between patients with and without intestinal ischemia [9].
Leukocyte count	- 0.80 (0.66-0.91)	- 0.50 (0.31-0.69)	0.54 (0.39-0.70) [10] - [14]	Other study shows no differences between patients with and without intestinal ischemia [13].
Base excess	0.74 (0.58-0.86)	0.42 (0.20-0.67)	- [14]	Other study shows no differences between patients with and without intestinal ischemia [13].
D-lactate	0.90 (0.71-1.00)	0.23 (0.12-0.34)	0.32 (0.22-0.43) [12]	Other study shows no differences between patients with and without intestinal ischemia [13].
D-dimer	0.96 (0.89-0.99) -	0.40 (0.33-0.47) -	- [6] 0.74 (0.63-0.85) [10]	Good correlation with the sequelae of intestinal ischemia [15].
Procalcitonin	0.72-1.00	0.68-0.91	0.77-0.92 [17]	Only one research group reports on this marker [16, 17].
Ischemia-modified albumin	Two pilot studies show that patients with intestinal ischemia have significant higher circulating ET-1 values than the patients without intestinal ischemia [19, 20].			
Endothelin-1	-	-	0.67 (0.58-0.75) [21]	Study in patients undergoing cardiac surgery with extracorporeal circulation developing NOMI.
I-FABP	0.80 (0.72-0.86)	0.85 (0.73-0.93)	0.86 (0.83-0.89) [22]	Meta-analysis of nine studies. Tissue specific.
a-GST	0.68 (0.55-0.80)	0.85 (0.76-0.92)	- [6]	Tissue specific.
CI: confidence interval; AUC: Area Under the curve; LDH: Lactate dehydrogenase; NOMI: non-occlusive mesenteric ischemia; I -FABP: Intestinal fatty acid binding protein; a-GST: a-Glutathione S-transferase				

Discussion

The diagnosis of intestinal ischemia is of importance in daily clinical practice for several reasons. First, intestinal ischemia is a disease entity itself mainly caused by arterial thromboembolism and venous thrombosis. Moreover, intestinal ischemia-reperfusion is also considered to be a crucial phenomenon involved in the onset of necrotizing enterocolitis, in the pathogenesis of small intestinal transplantation and rejection, and in the potential development of postoperative or posttraumatic complications, such as systemic inflammatory response syndrome, sepsis and multiple organ failure 1,2. Therefore, a broad spectrum of physicians and researchers will deal with these patients. Second, acute mesenteric ischemia is accompanied by high morbidity and mortality rates, which didn't change dramatically the last decades 1,2. Third, these high morbidity and mortality rates are partly attributed to the paucity in preventive and/or therapeutic options. Better insight into the pathophysiology of intestinal ischemia-related complications in man is imperative to create novel therapeutic strategies. Recent insights from human studies revealed some of the mechanisms underlying jejunal and colon ischemia-reperfusion 23,24. This may hopefully contribute to more specific treatment next years. Last, a delay in the diagnosis of patients with intestinal ischemia is often caused by its unspecific presentation and the lack of accurate markers 1,2.

The circulating markers for the diagnosis of intestinal ischemia are studied in different patient populations, since intestinal ischemia is such a broad phenomenon related to physiological conditions (e.g. exercise, stress) and to pathophysiological events (e.g. acute mesenteric ischemia, aortic surgery). In line, patients with different forms of intestinal ischemia are included in this review. This makes it sometimes difficult to compare the usefulness of different markers. However, this is the practice of every day in our research and clinic and it is striking that most promising markers performed best in almost all patients with various forms of intestinal ischemia.

It is noteworthy that none of the classically used markers for intestinal ischemia (lactate, LDH, CK, leukocyte count, base excess) has good diagnostic accuracy. True, this is not the first review to show this, but these markers are still used in everyday clinical practice, while we know that they are of no value in discriminating patients with intestinal ischemia from those without 8. This is also in line with the recommendations from the ESTES guidelines on acute mesenteric ischemia, which state that 'a normal serum lactate level does not exclude acute mesenteric ischemia and should not be used for diagnosis. Routine laboratory tests reflect disease progression in acute mesenteric ischemia, but should not be used for diagnostic purposes 4. Its widespread use is probably because of the availability of these measurements at most clinical chemistry departments, however we wonder how many patients that were managed erroneously by medical doctors relying on the results of these tests.

Table 3.2 Different phases of diagnostic tests²⁵

Phase	Question	How to study?
1	Do patients with the target disorder have different test results from normal individuals?	The answer requires comparison of test results among individuals with the disease and individuals that are disease-free.
2	Are patients with certain test results more likely to have the target disorder than patients with other test results?	This question is also answered by comparison of test results among patients with the target disease and healthy individuals, but now test characteristics as sensitivity, specificity and likelihood ratios are estimated.
3	Among patients in whom it is clinically sensible to suspect the target disorder, does the test result distinguish those with and without the target disorder?	To get the appropriate answer, a consecutive series of such patients should be studied.
4	Do patients who undergo the diagnostic test fare better (in their ultimate health outcomes) than similar patients who do not?	These questions have to be answered by randomising patients to undergo the test of interest or some other (or no) test.

Procalcitonin, an inflammatory marker, is a more promising test and we would encourage other researchers than Cosse et al. to study its usefulness in the diagnosis of patients with intestinal ischemia. This is also true for ischemia-modified albumin, which is only studied in two pilot experiments and deserves to be better investigated in a phase 2 diagnostic study, questioning whether patients with certain test results are more likely to have the target disorder than individuals with other test results (Table 3.2) 25. D-dimer, I-FABP and a-GST are the most promising markers for intestinal ischemia with I-FABP being studied most in depth. The advantage of I-FABP above a-GST and D-dimer is its tissue-specificity 26. High circulating D-dimer value may also result from other confounding inflammatory and infectious diseases, including other causes of acute abdominal complaints 6,15,16. a-GST may also be released by the liver, next to the intestine 27. To our surprise plasma I-FABP showed good diagnostic accuracy in distinguishing patients with and without the target disorder among patients in whom it is clinically sensible to suspect the target disorder (phase 3 question for diagnostic tests),

since theoretically elevated I-FABP plasma levels reflect intestinal mucosal damage and not specific ischemic damage 22. Moreover, a recent study showed its capability to differentiate mild, reversible jejunal ischemia from extensive, irreversible ischemia 3. This would have major therapeutic implications. Studies addressing phase 4 questions (Do patients who undergo the diagnostic test fare better (in their ultimate health outcomes) than similar patients who do not?) are needed for I-FABP. However, one should bear in mind that several aspects of the performance of the I-FABP measurement need to be addressed before multicenter study or worldwide use in clinical practice. The currently available tests has critical short-comings in that test results not are ready for at least three hours and that various I-FABP assays exists reporting different cut-off values with variable detection limits, ranging from picograms to nanograms.

How to continue? The main risk of intestinal ischemia is transmural infarction, leading to intestinal wall perforation, sepsis and death. Early detection of transmural damage is imperative because it mandates emergency surgery. Therefore, it would be interesting to find an additional biomarker reflecting transmural intestinal ischemic injury at an early stage. Evennett et al. recently reported that smooth muscle actin, measured semiquantitatively by Western blotting, was elevated in blood of rats undergoing intestinal ischemia-reperfusion and in it was detected in plasma of four neonates with severe surgical necrotizing enterocolitis, but not in neonates who were successfully conservatively managed 28. Next, an unbiased approach to identify novel host-response biomarkers for early and accurate diagnosis of intestinal ischemia could be executed by mass spectrometry-based proteomic profiling technology. This was already successfully applied in other biomarker discovery studies for different diseases 29. Last, high-quality, large-scale studies are still needed to assess the diagnostic accuracy of biomarkers for intestinal ischemia. We expect that a combination of markers, reflecting different types and stages of intestinal ischemia, is the key in future studies.

Practice points

- The most critical factor influencing clinical outcome in patients with intestinal ischemia is early, accurate diagnosis and treatment.
- Mortality rates of patients with intestinal ischemia has remained unchanged, 60-80%, the last decades, despite advanced in CT resolution and availability and vascular and endovascular intestinal revascularization.
- Classical, general markers, including lactate, leukocytes, base excess have little value in the diagnosis of acute mesenteric ischemia.
- Potential plasma biomarkers for intestinal ischemia are D-dimer, a-GST and I-FABP, reflecting activation of intravascular coagulation and intestinal mucosal damage, respectively.

Research agenda

- Creation of a rapid test for I-FABP with good diagnostic performance (low detection limit, clinical relevant cut-off).
- Study whether diagnostic accuracy of most promising biomarkers for intestinal ischemia remains high when analyzed in a phase 4 diagnostic study.
- Identify markers that provide information on different types and stages of intestinal ischemia.
- Clarify the pathophysiology of intestinal ischemia, which may lead to potentially new markers.

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Chapter 4

Plasma Intestinal Fatty Acid Binding Protein levels correlate with morphological epithelial intestinal damage in a human translational ischemia-reperfusion model

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Abstract

Background and Aim: Intestinal Fatty Acid Binding Protein (I-FABP) is a useful marker in the detection of intestinal ischemia. However, more insight into the test characteristics of I-FABP release is needed. This study aimed to investigate the relation between plasma I-FABP levels and the severity of ischemic mucosal injury, and define the clinical usefulness of systemic I-FABP following ischemia.

Methods: In a human experimental model 6 cm of jejunum, to be removed for surgical reasons, was selectively exposed to either 15, 30 or 60 minutes of ischemia (I) followed by 30 and 120 minutes of reperfusion (R). Blood and tissue was sampled at all time points. Arteriovenous (V-A) concentration differences of I-FABP were measured. Tissue-sections were stained for hematoxylin/eosin (HE) and villus height was measured to score epithelial damage.

Results: Histological analysis showed only minor reversible intestinal damage following 15I and 30I; however, severe irreversible epithelial damage was observed in jejunum exposed to 60I. I-FABP V-A differences paralleled the degree of tissue damage over time (7.79 (± 1.8) ng/mL versus 128.6 (± 44.2) ng/mL versus 463.3 (± 139.8) ng/mL, 15I, 30I and 60I, respectively). A good correlation was found between histological epithelial damage and V-A I-FABP ($r = -0.82$, $P < 0.001$). Interestingly, systemic I-FABP levels were significantly increased after 60 minutes ischemia of this short small intestinal segment.

Conclusions: This study demonstrates the relation between the duration of ischemia and the extent of tissue damage, which is reflected by I-FABP V-A plasma levels. In addition, systemic I-FABP levels appear valuable in detecting irreversible intestinal IR-damage.

Introduction

Intestinal ischemia-reperfusion (IR) is a severe clinical phenomenon accompanied by high morbidity and mortality ¹. Intestinal ischemia is a consequence of obstruction of mesenteric blood flow and of low flow states associated with major vascular or abdominal surgical procedures, hemorrhagic shock, major trauma, and sepsis ²⁻⁵. Prolonged periods of intestinal IR result in severe damage to the intestinal barrier, thereby allowing translocation of bacteria from the intestinal lumen to the systemic circulation ⁶. Therefore, late stages of prolonged intestinal IR are associated with severe systemic inflammation, which partly accounts for reported mortality rates up to 90% ^{1,2}. A major factor contributing significantly to the high IR-associated mortality rate is the delay in diagnosis of intestinal IR. This is mainly attributable to the non-specific clinical presentation and a paucity in clinically useful tests to detect intestinal IR ⁷. As a result of this diagnostic void, mortality of patients with intestinal IR has not improved over the past 70 years ¹.

As early detection of intestinal IR is essential to improve clinical outcome, we and others have studied the diagnostic value of biomarkers to detect enterocyte membrane integrity loss, an early phenomenon during intestinal ischemia ⁸⁻¹³. Intestinal Fatty Acid Binding Protein (I-FABP) is a potential marker for early enterocyte membrane integrity loss. I-FABPs are small (14-kDa) cytosolic proteins specifically present in mature enterocytes at the tip of the villus ¹⁴. They are released upon enterocyte membrane integrity loss into the circulation, which makes them useful as plasma markers for enterocyte damage ¹². Several studies showed that in patients suspected of intestinal ischemia, plasma I-FABP levels were significantly increased in those patients that eventually suffered from intestinal ischemia compared to patients with other diagnoses ^{13,15-17}.

Previous human studies revealed that plasma I-FABP increases within the early stages of intestinal ischemia ¹⁸. However, I-FABP concentrations did not allow discrimination between the stages of obvious ischemia resulting in full thickness necrosis and a temporary, mild ischemic hit only, resulting in reversible mucosal damage ¹⁶. In contrast, animal studies have demonstrated a direct correlation between serum I-FABP and the extent of histologic intestinal injury and recovery ¹⁹.

The aim of this study was first, to examine whether the duration of ischemia parallels the extent of intestinal tissue damage in a human intestinal IR model. The second objective of this study was to investigate the relation between plasma I-FABP levels and the severity of IR damage to the mucosa. The third goal of this study was to provide more insight into the clinical usefulness of systemic I-FABP after an ischemic hit, as it is of utmost importance that I-FABP levels should help to discriminate between patients with minor and severe intestinal ischemia.

Chapter 4

Using a newly developed experimental human IR model, we provide insight into the value of circulating I-FABP levels following intestinal ischemia and its association with intestinal damage during the development and progression of intestinal IR.

Materials and Methods

Ethics

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center and written informed consent of all patients was obtained. The proposed study was based on the principles of good clinical practice (GCP) and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul).

Patients and Surgical Procedures

A total of 32 patients (8F:24M) with a median age of 62 years (range 33–78y) undergoing pancreatic surgery were studied. All patients underwent a pancreaticoduodenectomy for benign or malignant disease. Twenty-seven patients were included in the experimental IR protocol, whereas 5 patients served as controls (see below). The experimental protocol was conducted as previously described²⁰. In short, during pancreaticoduodenectomy, a variable segment of the jejunum is routinely resected in continuity with the head of the pancreas and duodenum as part of the surgical procedure. The terminal 6 cm of this jejunal segment was isolated and subjected to either 15 minutes (15I, n=7), 30 minutes (30I, n=10) or 60 minutes (60I, n=10) of ischemia by placing 2 atraumatic vascular clamps over the mesentery. Meanwhile, the surgery proceeded as planned. After the period of ischemia, one third (2 cm) of the isolated ischemic jejunum was resected using a linear cutting stapler (GIA™; Covidien, Zaltbommel, the Netherlands). Next, clamps were removed to allow reperfusion, as confirmed by regaining of normal pink color and restoration of gut motility. Another segment of the isolated jejunum (2 cm) was resected similarly after 30 minutes of reperfusion (30R). The last part (2 cm) was resected after 120 minutes of reperfusion (120R). Simultaneously, 2 cm of the jejunum, which remained untreated during surgery, was resected, serving as internal control tissue. This segment underwent similar surgical handling as the isolated part of the jejunum, but was not exposed to IR. All tissue samples were immediately formalin fixed for immunohistochemistry.

To integrate I-FABP in daily practice, it is necessary that systemic circulating I-FABP levels are capable to discriminate between patients with or without intestinal ischemia and that they correlate with the grade and extent of IR damage of the intestine. As intestinal damage can also occur due to the nature of the surgery^{4,21} (eg, because of intestinal manipulation or intestinal hypoperfusion as a consequence of generalized blood loss), a control group that underwent same surgical interventions was included. The 5 patients in the control group underwent similar surgical handling while not being exposed to the experimental IR protocol.

Blood Sampling and Plasma Measurements

Arterial blood was sampled before ischemia, immediately after ischemia, and at 30 and 120 minutes after start of reperfusion. Simultaneous with each respective arterial blood sample, blood was drawn from the venule draining the isolated jejunal segment to assess concentration gradients across the isolated jejunal segment. For the control patients (ie, similar surgical procedure but not included in the experimental IR protocol), arterial blood was sampled at similar time points. All blood samples were directly transferred to prechilled EDTA vacuum tubes (BD vacutainer; Becton Dickinson Diagnostics, Breda, the Netherlands) and kept on ice. At the end of the surgical procedure all blood samples were centrifuged at 4,000 rpm, 4°C for 15 minutes to obtain plasma. Plasma was immediately stored in aliquots at -80°C until analysis.

I-FABP was determined in both arterial plasma and plasma derived from the vein directly draining the isolated jejunal segment, to allow for calculation of arteriovenous (V-A) concentration differences. Plasma I-FABP concentrations were measured by means of an in-house enzyme-linked immunosorbent assay (ELISA). This ELISA was developed to measure I-FABP in human plasma samples with rabbit polyclonal antibodies, using purified human I-FABP as standard. The assay is capable of measuring I-FABP in the range of 12.5 to 800 pg/mL. (Samples with a value of 800 pg/mL or more were diluted properly to determine the I-FABP level).

In addition, arterial samples were analyzed separately to investigate the systemic release of I-FABP following intestinal IR. Previous research showed that systemic plasma I-FABP levels correlate with a period of hypotension 30 minutes prior to sampling²¹. Therefore we measured I-FABP in the arterial sample that was obtained at the time point 30 minutes after the onset of reperfusion (i.e. 30R) for both the experimental groups and control group.

Histology

Tissue specimens were obtained at four different time points during the experimental protocol (control, after ischemia, after 30R and after 120R). Tissue samples were immediately immersed in 4% formaldehyde fixative (Unifix, Klinipath, Duiven, the Netherlands) and incubated overnight at room temperature. Next, tissue samples were embedded in paraffin, and 4 µm sections were cut. For morphologic analysis, sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water and stained with hematoxylin and eosin (HE). Villus height was measured using Lucia G (laboratory Imaging S.R.O., Prague, Czech Republic) in five representative microscopic fields (x 100) of stained control tissue and tissue exposed to ischemia with 120R.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Endogenous peroxidase activity was blocked using

0.6% hydrogen peroxide in methanol for 15 minutes. After blocking the nonspecific antibody binding with 5% bovine serum albumin in PBS, sections were incubated with in-house available polyclonal rabbit anti-human I-FABP as primary antibody for 60 minutes at room temperature. After washing, biotinylated swine anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) was used as secondary antibody and incubated for 30 minutes. This was followed by incubation with the streptavidin-biotin system (Vector laboratories, Burlingame, CA). Binding of primary antibody was visualized with 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO). Sections were counterstained with hematoxylin. No significant staining was detected in sections where primary antibody was omitted, indicating the absence of background staining. Stained sections were photographed by a Nikon eclipse E800 microscope with a Nikon digital camera DXM1200F.

Statistics

Statistical analysis was performed using Graphpad prism 5 for Windows (GraphPad Software Inc., San Diego, CA). Normality of all data was verified by Kolmogorov-Smirnov test. All data are presented as mean $\pm$ standard error (SEM). A Dunn post hoc test was used (after significant one-way ANOVA) to compare I-FABP V-A concentration differences, systemic I-FABP levels and loss of villus height at each time point in each model. For between group comparisons at similar time points a one-way ANOVA with a Dunn post hoc test was used as well. For comparison of the loss of villus height and systemic I-FABP levels between IR and controls in each model, a two-tailed Mann-Whitney U test was used. A *P* value below 0.05 was considered to be statistically significant.

Spearman rank correlation was used to correlate the loss of villus height to circulating I-FABP V-A plasma levels. These correlations were used to examine the relationship between intestinal ischemic damage and the amount of released I-FABP.

To find a potential cutoff point of systemic I-FABP levels that most accurately discriminate between patients with mild/temporary or severe intestinal ischemia (ie, 15I vs. 60I), a receiver operating characteristics (ROC) curve was drawn by plotting sensitivity against 1-specificity for all possible I-FABP thresholds. The overall accuracy of I-FABP in detecting intestinal ischemia was summarized using the area under the curve (AUC), with an AUC of 0.5 indicating no discrimination ability and an AUC of 1.0 indicating maximal discrimination ability. Ninety-five percent confidence intervals (CI) for sensitivity, specificity, positive and negative likelihood ratios (LR) were calculated for the best cut-off values, defined as the maximal sum of sensitivity and specificity.

Results

The degree of histological intestinal damage is related to the duration of the ischemic period.

Histology of the jejunum subjected to 15I showed the appearance of small subepithelial spaces as compared with the control tissue (Figure 4.1A). At 30 minutes of reperfusion, only few IR-damaged cells were pinched off into the intestinal lumen (Figure 4.1A). After 120R, no residual signs of intestinal damage were observed (Figure 4.1A), indicating full recovery.

In accordance with the previous studies from our group 22,23, histologic analysis after 30I showed the appearance of subepithelial spaces at the villus tips, whereas the epithelial lining remained intact (Figure 4.1A). At 30R, a constriction of opposing epithelial cells preventing denudation of the basal membrane was seen. At that stage, IR-damaged cells at the tips of the villi were pinched off into the lumen (Figure 4.1A). After 120R, the epithelial lining was resealed, with shedded cells still present in the debris in the lumen (Figure 4.1A). This shows that the human jejunum is capable of withstanding up to 30 minutes of ischemia without significant consequences for the intestinal epithelial lining.

After 60I, histologic evaluation of the jejunum showed disruption of the intestinal epithelial lining and appearance of vast subepithelial spaces (Figure 4.1A). During subsequent 30R, massive shedding of ischemically damaged villus tips into the lumen occurred, resulting in further disintegration of the epithelial lining (Figure 4.1A). After 120R, the epithelial lining was still disrupted, whereas marked shortening of the villi was

Figure 4.1 Damaged enterocytes are shed into the lumen during intestinal IR

A, Representative micrographs (x 100) of jejunal sections stained with hematoxylin and eosin after 15 (first row), 30 (second row) or 60 minutes (third row) of ischemia followed by 30 and 120 minutes of reperfusion. Staining shows an intact villus structure in control tissue. After 15I, the appearance of small subepithelial spaces was observed (**arrowhead**); 30I and 60I led to the appearances of large subepithelial spaces, whereas after 60I disruption of the epithelial lining was also observed (**arrowheads**). During reperfusion, damaged enterocytes were pulled together and were pinched off from the tip of the villi at 15I30R and 30I30R groups (**arrowheads**), while the epithelial lining remains intact. After 120 minutes of reperfusion the epithelial lining is restored. After 60I30R, damaged villus tips were shed into the lumen causing further disintegration of the epithelial barrier. The epithelial lining was not restored after 60I120R, with marked shortening of the villi. **B**, Mean villus height of the jejunum at control and after 120 minutes of reperfusion in patients submitted to 15, 30 and 60 minutes of ischemia. #Significant compared with control (30I). *Significant compared with control (60I). IR indicates ischemia-reperfusion; 15I, 15 minutes of ischemia; 30I, 30 minutes of ischemia; 60I, 60 minutes of ischemia.

observed (Figure 4.1A). These observations show that an ischemic period of 60 minutes in human small intestine is associated with massive enterocyte damage. Intestinal IR damage was characterized by shortening of villus height due to loss of the villus epithelium. In control HE sections, villus height was $445 (\pm 26) \mu\text{m}$, $452 (\pm 21) \mu\text{m}$ and $442 (\pm 21) \mu\text{m}$ in the 15IR, 30IR and 60IR groups, respectively (Figure 4.1B). After 15I120R, no significant loss of villus height was observed ($445 (\pm 26) \mu\text{m}$ in control vs. $435 (\pm 22) \mu\text{m}$ at 15I120R; Fig. 1B).

However, in jejunum subjected to 30I120R and 60I120R significant loss of villus height was observed compared with the control tissue (30IR: $452 (\pm 21) \mu\text{m}$ at control vs. $301 (\pm 20) \mu\text{m}$ after 120R, $P < 0.01$); 60IR: $442 (\pm 21) \mu\text{m}$ at control vs. $150 (\pm 7.0) \mu\text{m}$ after 120R, $P < 0.001$; Figure 4.1B).

Significant differences were observed between samples taken in the 15I120R group when compared to the 30I120R group and 60I120R samples. Loss of villus height was also significantly different between 30I120R compared to 60I120R (Figure 4.1B).

IFABP is a highly sensitive marker for intestinal ischemia

I-FABP release into the circulation was determined following three different periods of ischemia. In addition, the presence of I-FABP in the jejunum was investigated using

Figure 4.2 I-FABP V-A concentration differences reflect the degree of intestinal ischemic damage

A, Representative micrographs (x100) of jejunal sections stained for I-FABP in red (3-amino-9-ethylcarbazole, AEC) after 15 (first row), 30 (second row), or 60 minutes (third row) of ischemia followed by 30 and 120 minutes of reperfusion. I-FABP staining in the control tissue demonstrates the presence of I-FABP in mature enterocytes. 15I leads to the appearances of small subepithelial spaces (**arrowhead**), whereas the epithelial lining remains intact. Upon 30I, there is an abundant staining in the subepithelial space. 60I leads to disruption of the epithelial lining with appearances of large subepithelial spaces. I-FABP staining reveals leakage of I-FABP-positive intracellular components in both the subepithelial spaces (**arrowhead**) and intestinal lumen. After 15I30R, I-FABP stained sections of the jejunum show that IR-damaged cells at the tips of the villi are pinched off (**arrowheads**). Within 30I30R, damaged enterocytes are pulled together to cover the basal membrane. A decreased cytosolic staining is observed. 60I30R leads to massive shedding of damaged villus tips into the lumen. A decreased staining of I-FABP is observed in the remaining part of the villi with I-FABP-positive enterocytes present in the intraluminal debris. After 15I120R and 30I120R, I-FABP is still present in the epithelial cells and the epithelial lining appeared to be restored. After 60I120R, the epithelial lining was not restored (**arrowhead**) with marked shorting of the villi.

B, Mean I-FABP V-A concentration differences in patients submitted to 15, 30, and 60 minutes of ischemia followed by 30 and 120 minutes of reperfusion. *Significant compared with control (15I). #Significant compared with control (30I). *Significant compared with control (60I). I indicates ischemia; I-FABP, intestinal fatty acid-binding protein; R, reperfusion; V-A, arteriovenous concentration differences; 15I, 15 minutes of ischemia; 30I, 30 minutes of ischemia; 60I, 60 minutes of ischemia.

Figure 4.3 I-FABP arteriovenous concentrations differences correlate with villus height in jejunum exposed to ischemia with 120 minutes of reperfusion. (Spearman correlation: $r = -0.82$, $P < 0.001$). I-FABP indicates intestinal fatty acid-binding protein.

immunohistochemical analysis. It was apparent that I-FABP V-A levels closely paralleled the histological perturbations throughout the period of ischemia and reperfusion (Figure 4.2A, B).

In line with the data on morphology of the jejunum exposed to 15I with 0R, 30R and 120R, no differences in the intensity of the I-FABP staining were observed (Figure 4.2A). However, I-FABP V-A concentration differences significantly increased from $0.74 (\pm 0.28)$ ng/mL in controls to $7.79 (\pm 1.8)$ ng/mL directly after 15 minutes of ischemia ($P < 0.002$; Figure 4.2B). This demonstrates that even though morphological changes were minimal, and I-FABP staining remained unchanged, detectable amounts of I-FABP were released into the circulation.

Figure 4.4 Mean systemic I-FABP levels of control PPPD patients

Patients submitted to 15 minutes of ischemia, and patients undergoing 60 minutes of ischemia. Plasma I-FABP levels were not significantly different compared to each other before ischemia (ie, PreOp sample). After ischemia, no significant elevation of I-FABP was observed between 15I and the control group. Plasma I-FABP concentrations were significantly higher in patients after 60I compared with 15I as well as the control patients. *Significant compared with PreOp (60I). I indicates ischemia; I-FABP, intestinal fatty acid-binding protein; R, reperfusion; 60I, 60 minutes of ischemia.

After a 30-minute period of ischemia, I-FABP V-A concentrations were significantly elevated from 1.43 (± 0.57) ng/mL at control to 128.6 (± 44.24) ng/mL immediately after ischemia ($P < 0.001$; Figure 4.2B). During the 30 minutes period of reperfusion, I-FABP V-A concentration differences declined to 21.1 (9.38) ng/mL, which was still significantly increased ($P = 0.001$) compared with control (Figure 4.2B). At 120R, I-FABP V-A levels returned to baseline levels. Debris of apoptotic, shed epithelial cells was observed in the lumen, while the epithelial barrier seemed to be restored.

Directly after 60I, I-FABP V-A differences significantly increased from 1.27 (± 0.32) ng/mL before ischemia to 463.3 (± 139.8) ng/mL directly after ischemia ($P < 0.001$; Figure 4.2B). After 30R, I-FABP V-A differences remained significantly elevated at 119.4 (± 30.85) ng/mL (Figure 4.2B). Even at 120R, I-FABP V-A differences were 14.8 (± 5.0) ng/mL, and still significantly elevated compared with control (Figure 4.2B). These results show that I-FABP V-A differences only remain significantly enhanced when massive epithelial damage is induced (Figure 4.2B).

I-FABP V-A concentration differences are related to the extent of villus tip damage

The relation between I-FABP release and the severity of IR mucosal damage was studied by correlating I-FABP V-A concentration differences directly after ischemia with villus height at 120R. Spearman correlations showed a significant negative correlation between

Figure 4.5 Accuracy of systemic I-FABP in detecting relevant Intestinal IR-damage

Scatter plot for systemic I-FABP levels correlating with the time point after ischemia of patients with intestinal ischemia (ie, 15I and 60I) and patients who only underwent similar surgical handling (no IR). The dotted line represent the cutoff value of I-FABP (in ng/mL), as determined by Receiver Operating Characteristics (ROC) curve, that most accurately discriminate patients submitted to 60I and all other patients. IR indicates ischemia-reperfusion; I-FABP, intestinal fatty acid-binding protein.

I-FABP V-A concentrations and villus height at 120R ($r = -0.82$, $P < 0.001$; Figure 4.3). Thus I-FABP release into the plasma, a marker reflecting intestinal epithelial damage, correlates with morphologic intestinal injury as reflected by loss of villus height.

Irreversible intestinal IR damage in a short segment of the small bowel can be detected by measuring systemic plasma I-FABP levels.

To integrate measurements of I-FABP in daily practice, systemic I-FABP levels should be useful to estimate the extent of ischemia-induced enterocyte damage. Therefore, we analyzed the systemic increase of I-FABP levels in plasma of patients undergoing pancreatic surgery without being exposed to experimental intestinal ischemia versus plasma of patients with limited and reversible intestinal IR damage (15I) and extensive and irreversible intestinal damage (60I), which was induced in a small part of the jejunum (roughly 1% to 2% of small intestine).

Baseline I-FABP levels were not significantly different between the groups (preoperative sample). Fifteen minutes of ischemia did not result in a significant rise of systemic I-FABP levels ($P = 0.23$; Figure 4.4). In contrast, systemic I-FABP levels were significantly increased in patients with prolonged ischemia (60I) compared to the 15IR group and the control patients ($P < 0.001$; Figure 4.4).

These results show that systemic I-FABP-levels are capable to detect intestinal ischemia in a very short intestinal segment. These data also show that systemic I-FABP levels allow to distinguish extensive irreversible Ischemic damage from ischemia without obvious morphological damage.

In an attempt to find a possible cutoff point that permits differentiation between patients with or without irreversible intestinal IR damage, ROC curves were drawn. In our model, an I-FABP cutoff value of 1.3 ng/mL was associated with an AUC of 0.98 and should lead to an accurate discrimination between patients with mild or transient ischemia and patients with irreversible intestinal IR damage. (Figure 4.5 and Table 4.1). The sensitivity of this cut-off point was 90% with a specificity of 100% (Table 4.1).

TABLE 4.1. Ideal cutoff point to discriminate patients with 60IR out of all other patients : Sensitivity, Specificity, and overall accuracy (area under the curve, AUC) of systemic I-FABP

Cutoff Point (ng/mL)		Sensitivity (%)	Specificity (%)	AUC (95% CI)	P-value
60I	1.3	90	100	0.97 [0.89-1.03]	<0.001

AUC indicates area under the curve; CI, confidence interval; I-FABP, intestinal fatty acid-binding protein.

Discussion

The delay in diagnosis is a major factor associated with the high morbidity and mortality of patients with intestinal ischemia ². Although diagnostic accuracy of intestinal ischemia has improved with the development of computed tomography (CT) ^{24,25}, a laboratory test for clinical use is necessary ²⁶. I-FABP has emerged as a valuable marker in the diagnosis of intestinal damage at an early stage ^{12,13,15}. The current study provides additional information concerning the clinical utility of plasma I-FABP levels during intestinal IR.

First, we demonstrated the relation between the duration of ischemia and the extent of tissue damage. This was studied by inducing three different periods of ischemia in a well-defined small segment of human jejunum. The ischemic periods used, were based on previous studies from our group showing that a period of ischemia shorter than 30 minutes is associated with rapid reversal of IR-induced tissue damage and minor intestinal barrier integrity loss ^{22,23}. In contrast, periods of ischemia longer than 45 minutes are associated with both physical and immunological barrier function failure ^{27,28}, facilitating bacterial translocation, thereby triggering systemic inflammation ^{6,29}. For this reason, ischemia longer than 30 minutes leads to irreversible intestinal damage.

The second aim of the present study was to investigate the relation between plasma I-FABP levels and the degree of intestinal damage. Supported by animal studies of Gollin et al. ¹⁹, we show that, also in the human intestine, short ischemic periods are associated with considerably less I-FABP release into the circulation than long periods of ischemia. The results of this study show that the amplitude of increased arteriovenous I-FABP concentration differences significantly correlate with the extent of epithelial damage of jejunum exposed to IR in a standardized human experimental model. It is noteworthy that even in the situation of morphologic hardly noticeable intestinal ischemic damage, as observed in tissue exposed to only 15I, a tenfold increase in V-A concentration differences of I-FABP was observed. This emphasizes the high sensitive nature of I-FABP, and indicates that enterocyte membrane integrity loss alone is sufficient to cause increased plasma I-FABP levels.

The latter point raises challenges for the use of plasma I-FABP in clinical practice, as intestinal epithelial membrane integrity loss occurs regularly in patients suffering from a variety of diseases ^{4,5,8,16,30,31} and may not always require immediate (surgical) intervention. Therefore, the third goal of this study was intended to investigate the diagnostic value of systemic I-FABP levels during intestinal IR by comparing minimal IR induced damage (15I) and irreversible ischemic damage (60I). It was demonstrated that systemic I-FABP levels can differentiate between mild, reversible injury to a small amount of jejunal mucosa and more extensive, irreversible, mucosal damage. Intriguingly, 60I in only 6 centimeters length of jejunum (which comprises approximately 1-2% of the total

intestinal tract) led to significantly increased systemic I-FABP levels as compared to systemic I-FABP levels of patients without intestinal ischemia and patients submitted to 15I. In our model, systemic I-FABP levels could very well differentiate between irrelevant ischemic damage (following 15I) and clinically relevant ischemic periods (following 60I). Remarkably, the cutoff value of 1.3 ng/mL, found in our study resembles that of the cutoff point found by Vermeulen Windsant et al ¹⁷. They show that patients, developing postoperative intestinal necrosis after aortic surgery, display exceptionally high I-FABP levels at the end of surgery, which remain significantly elevated on the first postoperative day compared to all other patients. These results indicate that I-FABP is useful in the early identification and follow-up of patients suspected of intestinal ischemia. In our opinion, the latter category of patients requires follow up, as they are probably at risk of either developing inflammatory complications, or potentially intestinal damage might progress towards bowel necrosis.

Apart from these new insights into the value of I-FABP to detect ischemia in the human gut, we also obtained knowledge on the significance of dynamics in I-FABP levels. First, following a short exposure to ischemia (ie, 15I and 30I), I-FABP levels rapidly decreased over the course of a period of 120 minutes reperfusion, demonstrating rapid and full recovery of epithelial membrane integrity. In contrast, I-FABP remained significantly elevated during reperfusion of tissue exposed to 60I, even though large sheets of I-FABP containing epithelial cells had been shed into the lumen. This finding points towards a second mode of I-FABP uptake into the circulation: apart from direct leakage of I-FABP into the circulation from damaged epithelial cells, it suggests that (re)uptake of I-FABP, leaked from the damaged epithelial cells shed into the intestinal lumen, occurs through an IR-induced defective intestinal barrier (to the circulation). Declining but still elevated I-FABP levels could therefore mean that, although the ischemic cause has been resolved, intestinal barrier function loss still exists and careful follow up of the patient is required. The clinical value of these diagnostic properties has been demonstrated by Cronk et al ³². They showed that serial I-FABP levels may be very useful to help surgeons decide whether and when to operate.

The methods used in this study have several limitations that must be considered when evaluating the results. Although our methods enable us to study intestinal IR and evaluate (new) markers for the diagnosis of intestinal IR in a very controlled and save manner, the results must be translated into daily clinical settings. Therefore, additional work should be conducted to validate I-FABP in a variety of clinical settings and patients before it could be implemented in the daily practice. Second, different cutoff levels have been used in studies on I-FABP as a biomarker for intestinal ischemia ^{12,13,15,17}. This is a possible result of different I-FABP assays currently available. It is important to reach consensus at what cutoff point plasma I-FABP levels can be considered as elevated in patients with intestinal ischemia. It is necessary to address this issue first before I-FABP

can enter routine clinical practice. Finally, it is important to develop a rapid laboratory assay. The current assay used for the measurement of I-FABP takes 4 ½ hours to complete owing to the fact that it is for research purposes only, limiting the diagnostic use in the clinic.

Conclusions

This study provides new insight into the significance of plasma I-FABP following human intestinal IR. The results demonstrate that the extent of intestinal tissue damage is related to the duration of ischemia. Furthermore, the amplitude of increased V-A I-FABP concentration differences correlate with the degree of intestinal IR damage. In addition, systemic I-FABP levels can very well differentiate between mild, reversible IR damage and more extensive irreversible intestinal IR damage in only a short jejunum segment. Future studies should be conducted using I-FABP in the decision making during the diagnostic workup of patients suspected for intestinal ischemia, to integrate I-FABP in daily clinical practice.

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Chapter 5

SM22 a plasma biomarker for human transmural intestinal ischemia

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Abstract

Objective: To evaluate the diagnostic potential of smooth muscle protein of 22 kDa (SM22) as plasma biomarker for the detection of transmural intestinal ischemia.

Background: Acute mesenteric ischemia is an abdominal emergency requiring rapid diagnosis and treatment. Especially, detection of transmural damage is imperative because it mandates emergency surgery. Since early clinical and radiological signs are nonspecific, there is an urgent need for accurate biomarkers. SM22 is a potential marker for transmural damage because of its abundant expression in intestinal smooth muscles.

Methods: SM22 concentrations were measured using a newly built enzyme-linked immunosorbent assay. SM22 release was assessed in plasma and intestinal tissue of rats subjected to intestinal ischemia. Blood and tissue were sampled at baseline and following up to 24 hours of ischemia. Next, organ-specific SM22 arteriovenous concentration differences were studied in both rats and patients. Finally, plasma from patients with intestinal ischemia, other acute abdominal complaints, and healthy volunteers were tested for SM22.

Results: SM22 concentrations were significantly elevated in rats from 4 hours of ischemia onwards. Furthermore, SM22 plasma concentrations closely paralleled the histological increasing degree of intestinal smooth muscle damage. Arteriovenous calculations showed that SM22 was specifically released by the intestines and renally cleared. First data of SM22 release in man demonstrated that patients with transmural intestinal ischemia had significantly higher plasma SM22 levels than patients with only ischemic mucosal injury, other acute abdominal diseases or healthy controls.

Conclusions: This study shows that SM22 is released into the circulation upon severe ischemia of the intestinal muscle layers. Plasma levels of SM22 are potentially useful for the detection of transmural intestinal damage.

Introduction

Acute intestinal ischemia is a rare but critical abdominal emergency. It is a consequence of acute thromboembolic occlusion of the mesenteric blood supply or non-occlusive mesenteric ischemia secondary to diminished blood flow caused by intestinal strangulation, major surgery, shock, trauma and sepsis ^{1,2}. The main risk of intestinal ischemia is severe ischemia of the muscle layers, that is, transmural ischemia (TI), leading to intestinal wall perforation, sepsis and death ^{3,4}. Transmural ischemia necessitates prompt surgery to resect nonviable intestine ⁵. Early diagnosis and timely intervention are therefore key factors to reduce the high morbidity and mortality rates of 60% to 80% in patients suffering from intestinal ischemia ⁶. These rates remain as high as they were 70 years ago despite the introduction of new imaging techniques ^{3,7}. Consequently, there is a great need for a diagnostic test that timely detects transmural ischemia. Currently available biomarkers, including intestinal fatty acid binding protein (I-FABP) and α -glutathione S-transferase (α -GST), released by mature enterocytes upon intestinal ischemia, only diagnose mucosal damage ⁸⁻¹¹. Taking into consideration the relatively large proportion of smooth muscle cells in the outer layer of the gut, a potential diagnostic marker for transmural ischemia could reflect ischemic damage of the intestinal muscle layers. In this context Smooth Muscle protein of 22-kDa (SM22) is of interest. It is a small, water-soluble protein abundantly expressed by intestinal smooth muscle tissue ^{12,13}. The biological and physiological functions of SM22 remain unclear; it is hypothesized to play a role in the maturation and differentiation of smooth muscle cells ^{14,15}. The aims of this study were the following: the development and validation of a new reliable sandwich enzyme-linked immunosorbent assay (ELISA) for quantification of SM22; to investigate the release pattern of SM22 in plasma and the relation of plasma levels with the degree of intestinal damage in a rat model of intestinal ischemia; and to obtain pilot data on the diagnostic potential of SM22 plasma levels to detect severe ischemia of the muscle layers in man.

This study describes the first quantitative ELISA for SM22 and demonstrates SM22 to be a specific marker for severe ischemia of the muscle layers, leading to transmural intestinal ischemia, in both a clinically relevant rat model and in patients with acute intestinal ischemia.

Methods

Study approval

Animal experiments were approved by the Animal Ethics Committee at the Maastricht University. Clinical studies were approved by the Medical Ethics Committee of Maastricht University Medical Centre. Written informed consent was obtained from all participants, parents, or caretakers. The study was carried out according to the revised declaration of Helsinki (October 2008, Seoul).

Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories and housed under controlled conditions with unrestricted access to chow and drinking water. Rats were randomly divided into 8 groups of 6 animals that either underwent jejunal ischemia or sham-operation. Animals were anesthetized with isoflurane (induction 4%, maintenance 1.5%) and bupivacaine was used for analgesia.

Experimental rat model for occlusive intestinal ischemia

Animals were divided into groups based on the duration of intestinal ischemic injury that was used. After midline laparotomy, baseline blood samples were obtained from the inferior caval vein. Only the mesenteric blood supply (arteries and veins) to a 16-cm midjejunal segment was ligated (tied and cut) for 2, 4, 6, 8, 12 or 24 hours. During surgery, body temperature was maintained at 37° C using heat pads and rectal temperature control. To counteract fluid loss due to evaporation and lack of physical activity, rats were resuscitated with 3 mL normal saline injected subcutaneously. After ligation, the abdomen was closed and animals were allowed to recover from anesthesia. Twelve animals were sham-operated. In these groups, samples were obtained at 6 hours (n = 6) or 24 hours (n = 6) after laparotomy without intestinal ischemia.

At the end of each ischemic period, rats were again anesthetized according to the previously described protocol and the incision was re-opened. The renal vein, portal vein, hepatic vein, and inferior abdominal aorta (just above the bifurcation) were catheterized to study organ-specific SM22 release and clearance. A 25-gauge needle (BD Microlance, Becton Dickinson Medical, Breda, the Netherlands) fixed in a silicone tube (Silclear Medical Grade tubing, 0.020" x 0.037", MEDNET, Münster, Germany) was used for catheterization, and fixed with cyano-acrylate as previously described¹⁶. All catheters were filled with heparinized saline solution (20 U/mL) until blood sampling. Next, blood was collected from all catheters simultaneously and directly transferred to prechilled EDTA vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Breda, the Netherlands) and kept on ice. Samples were centrifuged at 3500 rpm at 4°C for 15 minutes to obtain plasma. Plasma was immediately stored in aliquots at -80°C until

analysis. At sacrifice, a segment of ischemic jejunum was resected, together with a proximal part of jejunum which remained untreated during surgery, serving as internal control tissue. Tissue samples were immediately snap frozen or formalin fixed for future analysis (Supplementary Figure 5.1).

Study participants

Twenty-two patients who were known to have intestinal ischemia using standard diagnostic procedures and therefore underwent emergency abdominal surgery were consecutively enrolled in this study. During routine laboratory tests an extra 2 mL of plasma was stored for further analysis. Forty patients presenting at the Emergency Department with other acute abdominal complaints were included to study, whether plasma SM22 allows differentiation between intestinal ischemia and other acute abdominal pathology. To study the renal clearance in man, arterial blood from the radial artery was collected simultaneously with blood drawn from the right renal vein by direct puncture from 10 patients undergoing major upper abdominal surgery. Fifty healthy volunteers were included in this study to obtain reference values for plasma concentrations of SM22 and I-FABP.

After inclusion, plasma was collected, transferred into multiple cryopreservation tubes and frozen at -80°C until analysis. All necessary clinical information was retrospectively collected from the electronic health record system of the participating patients, including radiological data, intraoperative findings, and histopathological examinations. To compare the studied markers with conventional markers to diagnose intestinal ischemia, plasma lactate level, white blood cell (WBC) count, and arterial pH were also retrospectively collected in the group of patients with histopathological proven intestinal ischemia.

Development of SM22 ELISA

Plasma concentrations of SM22 were measured using a newly developed in-house Sandwich ELISA. Two mouse monoclonal antibodies (mAbs), kindly provided by Dr. A. Chiavegato¹³, were used to select the best combination of mAbs against SM22 for an ELISA. These antibodies were raised in mice using purified native SM22 from porcine stomach as antigen¹⁷. A suitable antibody combination was selected by checking the immunoreactivity signal against recombinant SM22 protein (ATGen co., Ltd, Gentaur, Eersel, the Netherlands) and the assay procedure was optimized. The developed ELISA was then characterized by determination of the lower limit of detection, intra-assay and interassay coefficient of variation, dilutional linearity and recovery (see supplementary material). The ELISA was performed in 96-well microtiter plates (Greiner Bio-one, Alphen a/d Rijn, the Netherlands). The immunoreactive SM22 concentrations in samples were calculated by reference to the calibration curve. (see the supplemental data for details regarding these studies).

Plasma measurements

SM22 concentrations were measured using a newly built ELISA. Human I-FABP was measured using in-house available assay¹⁸. The ELISA was developed to measure I-FABP in plasma samples with rabbit polyclonal antibodies, using recombinant human I-FABP as standard. This assay is based on the sandwich principle with a working time of 3.5 hours. The detection range for human I-FABP is 12.5 to 800 pg/ml. Samples were assayed by trained laboratory technicians at the specialized laboratory facilities of the Department of General Surgery. The laboratory personnel were unaware of the final diagnosis concerning the patient samples or to which group the animals were allocated. Samples were run in duplicate, and a variability of 5% between sample duplicates was accepted.

Plasma lactate level, white WBC count, and arterial pH were determined as routine patient care by the clinical chemistry laboratory and were taken at the same time-point as the samples for SM22 and I-FABP measurement.

Calculations

Arteriovenous concentration differences were calculated to study the contribution of the intestines (portal vein minus artery) and the liver (hepatic vein minus (30% x artery + 70% x portal vein)) to systemic SM22 release. Renal clearance was calculated by dividing the arteriovenous concentration gradient (renal vein minus artery) by the arterial concentration (uptake/influx)¹⁹. This quotient was multiplied by the percentage of blood flowing through the kidney to calculate fractional plasma clearance. In rat, the renal blood flow per minute is approximately 25% of the total blood volume. Renal blood flow per minute in humans is to be estimated at 22%²⁰.

Intestinal tissue extracts

Snap-frozen tissue samples were homogenized in lysis buffer. Samples were centrifuged at 27,000g for 15 minutes at 4 °C. The resulting supernatants were centrifuged for a second time at 50,000g for 15 minutes. Protein concentration of the supernatants was determined using a BCA protein assay kit (Pierce). The SM22 concentration was measured using the newly developed ELISA. SM22 concentrations were expressed as nanograms SM22 per milligram total protein.

Immunohistochemistry for SM22

The sources of antibodies are listed in supplemental methods. Tissue samples were formaldehyde-fixed and cut at 4µm before blocking. Mouse anti-SM22 antibody was incubated with the sample overnight at 4°C. A biotiny conjugated rabbit anti-mouse antibody was used as the secondary.

Statistics

Analysis was performed using Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Normality was tested by Kolmogorov-Smirnov test. Results are presented as mean $\pm$ standard error of mean (SEM). Comparisons of two groups were performed by Mann-Whitney U test. For more than two groups, Kruskal-Wallis test with a Dunn post hoc test was used. Arteriovenous concentration gradients were tested versus a theoretical mean of zero using a Wilcoxon signed-rank test. $P \leq 0.05$ was considered statistically significant. (For more detailed information please see the Data Supplement).

Results

ELISA

The selected combination of mAbs was suitable for development of a sandwich ELISA to quantifying both human and rat SM22 concentrations. Figure 5.1 shows typical examples of standard curves of SM22 for this sandwich ELISA, which were prepared by plotting the absorbance of each standard solution (mean of 2 measurements) against standard of recombinant human SM22 ranging from 0 to 4 ng/mL (Figure 5.1A), a native human SM22-containing solution (diluted 1/500 to 1/3200, Figure 5.1B), or a native rat SM22-containing solution (diluted 1/100 to 1/6400, Figure 5.1C). These data show the usefulness of the ELISA for both human and rat SM22 measurements.

The basic validation features including a lower detection limit, reproducibility, dilution linearity, and recovery of this sandwich ELISA were assessed using different human plasma samples. The lower detection limit for SM22 in the ELISA was determined to be 62.5 pg/mL. The intra-assay and interassay coefficient of variation (CV%) ranged from 6.2% to 14.8% and from 4.9% to 16.3%, respectively (Supplementary Table 5.1). The assay showed dilutional linearity (Supplementary Figure 5.2) and the average recovery of the samples ranged from 93.2% to 108.6% (Supplementary Table 5.2). Similarly, the usefulness of the ELISA was investigated for rat plasma samples (data not shown).

The analytical performances of the SM22 ELISA indicate that the assay can be used for measurement of SM22 levels in both rat and human plasma samples.

Transmural ischemic damage in rats

First, the release pattern of SM22 was investigated throughout a 24-hour period in a rat model for transmural small intestinal ischemia by ligation of the mesenteric blood supply to a 16-cm jejunal segment. After 4 hours of ischemia SM22 levels were significantly increased in the systemic circulation (from 0.15 ± 0.03 ng/mL to 8.46 ± 1.76 ng/mL; $P < 0.001$; Figure 5.2A). The peak concentration was reached at 6 hours (11.20 ± 3.82 ng/mL; $P < 0.001$). Thereafter the values returned toward baseline. They were no longer significantly elevated after 12 and 24 hours of ischemia (Figure 5.2A). As expected, plasma SM22 levels remain low in the sham-operated animals throughout the entire experiment (Figure 5.2A). Next, we studied the relation between SM22 plasma release and the extent of smooth muscle damage using immunohistochemistry for SM22 in the smooth muscle layers. Damage of the longitudinal muscular layer of the intestinal wall became apparent in jejunum exposed to 6 hours ischemia compared with either control or sham (Figure 5.2C). This was marked by loss of nuclei and atrophy of the longitudinal muscle layer with pale SM22 staining, indicating that a major part of the SM22 protein had leaked out of the smooth muscle cells. As ischemia duration further increased, reduced intensity of staining revealed a further decrease in concentration or even total

Figure 5.1 Representative curves from different species of the SM22 sandwich ELISA

A, Recombinant SM22 with concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 ng/mL. **B**, Native human SM22-containing solution diluted 1/500 to 1/3200. **C**, Native rat SM22-containing solution diluted 1/100 to 1/6400. Each point represents the mean of duplicate measurements.

absence of SM22 protein in the muscular layers whereas the mucosa had disappeared completely (Figure 5.2C). Furthermore, in the late stages, lysis, separation and thinning of the circular and longitudinal layers of the muscularis externa was observed. There was no remarkable histological change throughout the experiment in sham-operated animals (Figure 5.2C). In line, the SM22 content measured by ELISA of extracts of jejunal tissue obtained after onset of ischemia was significantly diminished in jejunal tissue of rats subjected to ischemia, when compared with control at 8, 12 and 24 hours of ischemia (36.05 ± 2.56 ng/mg protein vs. 4.64 ± 1.67 ng/mg protein, 3.48 ± 0.38 ng/mg protein or 3.28 ± 0.58 ng/mg protein, respectively; $P < 0.05$) (Figure 5.2B). Importantly, SM22 plasma concentrations closely paralleled the increasing degree of intestinal transmural damage upon progression of the duration of ischemia. These data indicate SM22 to be a potential plasma marker for detection of severe ischemic intestinal damage of the muscle layers.

Source and fate of SM22 in rat and man

To investigate whether SM22 was specifically released from the intestines, arteriovenous concentration gradients of SM22 across the intestines were assessed in rat. Subtracting the arterial plasma concentration of SM22 from the concentration measured in the portal vein revealed that SM22 was specifically released from the intestine ($P < 0.05$ vs 0; Figure 5.2D). To clarify its clearance from the circulation and potential hepatic metabolism, arteriovenous concentration gradients of SM22 across the liver (hepatic vein minus (30% x artery + 70% x portal vein)) and the kidney (renal vein minus artery) were determined. We showed that SM22 was removed by the kidneys from circulation ($P < 0.05$ vs 0) without being primarily metabolized by the liver (Figure 5.2D). The renal fractional extraction rate (arteriovenous gradient/arterial concentration x 100%) was approximately 20%. Renal plasma SM22 clearance was calculated to be 5.0%/min (20% x 25%/min), resulting in a plasma half-life of approximately 14 min.

Figure 5.2 SM22 concentrations of plasma samples and jejunal extracts in rats subjected to intestinal ischemia compared with their histological perturbations

A, Plasma SM22 levels from rats undergoing intestinal ischemia or sham-operation. SM22 plasma levels becomes elevated after 4 to 8 hours of ischemia, and is significantly higher compared with $t = 0$ or sham ($*P < 0.001$). **B**, The SM22 content of jejunal extracts was diminished in rats subjected to ischemia compared to control or sham operated animals from 8 hours onwards ($*P < 0.05$). **C**, Histology revealed large subepithelial spaces at the villus tips (asterisk) with disruption of the epithelial lining (arrowhead) after 2 hours of ischemia. Complete disintegration of the villi was seen following 4 hours of ischemia. SM22 immunohistochemistry demonstrated necrosis of the longitudinal muscular layer of the intestinal wall in jejunum exposed to 6 hours ischemia (arrowhead). Next, staining showed a further decrease in intensity after 8 hours ischemia (arrowhead) or even total absence of SM22 protein in all muscular layers after 12 and 24 hours ischemia. In addition, in the late stages of lysis, separation and thinning of the circular and longitudinal layers of the muscularis externa was observed (arrowheads). There was no remarkable histological change throughout the experiment in sham-operated animals. **D**, Arteriovenous concentration gradients of SM22 across the intestines (portal vein minus artery), the liver (hepatic vein minus portal vein), and the kidney (renal vein minus artery), show that SM22 is released from the intestine and excreted renally. ($*P < 0.05$ compared with 0).

Figure 5.3 SM22 and Intestinal Fatty Acid Binding Protein (I-FABP) levels in plasma samples obtained from patients suffering from intestinal ischemia, other acute abdominal complains or healthy controls **A**, Plasma SM22 levels are only significantly elevated in patients suffering from transmural ischemic (TI) damage compared with patients with mucosal ischemic (MI) damage and healthy controls (Co) or patients with other acute abdominal complains (OAAC). **B**, Plasma I-FABP levels were significantly increased in both patients with MI and TI damage compared to Co and OAAC. No difference in I-FABP levels was apparent between MI and TI. **C**, The typical histopathology of patients with either MI or TI. The former is of a patient with only MI showing enhanced plasma I-FABP levels of 1200 pg/ml with normal plasma SM22 value. Whereas the TI patient (right panel) shows both elevated I-FABP and SM22 plasma levels. (* $P < 0.05$, *** $P < 0.01$).

To obtain data on the clearance of SM22 in man, we included ten patients undergoing major upper abdominal surgery from whom radial arterial blood was sampled simultaneously with blood drawn from the right renal vein. The fractional extraction rate of SM22 in humans was calculated to be approximately 11%. Plasma SM22 clearance was calculated to equal 2.4%/min (11% x 22%/min), leading to a plasma half-life of about 23 min. This short circulating half-life of SM22 allows detection of intestinal transmural damage to the intestinal muscular layers in patients without renal failure.

Translation to the clinical situation

Plasma SM22 levels were investigated in 22 patients diagnosed with intestinal ischemia undergoing surgery and compared with 50 healthy controls. The patients did not have renal failure. Eleven patients suffered from acute intestinal ischemia due to mesenteric occlusion or intestinal strangulation, nine patients as a complication of aortic surgery, and two as a result of sepsis accompanied by hypoperfusion. Nine of these patients were diagnosed by the pathologist with only mucosal infarction (MI), while 13 patients were diagnosed with transmural ischemia (TI). In patients with proven transmural ischemia, SM22 plasma levels were significantly higher (3.72 ± 0.91 ng/mL) compared with patients with only mucosal ischemic injury (0.45 ± 0.08 ng/mL; $P < 0.001$) or healthy controls (0.40 ± 0.07 ng/mL; $P < 0.001$) (Figure 5.3A).

Next, we measured the plasma concentrations of I-FABP, a small cytosolic protein exclusively present in mature enterocytes and rapidly released into the circulation upon damage. It is a highly sensitive marker for intestinal ischemia with levels already significantly elevated in the systemic circulation within 60 minutes of ischemia of only 1% to 2% of small intestine¹⁸. Previous research revealed that I-FABP has a short half-life of about 11 minutes²¹.

Mean plasma I-FABP levels were significantly increased in both patients with only MI and patients with TI when compared to healthy controls (699 ± 171.4 pg/mL or 952.0 ± 259.2 pg/mL vs 217.8 ± 13.6 pg/mL; $P < 0.05$, for both; Figure 5.3B). Interestingly we observed no differences in plasma I-FABP concentrations between the MI patients and TI patients ($P = 0.77$; Figure 5.3B). In addition, none of the classically used laboratory markers for intestinal ischemia (plasma lactate levels, WBC count, and arterial pH) could discriminate patients with transmural ischemia from patients with mucosal ischemic damage (lactate: 3.56 ± 0.92 mmol/L vs 4.77 ± 0.87 mmol/L; $P = 0.36$; WBC: $15.2 \pm 2.8 \times 10^9$ /L vs $20.3 \pm 4.0 \times 10^9$ /L; $P = 0.21$; pH: 7.36 ± 0.03 vs 7.28 ± 0.04 , $P = 0.16$). To further analyze the clinical potential diagnostic value of SM22 we investigated the SM22 plasma levels of 40 patients with other acute abdominal complaints (OAAC), divided into 5 diagnostic categories: acute appendicitis, diverticulitis, gynecological/urological pathology, gastroenteritis, and nonspecific abdominal pain (all $n = 8$). SM22 concentrations of all categories of OAAC patients were not significantly different from healthy controls or patients with MI (data not shown). Plasma SM22 concentrations were

0.33 ± 0.07 ng/mL in all patients with OAAC combined. In line, SM22 plasma levels of above mentioned patients suffering from TI were significantly higher when compared to patients with OAAC ($P < 0.001$; Figure 5.3A). Mean I-FABP plasma levels were 228.4 ± 28.4 pg/mL in patients with OAAC, which was significantly lower when compared to patients with intestinal ischemia ($P < 0.05$, Figure 5.3B). These data are consistent with observations from previous studies ^{8,22}.

Discussion

This study shows that SM22 is a potential plasma marker for the detection of severe ischemia of the intestinal muscle layers. There is currently no plasma marker of intestinal ischemia that might indicate transmural ischemia. Recently reported potential new biomarkers for intestinal ischemia, including I-FABP, only detect mucosal intestinal damage. Severe ischemia of the muscle layers, however, leads to intestinal perforation, sepsis, and death. It is imperative to diagnose patients suffering from this condition early, since it warrants prompt emergency surgery.

First, we set out to develop and validate a new sandwich ELISA to quantify SM22. Combinations of different mouse monoclonal antibodies were used to select the best pair of mAbs against SM22. These antibodies were raised in mice using purified native SM22 from porcine stomach as antigen. As both gene and protein sequences of SM22 are highly conserved across species ²³, the antibodies are known to specifically react with SM22 in smooth muscle tissue of different mammalian and avian origin, including rat, rabbit, guinea pig, chicken, and human ¹⁷. The quantitation range of our SM22 ELISA covers a SM22 value in the expected interval for most patients plasma. As reported in Supplementary Table 5.1, the values for interassay and intra-assay precision and accuracy are within current guidance limits of 20% for method acceptance ²⁴. Based on the analytical performance of the SM22 ELISA, we show that this assay can be used for measuring concentration levels of SM22 in both rat and human plasma.

Next, we studied the release pattern of SM22 from the intestine in plasma and the relation of plasma SM22 levels with the degree of intestinal damage in an experimental animal model of intestinal ischemia. This model of intestinal ischemia is well-characterized and results in histological damage to the intestine, including transmural damage ²⁵. We found that SM22 is rapidly released in plasma upon ischemic damage with peak concentrations at 6 hours. Thereafter, its plasma levels returned towards baseline. The latter was considered a consequence of the reduced leakage of smooth muscle cytosolic mass caused by the ischemic degradation of the intestinal muscle combined with the renal clearance of SM22 ²⁵. Therefore, we assessed the damage of intestine in the same model histologically by SM22 immunohistochemistry, and SM22 plasma clearance was studied by calculating AV-concentration gradients across the intestines, liver and kidney. Plasma SM22 marker levels agreed well with the histological findings. SM22 plasma concentrations are significantly elevated at 4 hours of intestinal ischemia in the rat compared with baseline or sham animals as is shown in Figure 5.2. Immunohistochemistry demonstrated no degradation of the muscle layers at this time point, but there is a trend to a significant decline in the concentration SM22 per mg protein of the tissue extracts of the same time point (Figure 5.2B). This shows that the transition of mucosal ischemic damage into transmural damage is around 4 hours of

ischemia, which is supported by others ²⁵. The decrease in plasma SM22 at 8, 12, and 24 hours after ischemia could well be explained by the muscle degeneration observed microscopically in corresponding specimens. We found that the content of SM22 per milligram of protein in the intestinal tissue was diminished after ligation of its blood supply, which indicated that the presence of SM22 in the circulation in this model resulted from leakage of the protein. However, active synthesis of SM22 by stressed cells, or a combination of synthesis and leakage, cannot be ruled out by these data.

Immediate SM22 release in the circulation after small intestinal ischemia shows that reperfusion is not necessary for SM22 to be released into the circulation. The results from our transorgan AV-concentration gradients show that SM22 was specifically released from the intestine in our rat model of intestinal ischemia. Furthermore, assessment of SM22 clearance in rats showed that kidneys are fully responsible for its clearance without involvement of the liver. Moreover, we reported a short half-life time of 14 minutes in rats and 23 minutes in man, which is equivalent with its molecular weight of 22kDa ²⁶. This short circulating half-life of SM22 is favorable for usage in daily clinical practice, since plasma measurements of SM22 reflect actual damage of the intestinal muscular layers without a cumulative effect. The results demonstrate the potential utility of SM22 as plasma marker of severe ischemia of the intestinal muscle layers.

Next, we translated the results to the human situation. Of the 22 patients with histologically proven intestinal ischemia, 13 had transmural ischemia and 9 suffered from mucosal damage. Increased SM22 plasma levels were specific for the patients with transmural intestinal ischemia in contrary to the intestinal mucosal damage marker I-FABP and the classical markers lactate, WBC count, and arterial pH, data which were supported by others ^{1,10,22}. The small intestinal mucosa, especially the mature enterocytes at the tips of the villi, is most vulnerable to ischemia. Transmural ischemia is therefore always associated with mucosal ischemia. As I-FABP is solely expressed by mature enterocytes, it is not surprising that there is no difference in I-FABP concentrations for mucosal and transmural ischemia. Furthermore, plasma levels of SM22 could also differentiate between patients with transmural intestinal ischemia and other causes of acute abdominal complaints, including acute appendicitis, diverticulitis, gynecological/urological pathology, gastroenteritis and nonspecific abdominal pain.

Although SM22 is never been used as a biomarker, it's family protein calponin was investigated as a potential diagnostic marker for aortic dissection ²⁷. In addition, the detection of smooth muscle proteins using the BB isoform of creatine kinase (CK-BB) and smooth muscle actine (SMA) during acute intestinal ischemic diseases was shown to be useful in both experimental animal studies and in humans ²⁸⁻³¹. Major concern of the studies using CK-BB is that CK-BB is located in all layers of the intestine since creatinin kinase isoenzymes are also present in the mucosa ²⁸. Furthermore, SMA was measured using only a semiquantitative manner, namely by Western blotting.

A potential drawback of this study is the fact that SM22 is not specific for intestinal tissue, since it is also expressed by other visceral smooth muscle tissue¹³. There are however, rarely diseases in which acute degradation of smooth muscle cells is part of the pathophysiology other than in the intestinal tract. Furthermore, the addition of plasma I-FABP measurement, which is solely expressed by enterocytes, ensures the detection of intestinal damage. This is in agreement with the findings of others^{10,22}.

It is also of clinical importance to determine whether patients suffer from focal intestinal ischemia or whether the ischemic damage is affecting the whole of the superior mesenteric artery territory. This would require another approach and determines whether the ischemia is survivable with resection. Unfortunately, to our knowledge, there is not such a marker. However we were able to find SM22 as marker to timely detect severe ischemic damage to the intestinal muscle layers, selecting which of the patients with mesenteric ischemia should undergo emergency surgery.

Future research needs to be conducted before SM22 can be applied in the clinic. First, in order to validate SM22, large prospective phase 3 to 4 clinical studies are required using patients suspected for intestinal ischemia. Phase 3 to 4 diagnostic studies answer the question whether, in patients in whom it is clinically sensible to suspect intestinal ischemia, the level of SM22 distinguish those with and without the target disorder and whether patients who undergo this diagnostic test fare better, in their ultimate health outcomes, than similar patients who do not. As it is impossible to perform a bowel resection on all patients suspected for intestinal ischemia, the endpoint for these clinical studies will be a biphasic multidetector computed tomography (MDCT) with intravenous contrast as the current gold standard for the detection of intestinal ischemia^{5,32}. Next, on the research agenda would be the creation of a rapid test for SM22, since the current ELISA takes 3.5 hours to conduct, limiting the use of SM22 in daily practice.

In conclusion, we show that assessment of plasma SM22 levels allows detection of severe ischemia of the muscle layers in patients. The combination of a marker for intestinal mucosal damage (I-FABP) and severe damage to the muscle layers (SM22) may help us in daily clinical practice to gain a more complete picture of the potential level of intestinal injury and to identify patients with severe ischemia of the intestinal muscle layers, in need of emergency surgery and patients suffering from mucosal ischemia solely, which is often reversible and in need of a different therapeutic strategy.

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Supplementary data

Methods

Study approval

Study protocols involving rats were approved by the Animal Ethics Committee at the Maastricht University and conducted in accordance with the Dutch guidelines for animal research. Human studies were approved by the Medical Ethics Committee of Maastricht University Medical Center and written informed consent was obtained from all patients, parents, or caretakers. During this study, the principles of good clinical practices were followed and carried out according to the revised declaration of Helsinki (October 2008, Seoul).

Animals

Forty-eight male Sprague-Dawley rats, weighing 300-350 g, were purchased from Charles River Laboratories (Wilmington, Massachusetts) and housed under controlled conditions of temperature and humidity with a 12:12h light-dark cycle. Throughout the experiments, rats had unrestricted access to standard rodent chow and drinking water. Rats were randomly divided in groups of 6 animals that either underwent jejunal ischemia or sham-operation. Animals were anesthetized with isoflurane (induction 4%, maintenance 1.5%) and bupivacaine was used for analgesia.

Experimental rat model for intestinal ischemia

Animals were divided into groups based on the duration of intestinal ischemic injury that was used. After midline laparotomy, baseline blood samples were obtained from the inferior cava vein. The mesenteric blood supply (arteries and veins) to a 16 cm jejunal segment was ligated, excepting collaterals, for 2, 4, 6, 8, 12 or 24 hours (h). During surgery, body temperature was maintained at 37° C using heat pads and rectal temperature control. To counteract fluid loss due to evaporation and lack of physical activity, rats were resuscitated with 3 ml normal saline injected subcutaneously. After ligation, the abdomen was then closed and animals were allowed to recover from anesthesia. 12 animals were sham-operated. In these groups, samples were obtained at 6h (N=6) or 24h (N=6) after laparotomy without intestinal ischemia respectively.

At the end of each ischemic period, rats were again anesthetized according to the previously described protocol and the incision was re-opened. The renal vein, portal vein, hepatic vein, and inferior abdominal aorta (just above the bifurcation) were catheterized to study organ-specific SM22 release and clearance. A 25-gauge needle (BD Microlance, Becton Dickinson Medical, Breda, the Netherlands) fixed in a silicone

tube (Silclear Medical Grade tubing, 0.020" x 0.037", MEDNET, Münster, Germany) was used for catheterization, and fixed with cyano-acrylate as previously described ¹. All catheters were filled with heparinized saline solution (20 U/ml) until blood sampling. Next, blood was collected from all catheters simultaneously and directly transferred to pre-chilled EDTA vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Breda, the Netherlands) and kept on ice. Samples were centrifuged at 3500 rpm at 4°C for 15 minutes to obtain plasma. Plasma was immediately stored in aliquots at -80°C until analysis. At sacrifice a segment of ischemic jejunum was resected, together with a

Supplementary Figure 5.1 Schematic representation for occlusive intestinal ischemia in anesthetized rats

After midline laparotomy, both the arterial and venous blood supply to a 16 cm midjejunal segment was ligated (tied and cut) for 2, 4, 6, 8, 12 or 24 hours. At the end of each ischemic period rats were again anesthetized and blood was drawn to study organ-specific SM22 release and clearance. At sacrifice a segment of ischemic jejunum was resected together with a proximal part of jejunum which remained untreated during surgery serving as internal control tissue.

proximal part of jejunum which remained untreated during surgery serving as internal control tissue. Tissue samples were immediately snap frozen or formalin fixed for future analysis. (Supplementary Figure 5.1)

Study participants

To study the diagnostic accuracy of SM22 in man, samples were collected from participants assigned to one of the four different patient populations all included at the emergency department (ED) or at the in-hospital ward of the Maastricht University Medical Center.

1: Intestinal ischemia

To investigate if SM22 could be detected in patients suffering from intestinal ischemia, 22 patients (11 males, 11 females; mean age 69 years, age range 46-83 years) who were known to have intestinal ischemia using standard diagnostic procedures and therefore underwent emergency abdominal surgery were consecutively enrolled in this study. During routine laboratory tests an extra 2 mL of plasma was stored for future analysis.

2: Other acute abdominal complaints (OAAC)

A group consisting of 40 patients (22 males, 18 females; mean age 44 years, age range 17-83 years) presenting at the ED with acute abdominal complaints were included to study whether plasma SM22 can differentiate between intestinal ischemia and other acute gastrointestinal pathology, including: acute appendicitis (n=8), diverticulitis (n=8), gynecological/urological pathology (n=8), gastroenteritis (n=8) and nonspecific abdominal pain (n=8).

3: Renal clearance

In addition to the organs-specific release and clearance in our animal model we studied the renal clearance in man by sampling arterial blood from the radial artery simultaneously with blood drawn from the right renal vein by direct puncture from ten patients undergoing major upper abdominal surgery (5 liver resections without Pringle maneuver, 5 pylorus preserving pancreaticoduodenectomies)

4: Healthy controls

To obtain reference values for plasma concentrations of SM22 and I-FABP, 50 healthy volunteers (25 males, 25 females; mean age 47 years, age range 12-84 years) without a previous history of gastrointestinal diseases were included in this study.

After inclusion blood samples were collected in a pre-chilled EDTA tube (BD Vacutainer, Becton Dickinson Diagnostics, Breda, the Netherlands). These samples were immediately centrifuged for 15 minutes at 3500 rpm and cooled down to 4°C. The plasma portion

was removed from each EDTA tube and transferred into multiple cryopreservation tubes and frozen at -80°C until analysis.

All necessary clinical information was collected retrospectively from the electronic health record system of all participating patients by two members of the research team, including radiological data, intraoperative findings and histopathological examinations. There were no missing data. To compare the studied markers with conventional markers to diagnose intestinal ischemia, plasma lactate level, WBC, and arterial pH were also collected in the group of patients with proven intestinal ischemia. These were determined as routine patient care by the clinical chemistry laboratory and were taken at the same time-point as the samples for SM22 and I-FABP measurement.

The final diagnosis of intestinal ischemia was confirmed by histopathology of the intestine as the gold standard. All patients with other acute abdominal complaints were successfully followed up at 24 hours and then by telephone at 3 months to determine the final diagnosis.

Development of SM22 ELISA

Plasma concentrations of SM22 were measured using a newly developed in-house Sandwich ELISA. Combinations of different mouse monoclonal antibodies (mAbs), kindly provided by dr. A. Chiavegato ², were used to select the best pair of mAbs against SM22. Antibodies were raised in mice using purified native SM22 from porcine stomach as antigen. As previous described ³, eight weeks-old Balb/c mice were injected intraperitoneally with the antigen emulsified in complete Freund's adjuvant for several times before fusion. Next, spleen cells of immunized mice were fused with mouse myeloma cell line NS-O using standard techniques ⁴. After fusion, the hybridomas were tested for the production of anti-SM22 antibodies by Western blotting and indirect immunofluorescence assays using homologous and heterologous tissues and cells.

A suitable antibody combination was selected by checking the immunoreactivity signal against recombinant SM22 protein (ATGen co., Ltd, Gentaur, Eersel, the Netherlands) and the assay procedure was optimized. The ELISA was performed in 96-well microtiter plates (Greiner Bio-one, Alphen a/d Rijn, the Netherlands). A capture mouse mAb was (3E11) coated in 1 x phosphate-buffered saline (PBS, 1.47mM KH_2PO_4 , 8.1mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 137mM NaCl, 2.68mM KCl, pH 7.4) to the plate and incubated overnight at 4°C . Remaining sites for protein binding were saturated with 150 μl /well 1% Bovine Albumin Serum (BSA) in 1x PBS and incubated at room temperature (RT) for 1h. Next, plates were washed using five times 250 μl /well of 1x PBS containing 0.1% Tween20 as washing buffer. Next, the standard of recombinant SM22 ranging from 0 to 4 ng/ml and samples were diluted in dilution buffer (1x PBS, 1% BSA) and applied to the plate in a volume of 100 μl /well and incubated for 1h at RT. Subsequently, plates were washed again for five times and 100 μl /well of a biotinylated detection antibody (1B8) was added. After incubation for 1h in the dark at RT the plate was washed again and 100 μl /well of

streptavidin poly-horseradish peroxidase (Strep-polyHRP, Pierce, Rockford, USA) in 1x PBS, 1% BSA and 0.1% Tween20 was added to the plate and incubated (1h, RT in the dark). After the final washing step, the HRP enzyme assay was initiated by adding 100µl/well of TMB sens solution (Kem-En-Tec Diagnostics, Windsor, USA). After incubating at RT for 15 min in dark, the reaction was terminated by adding 100µl/well of 1M sulfuric acid. Absorbance of each well was measured at 450 nm by a microtiter plate reader. The immunoreactive SM22 concentrations in samples were calculated by reference to the calibration curve.

The developed ELISA was then characterized by determination of the lower limit of detection, intra- and interassay coefficient of variation, dilutional linearity and recovery (see supplementary material).

Intestinal tissue extracts

Dissected jejunal segments were immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis. Tissue samples were homogenized in lysis buffer containing 200 mM NaCl, 10 mM Tris buffer, 5 mM EDTA and 10% glycerol using a Biospec mini-beadbeater (Bartlesvill, OK). Samples were centrifuged at 27,000 g for 15 minutes at 4 °C. The resulting supernatants were centrifuged at 50,000 g for 15 minutes and protein concentration of the resulting supernatants was determined using a BCA protein assay kit (Pierce, Thermo Scientific, Etten-Leur, the Netherlands). Equal amounts of total protein (5 mg) were diluted 1:300 with 1x PBS before ELISA so that their SM22 content was within the range of detection. SM22 content of extracts is expressed as nanograms SM22 per milligram total protein.

Immunohistochemistry for SM22

Tissue specimens obtained during experimental protocols were immediately immersed in 4% formaldehyde fixative and incubated overnight at room temperature. Next, tissue samples were embedded in paraffin and 4µm sections were cut.

Next, sections were deparaffinized in xylol and rehydrated in graded ethanol to distilled water. Endogenous peroxidase activity was blocked using 0.6% hydrogen peroxide in methanol for 15 min. Non-specific antibody binding was blocked with 5% BSA in PBS. Next, the slides were incubated overnight at 4°C with primary monoclonal anti-SM22 antibody (3E11). After washing, slides were incubated for one hour with biotinylated rabbit anti-mouse secondary antibody (DakoCytomation, Glostrup, Denmark) followed by incubation with the Streptavidin-Biotin System (Vector laboratories, Burlingame, CA) for 30 minutes. Binding of primary antibody was visualized with 3-amino-9-ethylcarbazole (AEC: Sigma, St. Louis, Missouri, USA). Sections were counterstained with haematoxylin. No significant staining was detected in sections where primary antibody was omitted, indicating the absence of background staining. Stained sections were

photographed by a Nikon eclipse E800 microscope with a Nikon digital camera DXM1200F at 100x magnification.

SM22 and I-FABP measurement

SM22 plasma and tissue concentrations were measured using the above-described sandwich ELISA protocol. Human I-FABP plasma concentrations were measured using in-house ELISA assay. The ELISA was developed to measure I-FABP in plasma samples with rabbit polyclonal antibodies, using recombinant human I-FABP as standard. This assay is also based on the sandwich principle with a working time of 3.5 hours. The detection range for human I-FABP was 12.5 to 800 pg/ml. Samples were assayed by trained laboratory technicians at the specialized laboratory facilities of the Department of General Surgery. The laboratory personnel were unaware of the final diagnosis concerning the patient samples or to which group the animals were allocated. Samples were run in duplicate, and a variability of 5% between sample duplicates was accepted.

Calculations

SM22 plasma concentrations were measured by means of ELISA and arteriovenous concentration differences were calculated to study the contribution of the intestines (portal vein minus artery) and the liver (hepatic vein minus (30% x artery + 70% x portal vein)) to systemic SM22 release. Renal clearance was calculated by dividing the arteriovenous concentration gradient (renal vein minus artery) by the arterial concentration (uptake/influx) ⁵. This quotient was multiplied by the percentage of blood flowing through the kidney to calculate fractional plasma clearance. In rat, the renal blood flow per minute is approximately 25% of the total blood volume ⁶, Renal blood flow per minute in humans is to be estimated at 22% ⁷.

Statistics

Statistical analysis was performed using Graphpad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Normality of all data obtained was verified by Kolmogorov-Smirnov test (all $P < 0.10$). Data are presented as mean with standard error of the mean (SEM). Means were compared between 2 groups by 2-tailed Mann-Whitney U test or among more multiple groups by Kruskal-Wallis test followed by a Dunn post hoc test. Arteriovenous concentration gradients were tested versus a theoretical mean of zero using a Wilcoxon Signed Rank Test. Differences with $P < 0.05$ were considered as statistically significant.

Validation

Lower detection limit

The lower detection limit was determined by comparing the mean + 3 standard deviation (SD) of the background absorbance and the mean absorbance – 3 SD of recombinant SM22 samples used for the calibration curve. The lowest SM22 concentration that did not overlap with the highest background value was designated to be the lower detection limit.

The absorbance values of recombinant SM22 samples at concentrations of 0, 62.5, 125 and 250 pg/ml were analyzed. Each sample was measured eight times and the mean $\pm$ 3 SD was calculated. The background absorbance of the assay was 0.188 ± 0.004 , revealing that the highest background absorbance was 0.2 (mean + 3 SD). For 62.5 pg/ml SM22 concentration, the lower absorbance represented by the mean – 3 SD was 0.218 (0.233 ± 0.005). These values did not overlap with the highest background value. The lower detection limit for SM22 in the ELISA was therefore determined to be 62.5 pg/ml.

Reproducibility

The intra-assay performance was analyzed by measuring four different human plasma samples (A, B, C, and D) in eight runs on one micro plate. The same four samples were used for the inter-assay analysis, in which each sample was analyzed in eight separate analytical runs. The mean, SD and coefficient of variation (CV%) were calculated. The coefficient of variation in the reproducibility test ranged from 6.2% to 14.8% in the intra-assay and 4.9% to 16.3% in the inter-assay tests. (Supplementary Table 5.1)

Recovery

The recovery tests were performed by measuring SM22 concentrations in two different undiluted human plasma samples (E and F) which were spiked with a known amount of recombinant SM22 in different concentrations (15, 7,50, 3.75, 1.88 and 0.94ng/ml). A division between the measured SM22 concentrations and the theoretical concentrations calculated recovery values. The average recovery of the samples was 108.6% and 93.2% (Supplementary Table 5.2).

Supplementary Table 5.1: Intra- and inter-assay precision and accuracy for SM22

Intra-assay					Inter-assay				
Sample					Sample				
Repeat	A	B	C	D	Repeat	A	B	C	D
1	4.8	2.6	0.6	0.2	1	4.2	1.9	0.5	0.2
2	4.7	2.2	0.6	0.2	2	4.4	1.6	0.4	0.2
3	4.3	2.2	0.5	0.2	3	4.1	1.7	0.4	0.1
4	4.6	2.0	0.6	0.2	4	4.6	1.8	0.5	0.2
5	4.1	2.2	0.4	0.2	5	4.5	1.9	0.5	0.2
6	4.6	2.2	0.5	0.3	6	4.7	1.9	0.5	0.2
7	4.8	2.3	0.5	0.2	7	4.3	1.9	0.4	0.2
8	4.2	2.2	0.5	0.2	8	4.2	1.6	0.4	0.2
Mean	4.5	2.2	0.5	0.2	Mean	4.4	1.8	0.5	0.2
S.D.	0.278	0.180	0.044	0.032	S.D.	0.212	0.136	0.053	0.028
%CV	6.2%	8.0%	8.5%	14.8%	%CV	4.9%	7.6%	11.9%	16.3%

To evaluate the intra-assay performance, 4 different human plasma samples (A, B, C and D) were simultaneously run 8 times on one microplate. The same samples were analyzed in eight separate analytical runs to evaluate the inter-assay variation.

CV= coefficients of variation, SD= standard deviation.

Supplementary Table 5.2: Recovery tests for determination of SM22 with the sandwich ELISA.

Sample	SM22 added (ng/mL)	Expected value (ng/mL)	Observed value (ng/mL)	Recovery (%)	Averaged recovery (%)
E	0	-	4.2		108.8
	0.94	5.1	5.8	114.1	
	1.88	6.0	6.9	115.2	
	3.75	7.9	7.9	100.2	
	7.5	11.7	13.3	113.9	
	15	19.2	19.2	100.4	
F	0.00	-	2.1		93.2
	0.94	3.0	2.8	92.3	
	1.88	4.0	3.5	88.9	
	3.75	5.8	4.9	84.9	
	7.50	9.6	9.5	99.7	
	15.00	17.1	17.1	100.2	

The recovery tests were performed by measuring SM22 concentrations in two different undiluted human samples (E and F) which were spiked with a known amount of recombinant SM22 in different concentrations. The recovery (%) was calculated using the ratio of the measured value and the theoretical value.

Dilutional linearity

Dilution tests were performed using three human plasma samples which were spiked by the addition of recombinant SM22 to the plasma. The linearity of dilution was then analyzed by testing samples using different levels of dilution. First two samples (G, H) were spiked with 8ng/mL and diluted 1:2 to 1:16. Sample I, spiked with 20ng/ml, was diluted 1:5 to 1:40 in dilution buffer to confirm linearity. A Linear dose-response curve was obtained for each of the plasma samples. The respective slopes, intercepts, linear regression correlation coefficients of the three samples were calculated. (Supplementary Figure 5.2)

Supplementary Figure 5.2

Three human plasma samples containing different SM22 concentrations were diluted 1:2 to 1:16 (G and H) or 1:5 to 1:20 (I). A Linear dose-response curve was obtained for each of the plasma samples. The respective slopes, intercepts, linear regression correlation coefficients of the three samples were; sample G: $y = 10.51 \cdot X - 0.200$, $r = 0.994$, sample H: $y = 8.348 \cdot X - 0.256$, $r = 0.998$, sample I: $y = 38.86 \cdot X - 0.05$, $r = 0.999$.

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Chapter 6

Response: "Early acute mesenteric ischemia: many rivers to cross"

Dirk H.S.M. Schellekens, Wim A. Buurman, Cornelis H.C. Dejong,

Joep P.M. Derikx

Annals of Surgery 2018

Reply

We highly appreciate the interest of Nuzzo et al¹ in our recently published paper on SM22 as a plasma biomarker for human transmural intestinal ischemia ².

First, we strongly agree with Nuzzo et al on the importance of early diagnosis of patients with acute mesenteric ischemia (AMI). This is underlined by our previous studies on potential new markers for the early diagnosis of patients suspected for intestinal ischemia, ^{3,4} which we also recently reviewed ⁵. Best diagnostic accuracy is described for circulating markers D-dimer, a-Glutathione S-transferase (a-GST) and Intestinal fatty acid binding protein (I-FABP), reflecting coagulation activity and mucosal damage, respectively. Further prospective studies need to be conducted to investigate the diagnostic value of these markers in clinical practice. However, the main risk of intestinal ischemia is severe ischemia of the muscle layers, leading to perforation, sepsis and death, ⁶ warranting prompt emergency surgery to resect non-viable intestine. This led us to evaluate the diagnostic potential of SM22 as a plasma marker for the detection of transmural intestinal ischemia. We were able to find SM22 as a marker to timely detect severe intestinal damage to the muscle layers, selecting patients in need of damage control surgery ².

Second, the specificity of increased plasma levels of SM22 for patients with intestinal ischemia needs to be addressed in future prospective human studies. We agree with Nuzzo et al¹ that elevated plasma SM22 values might also be present in patients with non-ischemic intestinal pathology. Therefore, we proposed to combine measurement of SM22 plasma levels with the intestinal mucosal derived marker I-FABP, which already showed good diagnostic accuracy for the detection of patients with intestinal ischemia ⁵. Interestingly, in our phase 1 to 2 human study SM22 was able to select which of the patients had irreversible transmural mesenteric ischemia and should undergo emergency surgery in contrary to the intestinal mucosal marker I-FABP and other classical markers such as lactate. Using both SM22 and I-FABP, it appears that it is possible to identify patients with severe ischemia of the intestinal muscle layers, which would be of great clinical benefit.

Furthermore, Nuzzo et al. question whether SM22 could differentiate between focal transmural ischemia and extensive non-transmural ischemia. The included patients with intestinal ischemia suffered from various lengths of intestinal ischemia and in these 22 patients we found elevated plasma SM22 levels only in patients with transmural ischemia and not in patients with extensive non-transmural ischemia, as is shown in Figure 5.3 ². Of course, this needs to be validated in a larger patient study.

Last, after the extensive approach to set-up a new reliable Enzyme-Linked Immunosorbent Assay (ELISA) for SM22 and to study the release pattern and clearance of SM22 in an experimental animal model, we performed a phase 1 to 2 human study

according to Sackett's 4 phases of diagnostic tests ⁷. These phase 1 to 2 studies question whether patients with certain test results are more likely to have the target disorder by determining the diagnostic accuracy among patients with the target disease and other patients and/or healthy individuals. Phase 1 to 2 studies are typically conducted among a group of patients known to have the disease and a group of people definitely known not to have it, or patients with known other diseases. Interestingly, we showed that in patients with various length of transmural intestinal ischemic injury SM22 plasma levels were significantly higher compared with patients with only mucosal ischemic injury, patients with other acute abdominal complaints (including acute appendicitis, diverticulitis, gynecological/urological pathology or gastroenteritis) and healthy controls. Follow-up studies need to be conducted before SM22 can be introduced into daily clinical practice. To validate SM22, we propose to perform a phase 3 to 4 clinical study, preferably multicenter, using patients suspected for intestinal ischemia. Phase 3 to 4 diagnostic studies answer the question whether, in patients in whom it is clinically sensible to suspect intestinal ischemia, the level of SM22 distinguishes those with and without the target disorder and whether patients who undergo this diagnostic test fare better, in their ultimate health outcomes, than similar patients who do not ⁷.

The correspondents have pointed out that the presence of three factors (serum lactate levels >2 mmol/l, organ failure, bowel loop dilation on CT) should warrant immediate surgery and absence of all three factors prefers a conservative treatment. It is noteworthy that revision of lactate metabolism shows that many factors may lead to increased serum lactate levels and that measuring lactate is merely an unspecific marker of tissue hypoperfusion and does not reflect early, specific intestinal ischemia ^{5,8}. Van der Voort et al ⁸ studied l-lactate and d-lactate showing no difference in blood levels between patients with proven ischemia and those without ischemia in 120 patients admitted to the intensive care unit with suspected intestinal ischemia. This is also in line with the recommendations from the European Society for Trauma and Emergency Surgery guidelines on acute mesenteric ischemia, which state that "a normal serum lactate level does not exclude AMI and should not be used for diagnosis."

In conclusion, we found that plasma levels of SM22 are potentially useful for the detection of transmural intestinal damage. The combination of a specific marker for early diagnosis of intestinal ischemia (I-FABP) and a marker for severe damage to the intestinal muscle layers (SM22) may help us in daily clinical practice to gain a more complete picture of the level of intestinal injury and to identify patients with transmural ischemia, in need of emergency surgery. However, future research needs to be conducted before SM22 can be applied in the clinic.

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A microscopic image of intestinal tissue, showing the characteristic villi and crypts, is positioned in the top-left corner of the page.

Chapter 7

Human small intestine is capable of restoring barrier function after short ischemic periods

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Abstract

Aim: To assess intestinal barrier function during human intestinal ischemia and reperfusion (IR)

Methods: In a human experimental model, 6 cm of jejunum was selectively exposed to 30 minutes of ischemia (I) followed by 30 and 120 minutes of reperfusion (R). A sham procedure was also performed. Blood and tissue was sampled at all-time points. Functional barrier function was assessed using dual-sugar absorption tests with lactulose (L) and rhamnose (R). Plasma concentrations of citrulline, an amino acid described as marker for enterocyte function were measured as marker of metabolic enterocytes restoration. Damage to the epithelial lining was assessed by immunohistochemistry for tight junctions (TJs), by plasma marker for enterocytes damage (I-FABP) and analyzed by electron microscopy (EM) using lanthanum nitrate as an electrondense marker.

Results: Plasma L/R ratio's were significantly increased after 30 minutes of ischemia (30I) followed by 30 minutes of reperfusion (30R) compared to control (0.75 ± 0.10 vs 0.20 ± 0.09 , $P < 0.05$). At 120 minutes of reperfusion (120R), ratio's normalized (0.17 ± 0.06) and were not significantly different from control. Plasma levels of I-FABP correlated with plasma L/R ratios measured at the same time points (correlation: 0.467, $P < 0.01$). TJs staining shows distortion of staining at 30I. An intact lining of TJs was again observed at 30I120R. Electron microscopy analysis revealed disrupted TJs after 30I with paracellular leakage of lanthanum nitrate, which restored after 30I120R. Furthermore, citrulline concentrations closely paralleled the histological perturbations during intestinal IR.

Conclusion: This study directly correlates histological data with intestinal permeability tests, revealing that the human gut has the ability of to withstand short episodes of ischemia, with morphological and functional recovery of the intestinal barrier within 120 minutes of reperfusion.

Introduction

The intestinal epithelial lining is one of the largest sites of the human body exposed to the external environment and holds a dual function: the absorption of nutrients and water from the lumen and at the same time providing an effective barrier against potentially harmful compounds such as bacteria, toxins and antigens ^{1,2}.

This is achieved by various lines of defense, including a physical barrier, formed by a mucus layer and a single rigid layer of enterocytes which are interconnected by tight junctions (TJs) and the subjacent adherens junctions (AJs) ³. The TJs act as a fence, regulating the passive diffusion of solutes across the paracellular pathway ⁴. They are composed of transmembrane proteins such as occludin and members of the claudin family, which are anchored to the cell cytoskeleton by zonula occludens proteins (ZO-1, ZO-2 and ZO-3) ⁵⁻⁸. AJs are important for connecting neighbouring cells and consist, amongst others, of the transmembrane protein E-cadherin. Formation of AJs is required prior to the assembly of the TJs ⁹.

Loss of intestinal barrier function is considered to play a key role in the development and/or progression of intestinal inflammation and the onset of systemic inflammatory response syndrome, and is associated with intestinal diseases including celiac disease, inflammatory bowel disease (IBD) and intestinal ischemia-reperfusion (IR) ^{1,10}. Intestinal IR is a frequently occurring phenomenon associated with high morbidity and mortality. It is caused by various pathological conditions that involve a critical reduction of blood flow to the intestine, such as acute obstruction of mesenteric arterial blood flow and hypoperfusion associated with major vascular or abdominal surgical procedures, hemorrhagic shock, major trauma and sepsis ¹¹⁻¹⁵.

Previous research of our group revealed the striking ability of the human small intestine to reduce and restore IR-induced epithelial lining damage. Using an experimental human intestinal IR model ¹⁶, it was observed that after periods of ischemia of up to 30 minutes, villous sloughing with epithelial lining disruption was rapidly followed by complete histological recovery during reperfusion ^{17,18}. The important remaining question however is whether functional barrier restoration with closure of paracellular permeability is achieved within this time span. This is in particular important as few human experimental studies exist that directly correlate barrier function to histological appearance. Here, we set out to study the relation between histological and functional barrier recovery using a human experimental small intestinal IR model. Damage to the epithelial lining was assessed by immunohistochemistry, plasma measurement of Intestinal Fatty Acid Binding Protein (I-FABP) and electron microscopy (EM). Functional intestinal barrier loss was evaluated using lanthanum nitrate as an electrondense marker together with the currently used gold standard for the measurement of intestinal permeability in clinical practice, the dual-sugar absorption tests (DST) ¹⁹. These tests rely on the differential

intestinal paracellular and cellular permeability of larger (lactulose) and smaller (L-rhamnose) molecules. Simultaneous measurement of lactulose and L-rhamnose are used as controls for gastric emptying, intestinal fluid volume, gastrointestinal transit time, and renal excretion which are thought to affect each molecule equally. The ratio of plasma concentrations reflects small intestinal permeability. Furthermore, plasma concentrations of citrulline, an amino acid produced by enterocytes that has been described as a marker for enterocyte mass/function ²⁰, were measured as a marker of functional, metabolic enterocyte restoration following 30 minutes of ischemia.

Materials and Methods

Ethics

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center (METC 06-3-044) and was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent of all patients was obtained.

Patients and Surgical Procedures

Patients

23 patients undergoing pancreatoduodenectomy for benign or malignant disease were included in this study. DST data were obtained from 13 patients (10F:3M) with a median age of 69 (range 48 to 85) years included in the experimental human intestinal IR model of these 3 patients underwent a sham procedure; their intestinal barrier function was assessed using DST as described below without being exposed to ischemia. DST data were compared to histology from 10 other patients (3F:7M), with a median age of 63 (range 47 to 78) years undergoing the same human intestinal IR model without applying the DST.

Human intestinal IR protocol

The experimental protocol was performed as previously described 16. In short, during pancreatoduodenectomy, a variable length of jejunum is routinely resected as part of the surgical procedure. The terminal 6 cm of this jejunal segment was isolated and subjected to 30 minutes of ischemia by placing two atraumatic vascular clamps across the mesentery (n = 20 of which 10 patients were included in the DST-protocol (see below)). Meanwhile surgery proceeded as planned. After ischemia, one third (2cm) of the isolated ischemic jejunum was resected using a linear cutting stapler (30I) (GIA™, Covidien, Mansfield, MA). Next, clamps were removed to allow reperfusion, as confirmed by regaining of normal pink color and restoration of gut motility. Another segment of the isolated jejunum (2 cm) was resected similarly after 30 minutes of reperfusion (30I30R) and 120 minutes of reperfusion (30I120R). Simultaneously, 2 cm of jejunum which remained untreated during surgery was resected and served as internal control tissue. Tissue samples were immediately snap frozen or formalin fixed for future analysis.

DST protocol

To study the consequences of IR on intestinal barrier function loss and recovery, a bolus of 10ml 0.9% NaCl containing the saccharides lactulose (1 mg/ml Lactulose, Centrafarm B.V. Etten-Leur, The Netherlands) and L-Rhamnose (0,5 mg/ml, Danisco Sweeteners, Copenhagen, Denmark) was directly injected into the lumen of the isolated part of

intestine of 10 patients before the induction of ischemia. The piece of jejunum with the injection site was stapled off, to prevent any possible leakage of intraluminal content towards the abdominal cavity. After the first blood sample was obtained (5 minutes after injecting the saccharides), the human IR protocol was initiated (see detailed protocol above). The only difference with the regular IR protocol was that no tissue was resected during IR to eliminate potential confounding effects of absorptive surface reduction and decrease in luminal presence of the saccharide solution on the outcome parameters. Three patients underwent a sham procedure during which the saccharide solution was injected in the isolated jejunum segment, without this being exposed to IR. Blood from all patients was drawn, centrifuged, aliquoted and stored according to the regular intestinal IR protocol (as mentioned below) until further analysis for plasma saccharide concentrations. In addition, luminal debris from the isolated segment was sampled at the end of the IR protocol to measure the remaining saccharide concentration and compare this to the concentration at the beginning of the experiment.

Blood Sampling

Arterial blood was sampled before ischemia, immediately on reperfusion (30I), and at 30 (30I30R) and 120 minutes (30I120R) after start of reperfusion. Simultaneous with each respective arterial blood sample, blood was drawn from the venule draining the isolated jejunal segment by direct puncture to assess concentration gradients across the isolated jejunal segment. All blood samples were centrifuged at 3500 rpm, 4°C for 15 minutes to obtain plasma. Plasma was immediately stored in aliquots at -80°C until analysis.

Measurement of intestinal barrier function loss

Arterial and venous plasma concentrations of lactulose and L-rhamnose were measured using High Performance Liquid Chromatography combined with Mass Spectrometry (HPLC-MS). In brief, sugars were separated using ion-exchange chromatography as described previously ²¹. After separation, the column effluent was mixed with an ammonia containing solvent, which enabled the electrospray ionisation to ammonia adducts. Detection, based on the mass to charge value (m/z value) of the ammonia adducts was then performed using mass spectrometry in positive mode. Arterial-venous concentrations differences and lactulose/L-rhamnose ratios (L/R-ratio) were calculated to investigate intestinal barrier function during IR.

Morphological assessment

Histology

Tissue specimens obtained during the experimental protocol were immediately immersed in 4% formaldehyde fixative (Unifix, Klinipath, Duiven, the Netherlands) and incubated overnight at room temperature. Next, tissue samples were embedded in paraffin and 4µm sections were cut. Sections were deparaffinized in xylene and

rehydrated in graded ethanol to distilled water and stained with haematoxylin and eosin (H&E).

Immunofluorescence

Cryostat sections (4µm) were cut and stained for ZO-1, occludin and E-cadherin. Briefly, slides were dried, fixed in 10% trichloroacetic acid for 15 minutes and permeabilized using 30mM glycine and 1% triton X-100 in phosphate buffered saline (PBS). Non-specific antibody binding was blocked using 30mM glycine and 3% Fetal Calf Serum in PBS for one hour. Next, slides were incubated with mouse anti-human ZO-1, rabbit anti human occludin (both Invitrogen, Eugene, OR) or mouse anti human E-cadherin (Abcam, Cambridge, UK). After washing, slides were incubated with goat anti-mouse Alexa 488, goat anti-rabbit CY3, or goat anti-mouse CY3 (all Invitrogen) secondary antibodies, followed by incubation with 4',6-diamino-2-phenylindole dihydrochloride (DAPI). Next, slides were washed and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized with an immunofluorescence microscope.

Electron microscopy

For EM, jejunal tissue was directly immersed in 2.5% glutaraldehyde and 2% paraformaldehyde fixative buffered in 0.1M cacodylate buffer at a pH of 7.4 for at least 3 days. Samples were then washed overnight in 0.1M cacodylate buffer with 7.5% sucrose and postfixed for 1 h at 4°C in 1% osmium tetroxide, containing 1% lanthanum nitrate buffered to pH 7.4 with 0.1M cacodylate. After washing in cacodylate buffer containing 7% sucrose at pH 7.4, dehydration was carried out rapidly in graded ethanol series followed by routine embedding in Epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate. A Philips CM 100 electron microscope was used to visualize the ultrastructure of the intestinal epithelial lining and the distribution of lanthanum nitrate particles. The presence of lanthanum nitrate inside the paracellular space of two adjacent enterocytes is associated with TJ and AJ function loss.

Measurement of intestinal mucosal cell damage

Damage to the epithelial lining was quantified using plasma levels of Intestinal fatty acid-binding protein (I-FABP). I-FABPs are small (14 kDa) cytosolic proteins specifically present in mature enterocytes at the tip of the villus²². They are released upon enterocyte membrane integrity loss into the circulation, which makes them useful as markers for enterocyte damage²³. I-FABP-levels were measured in both arterial and venous plasma samples, to allow for calculation of arteriovenous (V-A) concentration differences, by means of an in-house enzyme-linked immunosorbent assay (ELISA). This assay was developed to measure I-FABP in human plasma samples with rabbit polyclonal antibodies, using purified human I-FABP as standard (detection window: 12.5 to 800

pg/mL). Samples were run in duplicate, and a variability of 5% between sample duplicates was accepted.

Assessment of circulating amino acids

To study the metabolic activity of enterocytes following 30 minutes of ischemia and subsequent reperfusion, arterial and venous plasma concentrations of citrulline and glutamine were measured using HPLC (Model PU-1980 pump, Jasco Easton, MD) combined with MS (Model LTQ XL, Thermo Fisher Scientific, Waltham, MA) as previously described ²⁴. A 100 μ L plasma aliquot was pipetted into a 1.5 mL Eppendorf tube that already contained 5.5 mg solid sulfosalicylic acid (SSA), vortex-mixed immediately to deproteinize the plasma samples, snap frozen in liquid nitrogen and stored at -80°C until analysis.

Before analysis, deproteinized plasma samples were thawed, vortex-mixed and centrifuged for 10 min at 50,000 $\times$ g at 4°C in a Biofuge Stratos centrifuge (Heraeus, Haarlem, the Netherlands). Next, 5 μ L of the clear supernatant was diluted 100-fold in ice-cold water into a 1 mL WISP-style vial (Waters, Etten-Leur, the Netherlands). Amino acid analysis was performed by HPLC after automated pre-column derivatization using ophthalaldehyde (OPA). At the start of each cycle, 5 μ L of the diluted sample, stored in the pre-chilled sample compartment of a WISP autosampler, was automatically mixed with 5 μ L of OPA reagent. The resulting OPA-amino acid derivatives were separated on a 150 $\times$ 4.6 mm (inner diameter) Allsphere ODS 2 3 μ M High-Performance Liquid Chromatography (HPLC) column (Grace, Breda, the Netherlands), using an acetonitrile gradient against an aqueous citric acid buffer (25 mmol/L, pH=6.8) and detected by fluorescence (330 nm excitation, 440 nm emission). Arterial-venous concentration differences of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln A) reflect enterocyte functional and metabolic capacity.

Statistics

Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc. San Diego, CA) and SPSS 23.0 (SPSS, Inc., Chicago, IL). Normality was verified by using the Kolmogorov-Smirnov test. None of the parameters showed a normal distribution. A Dunn multiple comparison test was used (after significant one-way analysis of variance) to compare DST values and amino acid ratios over time. For between-group comparisons, the Mann Whitney U test was used. All data are expressed as mean $\pm$ standard error (SEM).

Linear mixed model regression was used to analyze correlations over time between plasma I-FABP and plasma L/R ratio. Within-person correlations were computed by normalizing both data sets. This enables the assessment of the pure association of both variables by calculating the correlation coefficient. Linear regression was used to visualize the correlation. A *p*-value below 0.05 was considered statistically significant.

Results

Thirty minutes of ischemia only moderately affects intestinal barrier function.

As described previously, the epithelial lining was microscopically intact directly after 30 minutes ischemia (30I) (Figure 7.1A, right panel) 18. Immunofluorescence staining of ZO-1, occludin and E-cadherin revealed structured ZO-1 and occludin staining at the apical pole of the epithelial cells and evenly distributed E-cadherin between the epithelial cells over the tips of the villi in both control tissue and jejunum after an ischemic period of 30 minutes (Figure 7.1A). To assess whether the ischemia-induced enterocyte damage resulted in increased intestinal permeability, the plasma Lactulose/L-rhamnose (L/R) ratio was determined. No statistically significant changes were observed in the plasma L/R ratio directly after 30I compared to control (0.16 ± 0.05 vs 0.20 ± 0.09 , $p = 0.80$; Figure 7.1C) or sham-operated patients (0.10 ± 0.07 vs 0.20 ± 0.09 , $p = 0.72$; Supplementary Figure 7.1). These data suggest that a short period of ischemia alone does not result in increased intestinal permeability. EM images of two adjacent enterocytes with microvilli showed that in control tissue the interconnecting TJs were intact (Figure 7.1B left panel, arrowhead) and the contrast dye lanthanum remains intraluminally. However, in contrast to the histological analysis, as shown by EM after 30I, lanthanum was able to penetrate the paracellular space, indicating intestinal barrier integrity loss (Figure 7.1B, right panel, arrowheads).

Enterocyte damage was quantified by measuring arteriovenous I-FABP concentration differences across the studied jejunum. I-FABP arteriovenous concentration differences significantly increased from $1.75 \text{ ng/mL} \pm 0.78 \text{ ng/mL}$ before ischemia to $126.6 \text{ ng/mL} \pm 65.53 \text{ ng/mL}$ on reperfusion ($P < 0.01$; Figure 7.1D).

The arterial-venous concentration differences of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln-A) ratio in 10 patients at control was 0.04 ± 0.009 . During the ischemic period, a significant decline in the Cit V-A/Gln-A ratio was observed (0.02 ± 0.004 , $P < 0.02$ compared to control, Figure 7.2).

This decreased plasma Cit-V-A/Gln-A ratio was the result of a decline in venous citrulline concentrations after ischemia. (see Supplementary Table 7.1) .

Initial reperfusion results in significant tight junction damage and functional intestinal barrier loss

After 30 minutes of ischemia followed by 30 minutes of reperfusion (30I30R), shedding of IR-damaged enterocytes from the villus tips towards the intestinal lumen was observed (Figure 7.3A, right panel, arrowhead). Short reperfusion led to compromised TJ integrity as immunofluorescence for ZO-1 and occludin showed an irregular staining pattern and loss of protein expression in the epithelial sheets that had lost contact with the basal membrane at the tips of the villi (Figure 7.3A, right panels, arrowheads). This

Figure 7.1 Changes in intestinal barrier function after 30 minutes of ischemia

A, HE staining showed an intact epithelial lining in control tissue. After 30 minutes of ischemia (30I) subepithelial spaces were visualized at the tips of the villi. Strong and evenly distributed immunofluorescent stainings for ZO-1, occludin and E-Cadherin were observed, over the tips of the villi, in both control tissue and jejunum subjected to 30I. **B**, Electron microscopic images of two enterocytes with interconnecting tight junctions demonstrate in control tissue that tight junctions are intact (arrow) and the contrast dye lanthanum remains intraluminally. On the contrary, after 30I, lanthanum is able to penetrate the paracellular space, indicating tight junction integrity loss (arrowheads). **C**, Plasma L/R ratio's however, remained unchanged after ischemia when compared with control. **D**, Mean plasma I-FABP V-A concentration differences show a significant release after ischemia when compared with control ($126.6 \text{ ng/mL} \pm 65.53 \text{ ng/mL}$ vs $1.75 \text{ ng/mL} \pm 0.78 \text{ ng/mL}$, $P < 0.01$), reflecting enterocyte damage. Electron microscopy scale bars = $0.5 \text{ }\mu\text{m}$ and $1 \text{ }\mu\text{m}$ respectively.

Figure 7.2 Mean Cit V-A/Gln A ratios in patients submitted to 30 minutes of ischemia followed by 30 and 120 minutes of reperfusion

Ratios were significantly decreased after 30I compared to control. During reperfusion ratio's return back to baseline values, which were no longer different compared to control. Results are presented as mean $\pm$ SEM. A *P*-value of < 0.05 was considered significant.

was accompanied by disruption of E-cadherin with diffuse staining in the cell cytoplasm across the villi (Figure 7.3A, right panel, arrowheads). The plasma L/R ratio significantly increased after 30I30R compared to control (0.75 ± 0.10 vs 0.20 ± 0.09 , $P < 0.01$; Figure 7.3C). Analysis also showed an increase of the plasma L/R ratio after 30I30R compared to sham (0.75 ± 0.10 vs 0.04 ± 0.01 , $P < 0.02$; Supplementary Figure 7.1). EM analysis revealed that lanthanum was still able to penetrate between two adjacent enterocytes demonstrating disruption of TJs and AJs after 30I30R (Figure 7.3B). These results show that the intestinal barrier function gets compromised during the early stages of reperfusion after a short ischemic hit.

I-FABP V-A concentrations declined after 30I30R but were still significantly increased compared to control (27.06 ± 2.73 ng/mL vs 1.75 ± 0.78 ng/mL, $P = 0.01$; Figure 7.3D), still indicating loss of enterocyte membrane integrity during the early reperfusion phase. The Cit V-A/Gln A ratio however, was increased after a short period of 30 minutes of reperfusion when compared with 30 minutes of ischemia (0.05 ± 0.09 vs 0.02 ± 0.004 , $P < 0.001$, Figure 7.2) and was no longer significantly different compared with control. This may indicate that the remaining enterocytes are already viable despite the compromised intestinal barrier.

The intestinal barrier restores within 120 minutes of reperfusion

After 30I120R, shedding of IR-damaged cells led to shortening of the villi, with an intact epithelial lining (Figure 7.4A, right panel). Immunofluorescence showed that both ZO-1 and occludin again formed a continuous staining pattern around the epithelial lining near the apical region of the enterocytes. In addition, E-cadherin staining appeared normal and was visible along the entire epithelium of each villus, rebuilding epithelial

Figure 7.3 30 minutes of reperfusion results in tight junction damage and functional intestinal barrier loss

A, After 30 minutes of reperfusion (30I30R) damaged epithelial cells were pinched off and shed into the lumen (arrowhead). Immunofluorescence for ZO-1 and occludin revealed irregular distribution patterns (arrowheads) and loss of expression in the epithelial sheets at the tips of the villi, together with the disruption of E-Cadherin showing only diffuse staining in the cell cytoplasm. **B**, Lanthanum was still able to penetrate between two adjacent enterocytes (arrowhead) demonstrating disruption of TJs and AJs at 30I30R. **C**, Plasma L/R ratio was significantly elevated at 30I30R compared to control (0.75 ± 0.10 vs 0.20 ± 0.09 , $P < 0.01$), indicating increased intestinal permeability after short reperfusion. **D**, Plasma I-FABP gradually reduce following 30I30R but was still significantly elevated compared to control (27.06 ± 2.73 ng/mL vs 1.75 ± 0.78 ng/mL, $P = 0.01$), indicating enterocyte membrane integrity loss. Electron microscopy scale bars = $0.5 \mu\text{m}$ and $2 \mu\text{m}$ respectively.

cell-cell integrity (Figure 7.4A, right panel). In line, the L/R ratio normalized at 30I120R to 0.17 ± 0.06 and was not significantly different from control or sham (Figure 7.4C). In EM images, lanthanum was no longer present in paracellular spaces after 30I120R (Figure 7.4B, right panel) indicating restored tight junction integrity.

I-FABP levels rapidly decreased over the course of a period of 120 minutes reperfusion to $14.77 \text{ ng/mL} \pm 5.46 \text{ ng/mL}$ and were no longer significantly elevated compared to control, demonstrating rapid and full recovery of epithelial membrane integrity (Figure 7.4D).

Remarkably, at 120 minutes of reperfusion the Cit V-A/Gln A ratio no longer differed from the value measured at control (Figure 7.2). The results demonstrate that, despite IR-induced reduction of enterocyte mass, the remaining epithelial cells are functional again after a short period of ischemia followed by 120 minutes of reperfusion.

To rule out the possibility that the low concentrations of saccharides measured at later time points were due to significantly decreased intraluminal saccharides as a consequence of uptake/leakage during early ischemia and reperfusion, we measured intraluminal concentrations of saccharides at 30I120R. Although the concentrations measured at 120R were approximately 3 times lower than the administered stock (29.16 ± 0.31 vs 11.47 ± 1.01 $P < 0.002$) and L-rhamnose (30.62 ± 1.80 vs 8.22 ± 1.30 $P < 0.002$, Supplementary Figure 7.2), the luminal concentrations of both sugars were still 100x higher than the observed plasma lactulose and L-rhamnose concentrations at 30I120R.

Figure 7.4 The intestinal barrier is restored after 30 minutes of ischemia followed by 120 minutes of reperfusion

A, At 120 minutes of reperfusion (30I120R) the epithelial lining appeared histologically intact compared to control tissue with ZO-1, occludin and E-Cadherin normally distributed across the villi. **B**, In addition, electron microscopy revealed that lanthanum was no longer present in the paracellular spaces, indicating restored tight junction integrity. **C**, Moreover, the observed restoration of the intestinal barrier seems to correlate with permeability with the plasma L/R ratio normalized to 0.17 ± 0.06 which was no longer significantly different from control. **D**, Plasma I-FABP levels, reflecting intestinal epithelial damage, also returned to $14.77 \text{ ng/mL} \pm 5.46 \text{ ng/mL}$ and were no longer significantly elevated compared to control. Electron microscopy scale bars = $0.5 \mu\text{m}$ and $2 \mu\text{m}$ respectively.

Intestinal barrier function in relation to intestinal damage

The IR-induced damage to the epithelial lining was measured using plasma I-FABP. This sensitive plasma parameter of enterocyte damage is rapidly released upon intestinal ischemia. This allows us to relate the structural damage to the mucosa with the permeability changes during intestinal IR. To this end within-person correlations were studied between arteriovenous concentration differences of plasma I-FABP and L/R ratios. Plasma levels of I-FABP correlated with plasma L/R ratio's measured at the same time points (correlation: 0.467, $P < 0.01$), indicating a relationship between structural damage to the intestinal mucosa and the observed changes in permeability (Figure 7.5).

Figure 7.5 Translated values of plasma levels of I-FABP and translated values of L/R ratio's in patients submitted to 30 minutes of ischemia followed by 30 and 120 minutes of reperfusion

I-FABP correlated significantly with L/R ratio's (correlation: 0.467, $P < 0.01$). Translations of both variables are specific for an individual in such a way that all within-person means correspond to the zeros in the plot. In this way the variation of individual levels are cancelled and the pure association of both variables remains.

Discussion

The current study demonstrated that the human intestinal epithelial barrier has the remarkable capacity to rapidly and functionally restore IR-induced tissue damage within 120 minutes of reperfusion. Second, we show in this study that the currently used golden standard to measure intestinal epithelial permeability i.e. the dual sugar test, reflects intestinal barrier function as objectified by electron microscopy in our model of acute intestinal damage and repair. These results are important for our understanding of the pathophysiology of human intestinal ischemia-reperfusion in general, and have implications for clinical practice. There is increasing evidence that the loss of intestinal barrier function after a period of ischemia or hypoperfusion is associated with the development of sepsis and multiple organ dysfunction in the acute setting, including trauma, hemorrhagic shock, following major (vascular) surgery or acute pancreatitis ^{2,25}. Furthermore, exposure of the intestinal epithelium to periods of ischemia followed by reperfusion results in sloughing of enterocytes at the tips of villi causing a breach in the gut barrier ^{12,26,27}. Disruption of epithelial integrity is associated with intestinal inflammation, bacterial translocation, the development of sepsis and multiple organ dysfunction ^{4,28,29}. In addition, loss of intestinal barrier function plays a role in the pathophysiology of various intestinal diseases including IBD and celiac disease ^{3,30}. Epithelial hypoxia can occur secondary to the inflammation, and might play a role in the pathophysiology ³¹ and etiology ³² of IBD.

The intestinal barrier function is dependent on an intact epithelial lining which is formed by epithelial cells, interconnected by cell to cell adhesion proteins at different levels of the intercellular junction ³. TJ complexes consist of transmembrane proteins, including occludin and claudins linked to the cell cytoskeleton by intracellular proteins, such as ZO-1. The TJ barrier controls the paracellular pathway via at least two routes allowing selective transport across the intestinal barrier. The pore pathway is a size- and charge-selective route for ions which is regulated by claudins. The leak pathway allows paracellular transport of large molecules, including proteins and bacterial lipopolysaccharides. Studies have shown that occludin and ZO-1 are mainly involved in the regulation of the leak pathway. Mainly defects in the leak pathway lead to increased epithelial permeability allowing leakage of bacterial products and antigens from the lumen into the systemic circulation ^{4,8}.

At 30 minutes of ischemia, ZO-1 and occludin appeared as continuous bands along the villous tips, similar as the immunofluorescent staining of control tissue. In line, the DST showed that the intestinal barrier was functionally still intact. Similar to the human setting animal studies have shown that the appearances of sub epithelial spaces during ischemia are not accompanied by increased permeability for lactulose ³³.

In contrast, shortly after reperfusion desquamation of the intestinal epithelium at the tips of the villi occurred. Although the gut possesses elegant pathways that maintain the intestinal barrier during cell shedding, ^{34,35} excessive rates of apoptosis make these pathways to fall short resulting in intestinal barrier loss ^{26,36}. Indeed, TJ derangements were observed and accompanied by increased L/R ratios.

These human data are in line with previous data from animal models demonstrating that the magnitude of ischemia and reperfusion-induced intestinal mucosal damage is a result of the duration of occlusion time. This was evaluated in standardized animal models of complete segmental arterial ischemia demonstrating that 30 minutes of ischemia followed by 30 minutes of reperfusion results in massive epithelial lifting with a few denuded villi ³⁷. Moreover, the mechanism of intestinal barrier repair during intestinal IR is also previously described ²⁶. This is a highly regulated event involving villus contraction, epithelial restitution and closure of the paracellular space. The latter is considered to account for the majority of barrier recovery after intestinal injury. The net result of the above mentioned repair mechanisms is a remarkably rapid closure of mucosal wounds in the mammalian intestinal epithelial lining to prevent the onset of sepsis. The observed time scale of intestinal barrier restoration within hours after ischemia, is also in agreement with previous studies ²⁶.

To study if the intestinal barrier is indeed functionally repaired, it is necessary to be able to actively and reliably measure intestinal barrier function. However, this has been a major challenge in clinical practice. Classically, DST are used based on the differential absorption of intraluminal sugars of different molecular weight across the intestinal barrier. Using these tests it has been shown that intestinal barrier function is involved in intestinal diseases including patients with IBD and celiac disease ^{38,39}. However, a pitfall of the DST is the lack of knowledge on how these tests correlate to epithelial cell damage and tight junction loss in vivo. In intestinal IR, loss of intestinal barrier function is seen as key explanation of intestinal dysfunction in patients leading to increased incidence of bacteria and toxin translocation from the intestinal lumen to the systemic circulation, causing infectious complications including sepsis and the multiple organ failure syndrome ²⁸. Using our in vivo human model, we were able to demonstrate the direct relationship between morphological barrier loss (epithelial cells and TJ proteins) and functional intestinal barrier loss (dual-sugar absorption test and leakage of lanthanum nitrate through defective tight junction complexes as visualized by EM).

I-FABP concentrations and release-patterns were comparable with previous published data from our group ²³. This shows that 30 minutes of ischemia is associated with rapid reversal of IR-induced structural tissue damage of the epithelial lining. Interestingly, the extent of intestinal damage, reflected by an increase in plasma I-FABP, significantly correlated with the permeability changes in the small intestine. This is in line with previous studies performed in humans undergoing splanchnic hypoperfusion because

of moderate-to-high intensity exercise demonstrating that intestinal cell damage result in permeability changes including restoration of the epithelial lining ⁴⁰.

Our results showed that the DST data closely reflect the morphological findings during intestinal IR. This is of importance as it supports the usefulness of DST ^{10,41,42}.

In addition, we provide support for the use of citrulline as a marker for enterocyte function. Measurement of gastrointestinal dysfunction, following small bowel diseases, is hard to establish ⁴³. Citrulline has been used for this purpose as it is an amino acid mainly produced by small intestinal enterocytes by the conversion of glutamine through the glutamate to ornithine pathway, without incorporation into proteins ⁴⁴. Loss of the intestinal epithelial lining has been shown to result in declined circulating levels of citrulline ⁴⁵. Several studies showed that there is a link between low plasma citrulline concentration and intestinal barrier function ⁴⁶⁻⁴⁸. Supply of glutamine to the enterocyte occurs from both arterial blood as well as from the intestinal lumen. As all patients were fasted, we hypothesized that the arterial-venous concentrations of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln A) ratios reflect enterocyte functional capacity. It is striking that the DST is at baseline level at 30I and only increased at 30I30R, whereas the Citr/Gln ratio is only decreased at the 30I time point and not during reperfusion. This discrepancy is in agreement with previous studies in patients with chemotherapy-induced mucositis and patients suffering from IBD, where permeability was not correlated with changes in plasma citrulline concentrations ^{49,50}. This may indicate that enterocytes do not require an intact intestinal barrier to be metabolically viable. Citrulline performed better as a marker for functional epithelial cell mass in these studies where it detected impairment of intestinal epithelium and small intestinal barrier integrity earlier compared with the DST and indicated recovery more accurate.

Potential limitations of this study may include the fact that the intraluminal concentration of lactulose and L-rhamnose show a decrease during the IR protocol. This drop in concentration however, is only partly relevant since the remaining concentrations in the lumen were still 100x higher than the observed plasma lactulose and L-rhamnose concentrations at 30I120R. Furthermore the hyperosmolality of the saccharide solution may affect the intestinal permeability for L-rhamnose directing the flux towards the intestinal lumen. To correct for this diffusion, the experimental IR protocol started 5 minutes after injection of the saccharides. In addition, dual-sugar absorption tests can be influenced by the presence of food-derived sugars in plasma and most of the studies of intestinal permeability do not report baseline excretion of the saccharides, which is perhaps most relevant with the use of DST. As baseline measurements were taken in the current study, this should have been accounted for.

Next, the larger part of the total paracellular conductance of intestinal epithelial lining is via a high linear density of TJs residing in the crypts ⁴. After villus contraction, caused by the IR injury, crypt epithelium accounts for the majority of surface area remaining.

As we did not focus on the role of the crypts in this study, the latter could be of influence on the results. Future studies, measuring mucosal barrier function within the crypts are warranted to investigate villus/crypt differences during IR injury.

Also, one should be careful generalizing the current findings to the whole length of the human gastro-intestinal tract. First, it is important to understand that epithelial permeability of the gastrointestinal tract needs to be evaluated in a site specific manner. Several saccharide probes are destroyed by digestion processes that take place in the lumen of different parts of the gut, which limits their capabilities to detect permeability changes throughout the whole intestinal tract. For example lactulose and L-rhamnose are destroyed in the caecum and therefore provide only information regarding the small intestinal epithelium³. Also the expression of epithelial tight junction proteins is region-specific along the gastrointestinal tract, which determine the properties of permeability in different regions. The proximal segments of the jejunum have a higher permeability than the distal ileum segments. Next, Takeyoshi et al. evaluated the mucosal regeneration of different parts of the small intestine during transplantation in dogs. They showed that the regenerative capacity was twice as fast in the jejunum than in the ileum⁵¹. This more pronounced recovery in jejunal tissue could have a beneficial effect on our data. Last, important to note is that the dual-sugar tests have not yet gained a place in everyday practice for diagnosis and follow up of the several patients groups, mainly because the detection methods are complex and not widely available.

Taken together, these findings reflect the ability of the intestine to withstand short episodes of ischemia, with morphological and functional recovery of the intestinal barrier within 120 minutes of reperfusion. These results explain why there are often no signs of inflammation or bacterial translocation after short periods of intestinal ischemia. Further exploration of the mechanisms responsible for this rapid morphological and functional recovery might impact treatment of intestinal diseases associated with barrier recovery loss. Next, data from the DST and citrulline glutamine ratios closely reflect the histological perturbations during intestinal IR highlighting the potential diagnostic value of these tests in the follow-up of patients with intestinal disease associated with intestinal barrier loss.

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Supplementary data

Supplementary Figure 7.1

In sham-operated patients no differences in plasma L/R ratio was observed throughout the experiment. Plasma lactulose/rhamnose (L/R) ratio was significantly elevated at 30I30R compared to control or sham-operated patients (0.75 ± 0.10 vs 0.20 ± 0.09 , $P < 0.01$ or 0.75 ± 0.10 vs 0.03 ± 0.01 , $P < 0.02$), indicating increased intestinal permeability after short reperfusion. At 30I120R, the ratio normalized to 0.17 ± 0.06 which was no longer significantly different from control or sham. * = $P < 0.01$.

Supplementary Table 7.1 Absolute arterial and venous glutamine and citrulline concentrations measurements

Amino acid Concentration ($\mu\text{mol/L}$)	Controle	30I	30I30R	30I120R
Glutamine arterial	567.11 $\pm$ 32.13)	578.64($\pm$ 36.54)	528.32($\pm$ 38.81)	512.86($\pm$ 19.90)
Glutamine venous	484.29($\pm$ 35.64)	510.57($\pm$ 29.92)	484.87($\pm$ 38.49)	394.59($\pm$ 34.71)
Citrulline arterial	26.18 ($\pm$ 2.80)	23.60 ($\pm$ 2.30)	23.27 ($\pm$ 2.93)	22.58 ($\pm$ 1.04)
Citrulinne venous	46.43 ($\pm$ 8.46)	30.55 ($\pm$ 3.36)	50.78 ($\pm$ 5.67)	47.40 ($\pm$ 6.42)

Data is presented as mean ($\pm$ SEM: standard error of the mean)

Supplementary Figure 7.2

Concentration of lactulose and L-rhamnose in the injected stock solution and in the luminal debris after completing the human experimental IR protocol.

A microscopic image of tissue, likely intestinal, showing a grid-like pattern of cells. It is positioned in the top-left corner of the page, partially overlapping the title area.

Chapter 8

Females are more resistant to ischemia-reperfusion induced intestinal injury than males: a human study

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Abstract

Objective: Sex differences in responses to intestinal IR have been recognized in animal studies. We aimed to investigate sexual dimorphism in human small intestinal mucosal responses to ischemia-reperfusion (IR).

Methods: In 16 patients (8 men and 8 women) undergoing pancreaticoduodenectomy, an isolated part of jejunum was subjected to IR. In each patient, intestinal tissue and blood was collected directly after 45 minutes ischemia without reperfusion (45I-0R), after 30 minutes of reperfusion (45I-30R), and after 120 minutes of reperfusion (45I-120R), as well as a control sample not exposed to IR, to assess epithelial damage, unfolded protein response (UPR) activation, and inflammation.

Results: More extensive intestinal epithelial damage was observed in males compared to females. Intestinal fatty acid binding protein (I-FABP) arteriovenous (V-A) concentrations differences were significantly higher in males compared to females at 45I-0R (159.0 [41.0-570.5] ng/ml vs 46.9 [0.3-149.9] ng/ml). Male intestine showed significantly higher levels of UPR activation than female intestine, as well as higher number of apoptotic Paneth cells per crypt at 45I-30R (16.4% [7.1-32.1] vs 10.6% [0.0-25.4]). The inflammatory response in male intestine was significantly higher compared to females, with a higher influx of neutrophils per villus at 45I-30R (4.9 [3.1-12.0] vs 3.3 [0.2-4.5]) and a higher gene expression of TNF- α and IL-10 at 45I-120R.

Conclusion: The human female small intestine seems less susceptible to IR-induced tissue injury than the male small intestine. Recognition of such differences could lead to the development of novel therapeutic strategies to reduce IR-associated morbidity and mortality.

Introduction

Sex disparities regarding pathogenic mechanisms, age of onset, progression and therapy suitability are common in human disease ¹. Sex differences in ischemia-reperfusion (IR) injury have, in particular, been investigated in animal studies, and provided evidence for sex differences in responses to IR in the heart ², brain ³, kidney ⁴, liver ⁵ and intestine ^{6,7}. Beneficial effects of the female hormone estrogen and/or deleterious effects of the male hormone testosterone are held responsible ⁸⁻¹³. For example, estradiol-treated male rats displayed less myocardial necrosis and inflammatory responses in a model for cardiac ischemia when compared to non-treated male rats ⁹. As an example of the negative effects of male hormones, orchidectomy in male rats prior to intestinal IR resulted in reduced IR-induced intestinal mucosal damage in comparison with non-orchidectomized males ¹⁰.

Even though the small intestine contains functional estrogen receptors and therefore is a potential estrogen-responsive organ ^{14,15} the impact of sex on the pathogenesis of human IR-induced intestinal epithelial injury, mucosal barrier dysfunction and inflammation remains unclear. Human intestinal IR is an important clinical problem. Intestinal IR-associated morbidity and mortality rates remain extraordinarily high with reported mortality rates of 60-80% ¹⁶. Previous studies from our group demonstrated that 45 minutes of human intestinal ischemia followed by reperfusion led to epithelial cell damage, inflammatory responses and Paneth cell apoptosis ^{17,18}. Intestinal IR is also a potent inducer of endoplasmic reticulum (ER) stress, which leads to the activation of the unfolded protein response (UPR) via three ER stress sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE-1), and activating transcription factor 6 (ATF6) ^{18,19}. The UPR, amongst others, is initially directed at cell survival, by inducing a translational block to reduce protein load to the ER and activating programs that increase protein folding capacity of the cell to cope with ER stress ^{20,21}. Prolonged or extensive ER stress will eventually lead to apoptosis ²². Several animal studies revealed that ER stress responses are higher in males compared with females, and that this may contribute to the observed sex differences in myocardial apoptosis, kidney injury and hypertension ²³⁻²⁵.

Although the consequences of IR in patients participating in our previous studies were consistent, we observed that some patients had considerably less intestinal IR-induced damage than others. In this study, we aimed to investigate whether such differences in disease susceptibility were sex-related. This is important since recognition of potential sex differences and elucidation of the underlying pathophysiological mechanisms is expected to be beneficial for treating both males and females with personalized therapies, and for the development of novel therapeutic approaches to reduce IR-associated morbidity and mortality.

Materials and Methods

Ethics

The study was approved by the Medical Ethical Committee of Maastricht University Medical Center+ and was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent of all patients was obtained.

Patients and experimental procedures

Patients

To investigate sex-related differences in human jejunal IR, 16 patients (8 males, 8 females) undergoing pancreaticoduodenectomy for pancreatic head tumors were included in this study after obtaining informed consent. Patients with underlying intestinal diseases were excluded.

Experimental procedures

The experimental protocol using a model of human small intestinal IR was conducted as previously described^{26,27}. In short, the model takes advantage of the fact that during pancreaticoduodenectomy, a variable length of healthy jejunum is routinely resected in continuity with the head of the pancreas and duodenum as part of the standard surgical procedure. Of all enrolled patients, the terminal 6 cm of this jejunal segment was isolated and subjected to 45 minutes of ischemia by placing two atraumatic vascular clamps across the mesentery. Meanwhile, surgery proceeded as planned. After ischemia, one third of the isolated jejunum was resected using a linear cutting stapler (GIA™, Covidien, Mansfield, MA). Next, the mesenteric clamps were removed to allow reperfusion, as confirmed by regaining of normal pink color and restoration of gut motility. Another segment of the isolated jejunum was resected similarly after 30 minutes of reperfusion and after 120 minutes of reperfusion. Simultaneously, a small part of jejunum not exposed to IR, was resected and served as internal control tissue.

Tissue and blood collection

Jejunal tissue samples were collected directly after 45 minutes of ischemia without reperfusion (45I-OR), after 45 minutes of ischemia followed by 30 minutes of reperfusion (45I-30R) and after 45 minutes of ischemia followed by 120 minutes of reperfusion (45I-120R). Directly after collecting the latter, a control sample was taken as well, resulting in a total of 4 tissue samples for every male (n=8) and every female (n=8). All samples were immediately snap-frozen for quantitative polymerase chain reaction (qPCR) or formalin-fixed for histology and immunohistochemistry. Arterial blood was sampled and collected in EDTA tubes before ischemia (control), at 45I-OR, 45I-30R and 45I-120R. Simultaneously with each respective arterial blood sample, blood was drawn from the

venule draining the isolated jejunal segment by direct puncture, resulting in 4 arterial blood samples and 4 venous blood samples for every male (n=8) and every female (n=8). All blood samples were centrifuged at 3500 rpm, 4°C for 15 minutes. Plasma was immediately stored in aliquots at -80°C until analysis.

Histology and immunohistochemistry

Tissue specimens were processed and stained with hematoxylin and eosin (HE) as described ²⁷. Paraffin-embedded sections were double-stained for cytokeratin 18 clone M30 (Peviva, Bromma, Sweden) and lysozyme to visualize apoptosis and Paneth cells, respectively, as described ¹⁸. Neutrophil influx was assessed by staining sections with rabbit anti-human myeloperoxidase (MPO staining; DakoCytomation, Glostrup, Denmark) as described ²⁷. All stainings were counterstained with hematoxylin. Sections were photographed using a Nikon eclipse E800 microscope equipped with a Nikon digital camera DXM1200F. The number of lysozyme-positive crypt cells (Paneth cells), lysozyme-positive M30-positive crypts cells (apoptotic Paneth cells) and MPO-positive cells (neutrophils), were quantified in a blinded way by 2 independent observers in 5 representative microscopic fields per section (200× magnification).

RNA isolation, qPCR, and XBP1 splicing assay

RNA was isolated from snap-frozen tissue samples followed by cDNA synthesis and qPCR as previously described, ²⁷ to analyze gene expression of interleukin-1 (IL-1),

Table 8.1 Human oligonucleotide primer sequences used for qPCR analysis

Gene Symbol	Forward Primer Sequence	Reverse Primer Sequence
<i>CYP4</i>	5'-CTCGAATAAGTTTGACTTGTGTTT-3'	5'-CTAGGCATGGGAGGGAACA-3'
<i>RPLP0</i>	5'-GCAATGTTGCCAGTGTCTG-3'	5'-GCCTTGACCTTTTCAGCAA-3'
<i>IL1</i>	5'- CTGAGCTCGCCAGTGAAATG-3'	5'- TTTAGGGCCATCAGCTTCAAA-3'
<i>IL6</i>	5'- TCCAGGAGCCCAGCTATGAA-3'	5'- GAGCAGCCCCAGGGAGAA-3'
<i>IL10</i>	5'- GGCGCTGTCATCGATTTCTT-3'	5'- TGGAGCTTATTAAAGGCATTCTTCA-3'
<i>TNFA</i>	5'-TCAATCGGCCCGACTATCTC-3'	5'-CAGGGCAATGATCCCAAAGT-3'
<i>XBP1</i>	5'-GGAGTTAAGACAGCGCTTGGGGA-3'	5'-TGTCTGGAGGGGTGACAAGTGGG-3'
<i>CHOP</i>	5'-GGAGCATCAGTCCCCCACTT-3'	5'-TGTGGGATTGAGGGTCACATC-3'
<i>GADD34</i>	5'-CCCAGAAACCCCTACTCATGATC-3'	5'-GCCCAGACAGCCAGGAAAT-3'
<i>BiP</i>	5'-CTGCTGTATCCTCTTACCAGTTG-3'	5'-TGACATTGAAGACTTCAAAGCTAAGA-3'

interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF- α), C/EBP homologous protein (CHOP), growth arrest and DNA damage-inducible protein 34 (GADD34) and binding immunoglobulin protein (BiP), using RPLP0 and CYPA as reference genes. XBP1 splicing was assessed as described by Grootjans et al ¹⁸. Primer sequences for qPCR are shown in Table 8.1.

Plasma I-FABP and cytokines

Plasma concentrations of I-FABP, IL-6 and TNF- α were measured using in-house developed enzyme-linked immunosorbent assay (ELISA). Plates (Greiner Bio-One, Kremsmunster, Austria) were coated overnight with rabbit anti-human I-FABP, or monoclonal antibodies 5E1 (IL-6) or 61E71 (TNF- α). Purified human I-FABP, human recombinant IL-6 and TNF- α were used as standard. After blocking, samples and standard dilution series were incubated for 1-2 hours. Biotinylated rabbit anti-human I-FABP and IL-6 were used as detection antibodies. For TNF- α , rabbit anti-human TNF- α was used as detection antibody, followed by incubation with peroxidase-labeled polyclonal goat anti-rabbit antibody (Jackson ImmunoResearch Europe, Suffolk, UK). Finally, 3,3',5,5'-tetramethyl-benzidine (TMB) was used as a substrate and the reaction was stopped by adding 1M H₂SO₄. Samples were analyzed spectrophotometrically (450 nm) using an automated ELISA reader. The detection limits for the assays were 25 pg/mL, 31.3 pg/mL and 78 pg/mL respectively. Samples were run in duplicate and a variability of 15% between sample duplicates was accepted. Plasma IL10 levels were measured using DuoSet ELISA (RnD Systems, Minneapolis, MN) according to manufacturer's protocol, with a lower detection limit of 62.6 pg/mL. Arteriovenous concentration differences across the intestinal segment were calculated by subtracting arterial plasma concentrations from venous plasma concentrations.

Statistics

Statistical analysis was performed using Prism 6.0 for Windows (GraphPad Software Inc. San Diego, CA). All data are presented as median and range as none of the parameters showed a normal distribution, according to Shapiro-Wilk test. Data were analyzed using two-way ANOVA with time points and sex as factors, with Sidak's post hoc test for multiple comparisons to identify the difference between males and females at the respective time points. Within group effects were tested with one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons versus control. A *P*-value below 0.05 was considered statistically significant.

Results

Patients characteristics and sample collection

A total of 16 patients (8 males, 8 females) were included in this study. Male and female patients were comparable regarding age (70.5 [65.3-79.0] vs 68.5 [55.8-80.5] respectively, $P>0.05$) and BMI (22.05 [20.63-29.95] vs 22.10 [20.7-23.6] respectively, $P>0.05$). The procedure was ended early in two males and one female, resulting in missing blood and tissue samples at 45I-120R. Moreover, blood sampling was unsuccessful at time point 45I-120R in one male and one female patient. One control tissue sample of a male patient was formalin-fixed only, hence no snap-frozen tissue was available.

Jejunal IR results in more extensive epithelial damage and higher plasma I-FABP levels in males compared to females

In control small intestine, an intact epithelial lining was visualized by HE staining in both males and females (Figure 8.1A and 8.1E, respectively). In males, 45I-0R was associated with epithelial damage at the villus tips (Figure 8.1B), which worsened at 45I-30R resulting in shedding of damaged enterocytes (Figure 8.1C). At 45I-120R, the villi were denuded in 83.3% of the male patients (Figure 8.1D). In contrast, in female intestinal IR, 45I-0R was particularly associated with the development of subepithelial spaces, a pathophysiological phenomenon of intestinal IR that we previously linked to short periods of intestinal ischemia of 15 and 30 minutes 28 (Figure 8.1F). Although epithelial shedding of villus tips was also observed at 45I-30R (Figure 8.1G), the epithelial lining appeared to be mostly intact in 86% of the female patients at 45I-120R (Figure 8.1H), in contrast to what was observed in male small intestine.

To provide further evidence for this apparent sex difference, we quantified epithelial injury following intestinal IR in the human intestine by measurement of plasma levels of I-FABP, a protein abundantly present in the cytoplasm of mature enterocytes, which is released into the circulation upon epithelial damage. Significantly higher AV-concentration differences across the isolated intestinal segment were measured in plasma of males than of females exposed to intestinal ischemia, at 45I-0R (159.0 ng/ml [41.0-570.5] versus 46.9 ng/ml [0.3-149.9] Figure 8.1I, $P<0.05$), and 45I-30R (82.5 ng/ml [43.3-97.2] versus 16.5 ng/ml [12.9-42.5]). AV-concentration differences were not significant at 45I-30R (85.0 ng/ml [13.1-752.3] versus 20.2 [10.3-61.7], $P=0.12$ and 45I-120R (10.1 ng/ml [6.4-32.1] versus 3.7 [0.0-23.4], Figure 8.1I, $P>0.99$). These results thereby provide further evidence that the female jejunum is less susceptible to IR-induced enterocyte damage than the male jejunum directly after ischemia.

Figure 8.1 More extensive epithelial damage and higher plasma I-FABP levels in IR-injured male small intestine compared to female small intestine

A, HE staining showed an intact epithelial lining in male jejunal control tissue. **B**, 45I-0R resulted in severe epithelial damage with disruption of the epithelial lining. **C**, 45I-30R resulted in shedding of damaged cells towards the lumen (arrows). **D**, Denudation of the basement membrane was observed at 45I-120R (arrows). **E**, An intact epithelial lining was observed in female jejunal control tissue. **F**, At 45I-0R, subepithelial spaces (asterisks) were observed, indicating basal membrane retraction. **G**, IR-damaged cells were pinched off into the lumen at 45I-30R (arrow), while no obvious disruption of the epithelial lining could be observed, thereby limiting exposure of the lamina propria to luminal content. **H**, Although the epithelial lining has a slightly irregular appearance, it appeared fully intact at 45I-120R. The histological features are representative for the tissue samples studied. **I**, Mean plasma I-FABP arteriovenous concentration differences are significantly higher in males after jejunal exposure to 45I-0R compared to females.

HE. Control: males n=8, females n=8; 45I-0R: males n=8, females n=8; 45I-30R: males n=8, females n=8; 45I-120R: males n=6, females n=7. **I-FABP.** Control: males n=8, females n=8. 45I-0R: males n=8, females n=8. 45I-30R: males n=8, females n=8. 45I-120R: males n=5, females n=6. Magnification 200x. **I** = ischemia. **R** = reperfusion. **I-FABP** = intestinal fatty acid-binding protein. **V-A** = arteriovenous concentration differences. * $P < 0.05$ compared to control, ** $P < 0.01$ compared to control.

Figure 8.2 More severe ER stress after jejunal IR in males compared to females

A, Upper electrophoresis bands represent unspliced *XBP1* mRNA; lower bands represent spliced *XBP1* mRNA. Splice products of *XBP1* mRNA were only observed in minor quantities at 45I-0R in either males or females, but were clearly present after 45I-30R. The electrophoresis bands are representative for the tissue samples studied. **B**, 45I-30R resulted in enhanced *XBP1* splicing in both males and females when compared to control. However, males displayed a significantly higher *XBP1*s/*XBP1*u ratio, when compared to females at the same time point. **C**, **D**, Relative *CHOP* and *GADD34* mRNA expression was significantly enhanced at 45I-30R and 45I-120R compared to control in male intestine, which was less evident in female intestine. Pro-apoptotic ER stress marker *GADD34* showed a significantly higher relative mRNA expression in males compared to females at 45I-30R. **E**, *BiP* mRNA expression was significantly upregulated in both the male and female jejunum at 45I-120R compared to control, although no expression difference was observed between the two groups.

Control: males n=7, females n=8. 45I-0R: males n=8, females n=8. 45I-30R: males n=8, females n=8. 45I-120R: males n=6, females n=7. *XBP1*u = unspliced X-box binding protein-1. *XBP1*s = spliced X-box binding protein-1. *CHOP* = C/EBP homologous protein. *GADD34* = growth arrest and DNA damage-inducible protein 34. *BiP* = binding immunoglobulin protein. * $P<0.05$ compared to control, ** $P<0.01$ compared to control, *** $P<0.0001$ compared to control. • = outlier. I = ischemia. R = reperfusion.

Intestinal IR in males is associated with increased levels of UPR activation compared to females

To explore UPR activation in the human small intestine, XBP1 mRNA splicing and mRNA expression levels of ER stress sensor BiP, and pro-apoptotic factors CHOP and GADD34 were assessed. Splice products of XBP1 mRNA were observed in only minor quantities directly after ischemia in both males and females, but were clearly present after 45I-30R (Figure 8.2A, representative subset of 2 male and 2 female patients). Quantification of the XBP1s/XBP1u ratios showed significantly higher ratios in males at 45I-30R compared to females (4.1 [2.0-7.5] versus 2.1 [0.9-3.8] respectively, Figure 8.2B, $P<0.001$), suggesting more pronounced ER stress in males. XBP1s/XBP1u ratios were not different between males and females at the other time points, 45I-0R and 45I-120R. At these time points, XBP1s/XBP1u ratios were not different from control within groups.

CHOP mRNA expression was significantly increased at 45I-30R and 45I-120R when compared to control in the male gut (3.9 [0.5-5.1] and 2.2 [1.0-4.6] versus 1.0 [0.5-1.6]), $P<0.0001$ and $P<0.01$, respectively), and at 45I-30R in the female gut (2.4 [0.9-5.0] versus 1.1 [0.7-1.5], $P<0.01$), Figure 8.2C). No significant differences were observed for CHOP mRNA expression between males and females at the different time points.

GADD34 mRNA levels were significantly upregulated both at 45I-30R and 45I-120R when compared to control only in males (8.7 [0.3-16.5] and 4.3 [1.7-14.5] versus 1.0 [0.6-1.4], $P<0.0001$ and $P<0.01$, respectively) Figure 8.2D) and not in females. A higher GADD34 mRNA expression was observed in males compared to females at 45I-30R (8.7 [0.3-16.5] versus 3.9 [0.2-8.1], Figure 8.2D, $P<0.01$). Furthermore, BiP mRNA expression was significantly upregulated at 45I-120R when compared to control in both the male and female gut (3.8 [0.9-5.1] versus 1.1 [0.3-1.4], and 3.0 [1.3-4.9] versus 1.3 [0.9-1.6], Figure 8.2E, both $P<0.0001$). Nevertheless, no significant differences were observed for BiP mRNA expression between males and females, which suggests that the activation of the IRE-1 rather than the PERK pathway might be sex-driven.

Paneth cells in the female intestine are more resistant to IR-induced apoptosis compared to the male intestine

Paneth cells are very susceptible to ER stress and sustained ER stress can lead to apoptosis. In control tissue, lysozyme staining showed presence of Paneth cells deep in the crypts, and Paneth cell apoptosis was absent in both the male and female small intestine (Figure 8.3A and 8.3E, respectively). Paneth cell apoptosis increased significantly in males and females at 45I-30R when compared to control (16.4% [7.1-32.1] and 10.6% [0.0-25.4] versus 0% [0.0-0.8] and 0% [0.0-0.0], Figure 3ACEGI, both $P<0.0001$). Both the male and female intestine showed significant Paneth cell apoptosis, and in line with the higher levels of UPR activation in the male intestine, as demonstrated by increased XBP1 splicing, the male small intestine also displayed considerably more Paneth cell apoptosis at 45I-30R, compared to the female small intestine (16.4% [7.1-32.1] versus 10.6% [0.0-

Figure 8.3 More Paneth cell apoptosis in male jejunum compared to less Paneth cell apoptosis in female jejunum

A, Paneth cells (lysozyme, blue staining) are visible in the crypts of male control jejunum. **B, C**, Paneth cell apoptosis is clearly present in male jejunal crypts at 45I-0R and 45I-30R (blue-brown double staining, arrowheads). **D**, At 45I-120R, Paneth cell apoptosis was still present, but to a lesser extent (blue-brown double staining, arrowheads). **E**, Female control jejunum displayed the presence of non-apoptotic Paneth cells. **F, G**, Only minor Paneth cell apoptosis was observed in female jejunal crypts at 45I-0R and 45I-30R (Blue-brown double staining, arrowheads). At 45I-120R, hardly any apoptotic Paneth cells were observed. Fragments: Magnification of jejunal crypts with healthy and apoptotic Paneth cells. The histological features are representative for the tissue samples studied. **I**, Quantification of the number of apoptotic Paneth cells showed a significant increase in Paneth cell apoptosis in both sexes at 45I-30R when compared to control. However, significantly more Paneth cell apoptosis was present in male small intestine exposed to 45I-30R compared to female small intestine.

Control: males n=8, females n=8; 45I-0R: males n=8, females n=8; 45I-30R: males n=8, females n=8; 45I-120R: males n=6, females n=7. Magnification upper panels 200x. *** $P < 0.0001$ compared to control. • = outlier. I = ischemia. R = reperfusion.

Figure 8.4 IR-induced inflammation is more pronounced in the male intestine than in the female intestine

A, MPO staining showed abundant presence of circulating neutrophils in the lamina propria of male small intestine upon exposure to 45I-30R (left panel, arrows), while female small intestine exhibited only minor neutrophil influx (right panel, arrows). The histological features are representative for the tissue samples studied. **B**, Quantification of MPO-positive cells showed a significant increase in neutrophil influx in male jejunum at 45I-0R and 45I-30R compared to control, but not in female jejunum. Male jejunal tissue displayed significantly more neutrophil influx at 45I-30R, compared to female jejunal tissue. **C**, Relative TNF- α mRNA expression significantly increased in male small intestine after 45I-30R and 45I-120R when compared to control. The male TNF- α mRNA expression levels were significantly higher when compared to females at these time points. **D**, Anti-inflammatory IL-10 mRNA expression was significantly increased in male small intestine at 45I-30R and 45I-120R. Males displayed significantly higher IL-10 mRNA expression when compared to females at 45I-120R. **E**, Relative IL-6 mRNA expression did not significantly increase during IR in either male or female jejunum. **F**, Arteriovenous concentration differences of IL-6 protein levels were significantly upregulated at 45I-30R and 45I-120R in males and at

45I-120R in females when compared to their respective controls. Protein levels approached significance between males and females during IR (overall sex effect $P=0.05$; post hoc comparisons not significant). MPO. Control: males $n=8$, females $n=8$; 45I-0R: males $n=8$, females $n=8$; 45I-30R: males $n=8$, females $n=8$; 45I-120R: males $n=6$, females $n=7$. Cytokine expression. males $n=7$, females $n=8$. 45I-0R: males $n=8$, females $n=8$. 45I-30R: males $n=8$, females $n=8$. 45I-120R: males $n=6$, females $n=7$. Magnification immunohistochemistry 200x. * $P<0.05$ compared to control, ** $P<0.01$ compared to control, *** $P<0.001$ compared to control, **** $P<0.0001$ compared to control. • = outlier. I = ischemia. R = reperfusion. IL-1 = interleukin 1. IL. TNF- α = tumor necrosis factor alpha. IL-10 = interleukin 10. IL-6 = interleukin 6.

25.4], Figure 8.3I, $P<0.05$). Paneth cell apoptosis was not significantly different between males and females at the other time points, 45I-0R and 45I-120R. At these time points, Paneth cell apoptosis was also not different from control within groups.

IR-induced inflammation is more pronounced in the male intestine than in the female intestine

Intestinal IR led to neutrophil infiltration in both male and female small intestine, which was only significant in males at 45I-0R and 45I-30R versus control (3.0% [1.2-5.8] and 4.9% [3.1-12.0] versus 1.5% [0.6-2.0] (Figure 8.4 A,B). Neutrophil influx was significantly more abundant in male intestinal villi compared to female intestinal villi after exposure to 45I-30R (Figure 8.4B, 4.9% [3.1-12.0] versus 3.3 [0.2-4.5], $P<0.01$). Neutrophil influx was not different between males and females at 45I-0R and 45I-120R. Although relative mRNA expression of pro-inflammatory cytokine IL-1 was not significantly different between the two groups (data not shown), relative TNF- α mRNA expression was significantly higher in males compared to females at 45I-30R (1.2 [0.1-1.9] versus 0.5 [0.3-1.2], $P<0.05$) and at 45I-120R (1.0 [0.4-1.7] versus 0.4 [0.1-1.5], $P<0.05$, Figure 8.4C). In addition, relative IL-10 mRNA was significantly higher in males compared to females at 45I-120R (1.3 [0.0-1.7] versus 0.2 [0.0-0.4], Figure 8.4D, $P=0.001$), indicative for a more activated inflammatory response in males. IL-6 mRNA levels were not significantly upregulated during IR in either males or females compared to their respective controls or between the two groups (Figure 8.4E). IL-6 protein AV- concentration differences however were significantly increased in males at 45I-30R and 45I-120R compared to control (41.2 pg/mL and 58.7 pg/mL vs 0.0 pg/mL respectively, Figure 8.4F, $P<0.05$ and $P<0.01$, respectively). In females, AV-concentration differences of IL-6 were only significantly upregulated at 45I-120R (0.0 versus 60.0 pg/mL, Figure 8.4F, $P<0.01$). A trend towards higher IL-6 protein expression level in males compared to females was observed (overall sex effect $P=0.05$; post hoc comparisons not significant). Plasma TNF- α and IL-10 protein levels were not detectable in both males and females (data not shown). These results may implicate that reperfusion times were too short for the generation of these proteins in the intestinal mucosa.

Discussion

It is well known that sexual dimorphism exists in trauma, shock and sepsis, cardiovascular diseases and cerebral damage after stroke ²⁹⁻³². Data on the influences of male or female sex on intestinal IR are scarce and only described in animal studies ²⁹⁻³². In this study, we compared the differences between the susceptibility of the human female and male small intestine to 45 minutes of ischemia followed by 0, 30 and 120 minutes of reperfusion. Our results demonstrate a clear sexual dimorphism in human small intestinal mucosal responses to ischemia and reperfusion, with females displaying less epithelial damage, less I-FABP release, less UPR activation and less inflammatory responses.

Our data, demonstrating the female small intestine to be more resistant to IR injury, are supported by clinical studies suggesting that women have an immunological advantage and decreased in-hospital mortality after abdominal surgery when compared to men ^{33,34}. Furthermore, it was demonstrated that despite comparable age and comparable surgical procedures being performed, a smaller number of female patients required intensive care after surgery and females who were admitted to the intensive care unit demonstrated a significantly lower incidence of severe sepsis or septic shock ³⁵. The exact mechanisms accounting for the differential response to intestinal IR in men and women remain to be determined. Sex differences have mainly been attributed to the effect of sex hormones. The deleterious effects of testosterone and the beneficial effects of estrogen on IR-induced injury in murine hearts, liver and kidneys and rat liver and small intestine have previously been demonstrated ^{10,36-42}. Estrogen has been shown to have anti-apoptotic and anti-inflammatory properties and strong anti-oxidative actions, thereby protecting tissues from oxidative damage ^{1,13,43-45}. Testosterone was demonstrated to have a pro-apoptotic effect ⁴⁶. Based on age, the majority of the women included in our study were post-menopausal. Nevertheless, the low levels of estrogen still present in the population studied here, could be enough to exert a protective effect. At the same time, testosterone could be held accountable for the higher disease susceptibility in males. Due to sampling limitations, we were not able to measure hormone levels in the blood of our patients in order to study the abovementioned hormonal effects in more detail. Nonetheless, other mechanisms responsible for the feminine intestinal resistance to IR could certainly be present.

It is known that female rats have a higher antioxidant capacity in a model of renal IR ⁴, and higher autophagy and anti-apoptotic activity compared to male rats in a model of cardiac ischemia, suggesting that female rats have stronger adaptation and repair abilities in response to nutrient and oxygen deprivation ^{2,13}. Additionally, it was shown that female rat intestinal segments produced more nitrogen oxide in a model of intestinal hypoxia, than male rat intestinal segments, resulting in improved tissue perfusion and less inflammation ^{41,47,48}. Leptin, secreted by adipocytes, and its receptors

have been found to be present in intestinal mucosa of rats, where it exerted protective effects in response to intestinal IR injury via anti-inflammatory and anti-oxidative processes ⁴⁹. It could be hypothesized that females exhibit more leptin activity, since females generally exhibit a higher percentage of body fat compared to males, resulting in mitigated intestinal injury in females after IR. These events and processes observed in rodents might also be present in human intestine upon IR exposure. On the other hand, the BMI's of our patients were not significantly different between the males and females, rendering the leptin-theory less likely as possible explanation for the observed sex differences in our study.

In the intestinal mucosa, a wide variety of cytokines are produced locally by the epithelium, the fibroblasts and immune cells present in the lamina propria. The epithelium itself may provide early signals for the acute mucosal inflammatory response via the release of proinflammatory cytokines and is demonstrated to be an important early source of IL-6 production or release in response to epithelial damage ⁵⁰. Our data demonstrate a significant increase of the pro-inflammatory IL-6 plasma protein levels after 30 and 120 minutes of reperfusion in males and after 120 minutes of reperfusion in females compared to control, while the IL-6 mRNA levels in both groups were not significantly upregulated during IR. In our study mRNA levels were measured in whole tissue, rather than only in epithelial cells, possibly explaining why IL-6 mRNA levels were not upregulated during IR.

In a previous study, it was demonstrated that small intestinal IR induces ER stress, resulting in activation of the unfolded protein response and subsequent XBP1 splicing ¹⁸. This XBP1 splicing was shown to be strongly correlated with Paneth cell apoptosis, which might be explained by the high secretory activity of these cells and the subsequent susceptibility to ER stress. In line with these results, our data demonstrate presence of ER stress and Paneth cell apoptosis in both males and females, especially after short reperfusion. Interestingly, male small intestine displayed a higher amount of ER stress with significantly more XBP1 splicing and a higher number of apoptotic Paneth cells per crypt than female small intestine, rendering them more prone to decreased production of antimicrobial proteins and subsequent bacterial translocation and systemic inflammation ¹⁸. These phenomena support the recent observations that differential ER stress responses in males and females may contribute to the sex differences in susceptibility to organ injury ²³⁻²⁵.

In conclusion, we showed that the human female small intestine appears to be better protected from IR-induced epithelial damage than the male small intestine. Correspondingly, the female intestine displayed less ER stress and inflammatory responses. Our results should raise awareness among clinicians and draw attention to the pathophysiological differences of intestinal IR between male and female patients, for example after admission to the intensive care unit or after elective aortic or major

abdominal surgery. Furthermore, our results indicate that future experimental studies on intestinal IR should consider sex differences and that the efficacy of therapeutic or preventive strategies should be tested in parallel in both sexes. Next to that, continued research into the cellular and molecular mechanisms responsible for these sex-based differences in response to intestinal IR is required. This research might ultimately provide targets for novel therapeutic interventions or preventive strategies, leading to improved outcomes for both male and female patients suffering intestinal ischemia and reperfusion in the near future.

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Chapter 9

The role of cyclooxygenase-2 in the development of ischemia-reperfusion induced small intestinal damage

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Abstract

Objective: To investigate the role of cyclooxygenase-2 (COX-2) in the development of tissue damage upon intestinal IR.

Background: Intestinal ischemia and reperfusion (IR) is a frequent occurring phenomenon associated with high mortality and morbidity rates. Intestinal IR leads to epithelial cell damage and severe inflammatory responses to the mucosa. As COX-2 plays a key function in intestinal epithelial wound healing, several animal studies suggest an important role for COX-2 in intestinal IR. However, controversy on whether COX-2 is beneficial and hence protective or, detrimental to the mucosa injury during intestinal IR remains. Using a COX-2 knockout mouse model, we hypothesize that COX-2 deficiency leads to aggravation of the IR induced intestinal damage and inflammation.

Methods: Wild-type and COX-2 knockout (COX-2 KO) mice were subjected to intestinal IR by selectively exposure of a 6-cm jejunal segment to either 30 (30I) or 60 (60I) minutes of ischemia followed by 30 minutes (30R) or 24 hours (24hR) of reperfusion. Tissue was sampled at all time points together with an untreated part of jejunum serving as internal control. Tissue sections were stained with hematoxylin and eosin (H&E) for morphological analysis. Intestinal damage as a result of IR was scored using the Chiu/Park Scoring System. Neutrophil influx was studied using myeloperoxidase (MPO) staining. Neutrophil influx was quantified by counting all MPO-positive cells per villus-crypt axis.

Results: COX-2 KO mice exhibited more severe IR induced intestinal injury directly after 30I and upon 30I30R when compared to wild-type mice. Histological grading scores at 30I followed by 30R were significantly higher in COX-2 KO mice compared to wild-type mice ($P<0.05$) signifying more intestinal damage). Using histological grading scores, no difference was seen between wild-type and COX-2 KO mice after 60I followed by reperfusion. 24hR resulted in restoration of the intestinal mucosa and normal intestinal injury scores in both the 30I and 60I group. COX-2 deficiency markedly increased neutrophil influx after 30I30R compared to wild-type mice ($P<0.05$). However, MPO activity in COX-2 KO mice was similar to that in wild-type mice in the 60I group. After 24 hours of reperfusion the neutrophil influx was reduced towards the levels of control tissue in all groups.

Conclusions: This short communication suggests that depletion of COX-2 led to the aggravation of intestinal damage and inflammatory response in small intestine directly after short (30I) periods of ischemia and followed by 30 minutes of reperfusion. Although prolonged periods of 60I leads to more intestinal damage and neutrophil influx, no difference were observed between wild-type and COX-2 KO mice, suggesting that this ischemic hit is too grave, failing all restorative cellular mechanisms.

Further studies aimed at identifying specific pathways that are responsible for COX-2 induction are needed to develop useful therapies for intestinal IR injury.

Introduction

Small intestinal ischemia and reperfusion (IR) is an important clinical problem associated with high mortality and morbidity in trauma and surgical patients, and is caused by temporary interruption of the blood flow to the gut. Previous studies demonstrated epithelial cell damage, apoptosis and inflammatory responses after both human and animal intestinal IR ^{1,2}. During short periods of ischemia, the intestine is able to fully recover after reperfusion ^{3,4}. In contrast, prolonged intestinal ischemia results in physical and immunological barrier function loss causing severe inflammation during reperfusion. Although several mechanism leading to the loss of intestinal barrier function are elucidated in previous studies, the key mechanism still need to be unraveled ⁵.

As Cyclooxygenase-2 (COX-2) plays a key function in intestinal wound healing, several animal studies suggest an important role for COX-2 in intestinal IR ^{6,7}. However, controversy on whether COX-2 is beneficial and hence protective or, detrimental to the mucosa during intestinal IR remains. Chen et al. showed that commensal depletion led to enhancement of IR-induced damage through TNF α signaling in a mouse model of intestinal IR, with TLR4 playing an important role ⁸. Addition of TLR4 ligand LPS after commensal depletion led to decreased IR-induced damage. LPS and TNF α are known to upregulate COX-2 expression and subsequently PGE2 production and this study implicated that COX-2 upregulation through TLR4 is protective of IR-induced damage 8-10. Additionally, Fredenburgh et al. showed that COX-2 deficiency led to intestinal barrier dysfunction, increased bacterial translocation and inflammation in a murine model for polymicrobial sepsis, emphasizing the important protective function of COX-2 in the intestine ⁷. Furthermore, several studies have shown that selective COX inhibition, with the use of NSAIDs, resulted in gut damage in both animal models of IR and models for enterocolitis ¹¹⁻¹³. In contrast, Moses et al. showed that TLR4-mediated COX-2 expression increased intestinal IR-induced damage despite being unable to identify a possible TLR4 ligand, thus admitting to the involvement of additional mechanisms to IR-induced damage ⁶.

In patients undergoing intestinal surgery, non-steroidal anti-inflammatory drugs (NSAIDs) led to an impaired intestinal wound healing, resulting in a higher incidence of anastomotic leakage and subsequent complications ¹⁴⁻¹⁶. Furthermore, inhibition of COX-2 by the use of NSAID's led to increased intestinal damage in healthy volunteers during exercise-induced small intestinal injury and induces gut barrier dysfunction ¹⁷.

This led us to investigate the controversial role of COX-2 in intestinal IR. Mice underwent laparotomy and segmental ischemia of different duration was induced in both wildtype and COX-2 knockout mice, comparable to mesenteric ischemia in patients with the potential to recover from reversible intestinal damage. Our hypothesis was that COX-2 is of importance in restorative capacity of the gut after short periods of small intestinal

ischemia followed by reperfusion and that this restorative mechanism fails during prolonged periods of ischemia.

Materials and Methods

Ethics statement

All animal experiments were approved by the Maastricht University Animal Experiments Committee (DEC 2010-176).

Animal studies

COX-2^{+/-} mice were obtained from the Jackson Laboratory (Bar Harbor, United States) to produce littermate wild type (WT) and COX-2^{-/-} (KO) mice. For all experiments, COX-2^{-/-} mice were used simultaneously with their wild type littermates resulting in various group sizes. Mice were housed under controlled conditions of temperature and humidity. During all experiments, mice had unlimited access to chow and drinking water. 10-12 weeks old male and female mice were randomly divided into eight groups of 10 to 14 mice and exposed to the experimental protocol, as described below. Animals were anesthetized with isoflurane (induction 4%, maintenance 1,5%), and bupivacaine was used for analgesia. Body temperature was maintained at 37°C by a heating lamp until animals had recovered from anesthesia.

Experimental IR-protocol

Following midline incision, a 6 cm-jejunal segment was isolated and ligated at both ends. The segment was subjected to 30 (30I) or 60 (60I) minutes of ischemia by placing atraumatic vascular clamps across the mesentery. After ischemia, the animal was sacrificed and the isolated segment resected (30I/60I), or the clamp was removed to allow reperfusion for 30 minutes (30R) or 24 hours (24hR). Reperfusion was confirmed by regaining of the normal pink color and restoration of gut motility. A proximal jejunal segment which remained untreated during the I/R experimental protocol was resected and served as internal control tissue (N=12). The researchers were blinded for the genotypes at the time of surgery.

Histology

Tissue specimens obtained during the experimental protocol were immediately immersed in 4% formaldehyde fixative (Unifix, Klinipath, Duiven, the Netherlands), incubated overnight at room temperature and stored in 70% ethanol. Next, tissue samples were embedded in paraffin and 4-μm sections were cut. For morphological analysis, sections were stained with hematoxylin and eosin (H&E) to determine small

intestinal damage. To investigate neutrophil influx in the small intestine, myeloperoxidase (MPO) staining was performed on tissue sections.

Hematoxylin and eosin staining

Slides were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Next, slides were stained with H&E, dried and mounted in Entellan (Merck, Darmstadt, Germany). Stained sections were photographed using a Nikon eclipse E800 microscope equipped with a Zeiss Digital Camera AxioCam MRc5 (200x magnification). Intestinal epithelial damage was scored according to the Chiu/Park Scoring System ¹⁸. This was performed by two blinded independent members of the research team in five representative microscopic fields per slide (200x magnification).

Immunohistochemistry

As influx and accumulation of neutrophils beneath the damaged epithelial lining is one of the hallmarks of intestinal IR induced injury and inflammation ⁵, MPO staining, a marker abundantly expressed in neutrophils, was performed to analyze differences in inflammation between the groups.

After deparaffinization and rehydration of paraffin-embedded sections, endogenous peroxidase activity was blocked using 0,6% hydrogen peroxide in methanol for 15 minutes. Non-specific binding was blocked by 10% normal goat serum (NGS) in Phosphate buffered saline (PBS) and 1% bovine serum albumin. The following primary antibodies were used: polyclonal antibody rabbit anti-human myeloperoxidase (MPO) (1:500 dilution; Hycult Biotech, Uden, The Netherlands). Both antibodies showed cross-reactions with mice. After washing, the biotin-labeled polyclonal secondary antibody swine anti-rabbit (1:500 dilution; Dako, Glostrup, Denmark) was used followed by incubation with the streptavidin-biotin system (1:50, DakoCytomation). Staining was visualized with 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO) and counterstained with hematoxylin (Merck). Stained sections were photographed using a Nikon eclipse E800 microscope equipped with a Zeiss Digital Camera AxioCam MRc5. No staining was detected in sections incubated without primary antibody. Neutrophil influx was quantified by 2 independent blinded observers counting all MPO-positive cells per villus-crypt axis in five representative microscopic fields per slide (200x magnification) of stained control tissue and tissue exposed to IR.

Statistics

Statistical analysis was performed using Prism 5.0 for Windows (GraphPad Software Inc. San Diego, California, USA). Normality of all data was verified by the Kolmogorov-Smirnov test. All data are presented as mean $\pm$ SEM. A Dunn post hoc test was used (after significant 1-way ANOVA) to compare differences in intestinal injury score and MPO positive cells count over time in each genotype (WT or COX-2 KO mice) and each

model (30I or 60I). For between-group comparisons at similar time points, a 1-way ANOVA with a Dunn post hoc test was used as well. For comparisons between WT and COX-2 KO mice at similar time points in each model, a 2-tailed Mann Whitney U test was used. A *P* value <0.05 was considered statistically significant.

Results

COX-2 status and the severity of IR injury of the small intestine

In wild-type mice, IR induced severe small intestinal damage was characterized by the appearance of sub-epithelial spaces (30I) or disruption of the mucosal epithelium (60I) directly after ischemia. During 30 minutes of reperfusion, damaged epithelial cells were shed into the lumen, which resulted in the breakdown of the epithelial lining in both the 30I and the 60I group. After 24 hours of reperfusion, the epithelial lining was restored with some shortening of the villi in both groups (30I and 60I). No mucosal damage was observed in control tissue (Figure 9.1A).

Compared to wild-type mice, COX-2 KO mice exhibited more severe IR induced intestinal injury, with some degree of intestinal damage already noticeable in control-tissue. After 30I, large sub-epithelial spaces were observed at the villus tips whereas 60I already led to complete disruption of the intestinal epithelial lining. 30 minutes of reperfusion caused massive shedding of ischemically damaged villus tips into the lumen leading to further destruction of the villi in COX-2 KO mice. After 24 hours of reperfusion the epithelial lining was still disrupted in the 60I group (Figure 9.1A), whereas the 30I group showed recovered epithelial lining.

Intestinal damage as a result of IR was scored using the Chiu/Park Scoring System in at least five representative microscopic fields per slide (200x magnification) .

Statistical evaluation of the histological grading scores showed no differences between wild-type and COX-2 KO mice in control tissues (Figure 9.1B). Histological grading scores at 30I were significantly higher in COX-2 KO mice compared to wild-type mice (1.40 ± 0.20 vs. 1.95 ± 0.05 , $P < 0.05$; Figure 9.1B). Remarkably no significant difference was seen between wild-type and COX-2 KO mice at 60I.

Next, during 30I30R COX-2 KO mice again showed statistically significant more histological damage compared to wild-type mice (1.95 ± 0.10 vs. 3.07 ± 0.67 , $P < 0.05$; Figure 9.1B). This difference was not seen during 60I30R. After 24 hours of reperfusion no significant differences were observed between COX-2 KO mice and wild-type mice in both the 30I and 60I group.

Duration of ischemia and the extent of tissue damage

After 30I, no significant increase of the intestinal injury score was observed when compared with their own control tissue in wild-type as COX-2 KO mice (Figure 9.1B).

Figure 9.1 Evaluation of IR-induced small intestinal injury in wild-type and COX-2 KO mice during short (30I) and prolonged (60I) periods of ischemia

A, Hematoxylin and eosin (H&E) staining showed intact villi at control. IR-induced intestinal damage was characterized by the appearance of sub-epithelial spaces (30I, arrowhead) or disruption of the mucosal epithelium (60I, arrowhead) directly after ischemia. During 30 minutes of reperfusion, damaged epithelial cells are shed into the lumen, which result in de breakdown of the epithelial lining in both the 30I as the 60I group (arrowheads). After 24 hours of reperfusion, the epithelial lining is restored with some shortening of the villi in both groups **B**, Comparison of the intestinal injury scores between wild-type and COX-2 KO mice that were subjected to IR-injury. Each column represents the mean $\pm$ SEM. * Significant compared with control (30I wild-type). # Significant compared with control (30I COX-2 KO). ^ Significant compared with control (60I wild-type). † Significant compared with control (60I COX-2 KO). I indicates ischemia; Rep: reperfusion; 30I: 30 minutes of ischemia; 60I: 60 minutes of ischemia; WT: wild-type; -/-: knock-out.

Figure 9.2 Neutrophil influx during IR-induced intestinal injury in wild-type and COX-2 KO mice

A, Neutrophil influx was visualized by MPO staining after IR. Control tissue showed no neutrophil influx. At 30I and 60I, little influx of neutrophils was observed (arrowheads). At 30R, neutrophil influx was increased (arrowheads). After 24hR, the intestine was slightly recovered from the IR-induced damage and neutrophil influx was normalized. **B**, Quantification of neutrophil influx between wild-type and COX-2 KO mice that were subjected to intestinal IR injury. Each column represents the mean $\pm$ SEM. # Significant compared with control (30I COX-2 KO). ^ Significant compared with control (60I wild-type). † Significant compared with control (60I COX-2 KO). I indicates ischemia; Rep: reperfusion; 30I: 30 minutes of ischemia; 60I: 60 minutes of ischemia; WT: wild-type; -/-: knock-out.

During 30I30R, both wild-type and COX-2 KO mice showed increased intestinal injury scores when compared with control (0.01 ± 0.00 vs. 1.95 ± 0.10 and 0.47 ± 0.07 vs. 3.07 ± 0.67 respectively, $P < 0.05$; Figure 9.1B). At 30I24hR, no differences in intestinal injury scores were observed.

60I led to increased histological grading scores in both wild-type as COX-2 KO mice when compared with control tissue (0.01 ± 0.00 vs. 3.50 ± 0.22 and 1.33 ± 0.62 vs. 3.83 ± 0.17 respectively, $P < 0.05$; Figure 9.1B). After 60I30R, intestinal injury scores continued significantly increased at $4.00 (\pm 0.32)$ in wild-type mice and $3.97 (\pm 0.03)$ in COX-2 KO mice (Figure 9.1B). At 24hR no significantly elevated intestinal injury scores were seen compared with control (Figure 9.1B).

Wild-type mice in the 60I group suffered from more intestinal damage compared to the wild-type mice in the 30I group (1.40 ± 0.20 vs. 3.50 ± 0.22 , $P < 0.05$; Figure 9.1B). This effect was also observed during 30 minutes of reperfusion (1.95 ± 0.10 vs. 4.00 ± 0.32 , $P < 0.05$; Figure 9.1B). 24hR resulted in restoration of the intestinal mucosa and normal intestinal injury scores.

Interestingly, no differences were noticed between COX-2 KO mice subjected to either 30 or 60 minutes of ischemia at all time points.

COX-2 status and neutrophil infiltration during development of small intestinal IR injury MPO activity, a marker of neutrophil infiltration, was visualized using immunohistochemistry (Figure 9.2A). Control tissue showed no neutrophil influx. At 30I and 60I, little influx of neutrophils was observed. 30 minutes of reperfusion led to increased neutrophil influx in the villi. At 24hR, the intestine was recovering from the IR-induced intestinal damage and neutrophil influx was again normalized.

Neutrophil infiltration was quantified by two independent blinded observers counting all MPO-positive cells per villus-crypt axis in five representative microscopic fields of stained control tissue and tissue exposed to IR. Statistical analyses revealed no significant differences between wild-type and COX-2 KO mice at control and directly after 30 and 60 minutes of ischemia (Figure 9.2B). COX-2 deficiency markedly increased intestinal MPO-positive cells per villus-crypt axis after 30I30R compared to wild-type mice (1.86 ± 0.15 vs. 4.65 ± 0.48 , $P < 0.05$; Figure 9.2B). On the other hand, MPO activity in COX-2 KO mice was similar to that in wild-type mice during 60I30R, showing similar numbers of neutrophils infiltrating the intestinal mucosa (Figure 9.2B). After 24 hours of reperfusion the neutrophil influx was reduced towards the levels of control tissue in all groups.

Duration of ischemia and neutrophil infiltration

Quantification of MPO-positive cells showed no significant increase of neutrophil influx in wild-type mice subjected to a short period of 30 minutes throughout the IR-experiment when compared with control tissue (Figure 9.2B). In COX-2 KO mice only

30I30R led to an increase of neutrophils per villus-crypt axis when compared with their own control tissue (0.48 ± 0.32 vs. 4.65 ± 0.48 , $P < 0.05$; Figure 9.2B). 60I30R causes a significant increase of infiltrating neutrophils in both wild-type as COX-2 KO mice in comparison with control (1.87 ± 0.79 vs. 8.10 ± 2.01 and 1.89 ± 0.52 vs. 6.41 ± 2.12 respectively, $P < 0.05$; Figure 9.2B).

Prolonged ischemia of 60 minutes resulted in an increased neutrophil influx in wild-type mice after 30 minutes of reperfusion in comparison with wild-type mice subjected to 30I30R (1.86 ± 0.15 vs. 8.10 ± 2.01 , $P < 0.05$; Figure 9.2B).

Discussion

This is a pilot report on the role of COX-2 during intestinal IR in a mouse model. For the first time, differences in IR-induced damage and inflammation between wild-type and COX-2 KO mice subjected to either short or prolonged periods of ischemia have been investigated. Results in this study indicate that COX-2 deficiency aggravates intestinal damage after a short period of 30 minutes of ischemia. Following 30 minutes of reperfusion a lack of COX-2 exhibited even more intestinal injury than was seen in wild-type mice. At this time-point, the amplified damage was accompanied by increased neutrophil infiltration in COX-2 KO mice compared to wild-type mice. Remarkably, although prolonged periods of 60 minutes of ischemia led to markedly more intestinal damage and neutrophil influx, no difference were observed between wild-type and COX-2 KO mice. Thus, these results support the hypothesis that COX-2 is of importance in restorative capacity of the gut after short periods of small intestinal ischemia followed by reperfusion and that this restorative mechanism fails during prolonged periods of ischemia.

The role of COX-2 is somewhat disputed with various studies demonstrating a protective function for COX-2 during intestinal IR, as well in models of sepsis and colitis ^{7,8,19,20}. In other models of intestinal IR, COX-2 inhibition led to attenuation or no differences on intestinal damage and inflammation ⁶. One reason for this different outcomes is the complexity of the COX pathway. Knockout of one of the COX enzymes may result in compensatory upregulation of the other COX isoform. Also, Blikslager et al. used a prolong ischemia model of 45 minutes. When compared with our data of the 60 min ischemia group, the duration of ischemia could very well explain why there is no effect of COX-2 deficiency on IR-induced injury and inflammation in mouse intestine seen at this time-point. The ischemic hit seems a little too grave, failing all restorative cellular mechanisms. Previous studies have shown that the degree of intestinal damage is related to the duration of the ischemic period, with irreversible intestinal damage after more than 30 minutes of ischemia ^{1,21}. In line with these studies, the current data demonstrate that 60 min of ischemia indeed increase the amount of intestinal histological damage and neutrophil influx compared to the short period of 30 min of ischemia in wild-type mice.

Next, previous studies suggest that the role of COX-2 in intestinal recovery from ischemic injury lies in the COX-elaborated prostanoids as prostaglandin E2 (PGE2). Interestingly, exogenous PGE2 administration reduced the severity of injury to baseline levels, indicating COX-2 derived PGE2 is protective against IR injury of the small intestine ²⁰. This could be a starting point in the development of new medical therapies that can limit intestinal IR damage.

An important limitation of the current study is that the preliminary results we presented contain a lot of variation. Since this is a pilot study with relatively small group sizes and limited conducted experiments, this could clarify the observed variations. For now, conclusions should be drawn with caution before follow-up experiments have been conducted.

In conclusion, this short communication suggest that depletion of COX-2 led to the aggravation of intestinal damage and inflammatory response in small intestine after IR. Further studies aimed at identifying specific pathways that are responsible for COX-2 induction are needed to develop useful therapies for intestinal IR injury.

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Chapter 10

Cyclooxygenase-2 is essential for colorectal anastomotic healing

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Abstract

Objective: To study the effects of COX-2 on colonic surgical wound healing.

Background: Cyclooxygenase-2 (COX-2) is a key enzyme in gastrointestinal homeostasis. COX-2 inhibitors have been associated with colonic anastomotic leakage.

Methods: Wildtype, COX-2 knockout and COX-2 heterozygous mice were subjected to a model of colonic anastomotic leakage, and were treated with vehicle, diclofenac, or prostaglandin E2 (PGE2), the most important COX-2 product in the intestine. We assessed anastomotic leakage, mortality, angiogenesis, and inflammation. Furthermore, we investigated the association between anastomotic leakage and a human polymorphism of the COX-2 gene resulting in low COX-2 levels.

Results: Diclofenac, a nonsteroidal anti-inflammatory drug inhibiting COX-2, increased anastomotic leakage compared to vehicle-treated mice (100% vs 25%, respectively). Similarly, 92% of COX-2-deficient mice developed anastomotic leakage ($P=0.003$) compared to WT. PGE2 partly rescued this severe phenotype because only 46% of PGE2-administered COX-2 knockout mice developed anastomotic leakage ($P=0.02$). This may be related to decreased neovascularization, because decreased CD31 staining, indicating less blood vessels, was observed in COX-2^{-/-} mice (2 vessels/mm² vs. 6 vessels/mm² in controls ($P=0.03$)). This effect could partly be reversed by administration of PGE2 to COX-2^{-/-} mice. No significant differences in inflammation were found. PTGS2-765G>C polymorphism in humans, associated with reduced COX-2 expression, was associated with higher anastomotic leakage rates.

Conclusions: COX-2-induced PGE2 production is essential for intestinal wound healing after colonic surgery, possibly via its effects on angiogenesis. These data emphasize that COX-2 inhibitors should be avoided after colonic surgery, and administration of PGE2 might be favourable for a selection of patients.

Introduction

The enzyme cyclooxygenase-2 (COX-2), also known as prostaglandin-endoperoxide synthase 2 (Ptgs2), plays an important role in gut homeostasis. In general, cyclooxygenase regulates the conversion of arachidonic acid into prostaglandins, of which prostaglandin E2 (PGE2) is reported to restore intestinal integrity in experimental models of intestinal inflammation and damage ¹⁻⁵. COX-2-induced production of prostaglandins in mesenchymal stem cells in the colon have an immunomodulatory role ², and these mesenchymal stem cells may thereby act as monitors of the colonic environment. In addition, they might have important functions in colonic wound healing ⁵. Furthermore, COX-2 expression is induced in macrophages and myofibroblasts upon exposure to proinflammatory cytokines and bacterial products, leading to proliferation and protection against apoptosis ^{2,3,6}. Lastly, COX-2 and PGE2 production by endothelial cells are critically involved in vascular endothelial growth factor (VEGF)-induced angiogenesis ^{7,8}.

Data from human studies underline the importance of COX-2 in colonic wound healing as the use of nonsteroidal anti-inflammatory drugs (NSAIDs), especially those with strong COX-2-inhibiting properties, is correlated with the development of anastomotic leakage after colorectal surgery ⁹⁻¹². The incidence of anastomotic leakage requiring re-intervention is around 7% ¹³, making anastomotic leakage the primary complication requiring re-intervention after colonic surgery. Anastomotic leakage carries a high mortality of 15% ¹⁴. The pathophysiology of anastomotic leakage remains largely unknown and is supposedly multifactorial.

Studies showing increased anastomotic leakage due to NSAID use are, however, retrospective in nature and a recent meta-analysis could not prove an unambiguous detrimental effect of NSAIDs ¹⁵. Although these studies suggest to restrict NSAID use in patients with intestinal anastomoses, omission of NSAIDs from postsurgical care in these patients is not yet standard clinical practice ^{16,17}.

Because anastomotic healing requires adequate perfusion of the anastomosis and anastomotic healing seems to be affected by the use of NSAIDs, we set out to investigate the role of COX-2 in the development of anastomotic leakage post colonic surgery. We provide evidence that COX-2 is essential for neovascularization of the colonic anastomosis and thereby plays a crucial role in colonic anastomotic wound healing. Both pharmacological and genetic disruption of COX-2 function, by use of NSAIDs or COX-2^{-/-} mice respectively, increased colonic anastomotic leakage significantly after colonic surgery in mice, which was associated with severely decreased survival.

Importantly, we additionally show that humans with decreased COX-2 expression due to a COX-2 gene polymorphism show increased risk for developing anastomotic leakage. These results indicate that COX-2 is essential in colonic anastomotic healing.

Methods

Mice

All animal experiments were approved by the Maastricht University Animal Experiments Committee. Mice heterozygous for COX-2 were ordered from Jackson Laboratory (Bar Harbor, ME) to obtain littermate wildtype (WT, $n=32$), COX-2^{+/−} ($n=17$) and COX-2^{−/−} ($n=25$) mice. For all experiments, COX-2^{−/−} mice were used simultaneously with their wildtype and/or heterozygous littermates.

Model

A previously described murine model of colonic anastomotic leakage was used and adapted to reach an anastomotic leakage rate of 25% to 33%¹⁸. Briefly, 10- to 12 week-old mice were anesthetized using isoflurane and were given buprenorphine as analgesic. After a 1 cm midline laparotomy, the cecum was exteriorized and the right colon was microscopically transected, without damaging blood supply. An end-to-end anastomosis was performed with 7 interrupted sutures (Prolene 8–0, Ethicon, Somerville, NJ). The colon was repositioned and the abdomen was closed in 2 layers of interrupted sutures (Vicryl 4–0, Ethicon, and Ethilon 4–0, Ethicon, respectively). The colon was kept moist with sterile saline during the procedure. Two medically trained researchers that had equal skill and experience with the surgical procedure performed the surgical procedure. There was no difference in the rate of anastomotic leakage between the 2 surgeons. The researchers were blinded for the genotypes at the time of surgery.

Study design

WT, COX-2^{+/−} and COX-2^{−/−} mice underwent laparotomy with colonic anastomosis, and received vehicle Phosphate-buffered saline (PBS), 16,16-dimethyl PGE2 (dmPGE2, Cayman Chemical, Ann Arbor, MI), a stable analogue of PGE2, or diclofenac sodium (Cayman Chemical) by intraperitoneal injection. Dosage of dmPGE2 was 100mg/kg bodyweight, twice daily, and dosage of diclofenac was 10 mg/kg bodyweight, twice daily^{5,19}. This diclofenac dosage was comparable to dosages used in humans when using the dose translation formula published by Reagan-Shaw et al²⁰. Vehicle, dmPGE2 and diclofenac treatment was started 1 day prior to laparotomy and continued until the end of the experiments (Figure 10.1). Mice were killed at 5 days postoperatively to ensure detection of anastomotic leakage, or when humane endpoints were reached.

Endpoints

Anastomotic leakage was defined as either fecal peritonitis or abscess formation around the anastomosis. Two independent investigators who were blinded for genotype at the time of evaluation diagnosed anastomotic leakage.

Tissue preparation

A 1 cm segment of colonic tissue surrounding the anastomosis was dissected in longitudinal direction, thereby dividing it into equal parts. One part was fixed overnight in 10% formalin and embedded in paraffin using standard techniques. The second part was snap frozen in liquid nitrogen and used for mRNA isolation.

Immunohistochemistry

For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Endogenous peroxidase activity was blocked using 0.6% hydrogen peroxide in methanol for 30 minutes. Nonspecific antibody binding was blocked using 5% bovine serum albumin in PBS [myeloperoxidase (MPO) and CD31 staining] or 10% normal rabbit serum (MAC-3 staining). Antigen retrieval was required for CD31 immunohistochemistry (IHC) [0.1% trypsin (Difco Laboratories, Detroit, MI) in 0.1% CaCl₂ solution for 20 minutes at 37°C] and MAC 3 IHC (10mM sodium citrate, pH 6.0). Sections were incubated with primary antibodies rabbit antihuman MPO antibody (MPO, Dako-Cytomation, Glostrup, Denmark), which cross-reacts with mouse, rat anti-mouse CD31 (BD Pharmingen, Breda, The Netherlands), or rat anti mouse MAC-3 (BD). After washing, biotin-conjugated swine anti-rabbit IgG (DakoCytomation), biotinylated rabbit anti-rat IgG (DakoCytomation), or biotin-conjugated rabbit anti-rat IgG (Jackson ImmunoResearch, West-Grove, PA) was used for MPO, CD31, and MAC-3 IHC, respectively, followed by incubation with the streptavidin-biotin-HRP system (DakoCytomation), Brightvision poly HRP anti-rabbit IgG (Immunologic, Duiven, The Netherlands), or streptavidin-biotin-HRP system (Vector Laboratories, Burlingame, CA), respectively. Binding of primary antibody was visualized with 3,30-diaminobenzidine-tetrahydrochloride-dihydrate (Sigma, St Louis, MO) and counterstained with hematoxylin. No staining was detected in slides incubated without primary antibody. Stained sections were photographed by a Nikon eclipse E800 microscope with a Nikon digital camera DXM1200F. The CD31 staining was quantified in a blinded way by 2 observers by counting all CD31 positive vessels at 200x magnification and expressed as

the number of vessels per total area (mm²) using ImageJ (NIH Software, Bethesda, MD). Staining of MPO was quantified by ImageJ as well and staining of MAC-3 was quantified by 2 independent blinded observers using computerized morphometry (Leica QWin V3, Cambridge, UK). The results were presented as neutrophils per field of view (MPO) or percentage of positive cells per total tissue area (MAC-3).

Real-time Quantitative Polymerase Chain Reaction

RNA was isolated from snap-frozen anastomotic tissue samples with AllPrep DNA/RNA/Protein kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In short, samples were crushed with a pestle and mortar in liquid nitrogen. Disruption and homogenization of the tissue was performed using an Ultra Turrax Homogeniser (IKA Labortechnik, Staufen, Germany) in lysis buffer containing β -mercaptoethanol (Promega, Madison, WI). RNeasy spin columns were used to bind RNA. Samples were treated with DNase (Promega) to ensure complete removal of genomic DNA. Columns were washed and RNA was eluted in RNase-free water. RNA quantity was measured using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Only RNA samples with a clearly visible S28 and S18 on agarose gel were considered as intact RNA and were used. Total cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCR reactions were performed on 10 ng cDNA with 300 nM of gene-specific forward and reverse primers and 1X Absolute qPCR SYBR Green Fluorescein Mix (Bioline, London, United Kingdom) using the MyIQ system (Bio-Rad). Sequence of primers for VegfA were: forward TATTCAGCGGACTCACCAGC, reverse CCTCCTCAAACCGTTGGCA). Gene expression levels were calculated with IQ5 software using a Δ Ct relative quantification model. The geometric mean of 2 internal control genes (β 2-microglobulin and cyclophilin A) was calculated and used as a normalization factor.

Human tissue collection

From 148 consecutive patients who underwent oncologic colorectal surgery, paraffin-embedded colonic or rectal tissue sections were collected from the pathology department's database for DNA extraction. In all cases, surgery with primary anastomosis was performed in a single non-academic centre with a dedicated team of colorectal surgeons from January 2010 until August 2011. Data on clinical outcome, including development of anastomotic leakage, had been electronically and prospectively registered in detail for nationwide audit purposes.

DNA isolation from human tissue

Genomic DNA was obtained from formalin-fixed paraffin-embedded tissue using either a QIAamp DNA FFPE Tissue Kit (Qiagen) or an in-house protocol. In short, 4 tissue sections of 8 μ m per sample were deparaffinized, and samples were lysed under

denaturing conditions with proteinase K (at least 3 h at 55°C). After centrifuging multiple times, pure genomic DNA was obtained and concentration was determined using Nanodrop. To obtain more purified DNA, all samples were twice precipitated with sodium acetate, both after the amplification and the restriction step.

PTGS2-765G>C Genotyping

A common promoter variant in the COX-2 gene, -765G>C, leads to lower expression of COX-2 as the -765C allele, compared with the -765G allele, reduces promoter activity²¹. In this study, it was also demonstrated that the -765C allele correlates with lower C reactive protein levels. Therefore, the presence of -765G>C may have significant clinical implications. We hypothesized that the PTGS2-765G>C polymorphism would be associated with an increased risk of anastomotic leakage through lower promoter activity of the COX-2 gene. Amplification of this region was performed using specific forward and reverse primers (CCGCTTCCTTTGTCCATCAG and GGCTGTATATCTGCTCTATATGC, respectively). Subsequently, AclI restriction enzyme (New England Biolabs, Ipswich, MA) was applied (60 min incubation at 37°C, 20 min inactivation at 65°C) resulting in both a 188 base pair product and a 118 base pair product in case of a -765G allele, or an uncut 306 base pair product in case a -765C allele. The presence of homozygous PTGS2-765G>C polymorphism (-765CC) was then correlated to anastomotic leakage.

Statistics

Statistical analysis was performed using Prism 5.0 for Windows (Graphpad software, Inc, San Diego, CA) and SPSS 20.0 for Windows (SPSS Inc, Chicago, IL). Normality was tested using Kolmogorov-Smirnov. All continuous variables are presented as mean and standard error of the mean and compared using student t test. Dichotomous variables were compared using chi-square test. Survival was analyzed by logrank test.

Results

Diclofenac increases colonic anastomotic leakage in an experimental colonic surgery model

To study whether the observed retrospective human data on colonic anastomotic leakage in patients receiving COX-2 inhibitors were also observed in mice, anastomotic leakage rates in mice receiving either vehicle or NSAIDs were determined. Three of 11 mice in the wildtype group developed anastomotic leakage (27%), which is consistent with previous studies using this experimental model. Intriguingly, all mice in the diclofenac group ($n=9$, 100%), however, developed anastomotic leakage, $P=0.001$ (Figure 10.2A). As expected, survival was reduced in the group receiving diclofenac compared with the group receiving vehicle [hazard ratio (HR) 17.9 (95% confidence interval (CI), 3.7–87.4), $P<0.001$, Figure 10.2B). These data confirm that NSAIDs in this experimental mouse model also have detrimental effects on anastomotic healing, making it a useful model to study the deleterious effects of NSAIDs in more detail.

Figure 10.2 Effect of diclofenac on experimental anastomotic healing

A, Incidence of anastomotic leakage in mice treated with vehicle ($n=11$) and mice treated with diclofenac ($n=9$). **B**, Survival of mice treated with vehicle and mice treated with diclofenac. Mice treated with diclofenac showed higher anastomotic leakage incidence and mortality rates.

Cyclooxygenase 2 is critically involved in colonic anastomotic healing

Because we hypothesized that the adverse effects of NSAIDs were particularly a consequence of COX-2 inhibition, we further investigated the selective effects of COX-2 in anastomotic healing using COX-2^{-/-} mice. Eleven of 12 COX-2^{-/-} mice (92%) had macroscopic leakage compared with only 27% of WT mice ($P=0.003$, Figure 10.3A). Survival was reduced in COX-2^{-/-} mice compared to WT mice [HR, 3.5 (95%CI, 1.2–10.3), $P=0.02$, Figure 10.3B). Because COX-2 is essential for the production of PGE₂, which is an important mediator in gut homeostasis, we investigated whether PGE₂ administration

could rescue COX-2^{-/-} from anastomotic leakage. Indeed, supplementation with dmPGE2 halved the anastomotic leakage rate in COX-2^{-/-} mice from 92% (11 of 12 mice) to 46% (6 of 13 mice), *P*=0.02 (Figure 10.4).

Figure 10.3 Effect of genotype on experimental anastomotic healing
A, Incidence of anastomotic leakage in wildtype (n=11), COX-2^{+/-} (n=17), and COX-2^{-/-} (n=12) mice. **B**, Survival of wildtype and COX-2^{-/-} mice. COX-2^{-/-} mice showed higher anastomotic leakage and mortality rates compared to wildtype mice.

Figure 10.4 Effect of PGE2 administration on anastomotic leakage rates in wildtype (n=12) and COX-2^{-/-} (n=13) mice PGE2 administration decreased anastomotic leakage rates in COX-2^{-/-} mice.

Lack of COX-2 does not lead to inflammatory changes in anastomotic tissue

Having determined a clear role for COX-2 in anastomotic healing, we set out to dig further into the mechanisms involved in COX-2-dependent impaired anastomotic healing. One of the obvious mechanisms that could be disturbed by blocking COX-2 function is the inflammatory response, which is critical in the first phases of wound healing. In the early phase of wound healing, neutrophils are the predominant cell type; however, no significant differences were observed in influx of MPO-positive cells between WT and COX-2^{-/-} mice (Figure 10.5A). On average 148 cells/field of view were found vs. 188 cells/field of view at 100x magnification, WT vs. COX-2^{-/-}, respectively (Figure 10.5B). Apart from neutrophils, macrophages are important COX-2 expressing inflammatory cells that also play a major role in wound healing. We observed no significant differences in the presence of MAC-3-positive cells in colonic anastomotic tissue of WT vs. COX-2^{-/-} mice. (Figure 10.5A).

Figure 10.5 Immunohistochemical staining of MPO

A, Sections of colonic anastomotic tissue stained with MPO. Upper panel: wildtype; lower panel: COX-2^{-/-}. Magnification 40x, inset 100x. **B**, Quantification of MPO staining of wildtype and COX-2^{-/-} mice. Data are presented as mean with SEM (number of positive cells per field of view at 100x magnification). We observed no differences in MPO staining between wildtype and COX-2^{-/-} mice.

On average a 25% vs. 23% ratio of positive staining/total tissue area, WT vs. COX-2^{-/-}, respectively (Figure 10.6B).

These data indicate that local cellular inflammatory responses after colonic surgery are not affected in COX-2^{-/-} mice, which may point toward a different underlying factor involved in the impaired anastomotic healing in COX-2^{-/-} mice.

Angiogenesis is impaired in anastomotic tissue of COX-2 knockout mice

Ischemia of the anastomosis is an important factor associated with the occurrence of anastomotic leakage, and (neo)vascularization of the wound region is critical for adequate wound healing²². Because COX-2 is well known to be involved in angiogenesis, we next assessed whether vascularization of the wound region was impaired in COX-2^{-/-} mice. Staining of CD31, an endothelial marker, was markedly reduced in anastomotic tissue of COX-2^{-/-} mice compared to control. Quantification of the amount of blood vessels showed that only 2 vessels/mm² were stained in

Figure 10.6 Immunohistochemical staining of MAC-3

A, Sections of colonic anastomotic tissue stained with MAC-3. Upper panel: wildtype; lower panel: COX-2^{-/-}. Magnification 40x, inset 100x. **B**, Quantification of MPO staining of wildtype and COX-2^{-/-} mice. Data are presented as mean with SEM (percentage of positive cells per total tissue area). We observed no differences in MAC-3 staining between wildtype and COX-2^{-/-} mice.

anastomotic tissue of COX-2^{-/-} mice compared to 6 vessels/mm² in wildtype mice, 5 days after surgery ($P=0.03$, Figure 10.7A, 10.7B). This effect could partly be reversed by administration of dmPGE2 to COX-2^{-/-} mice, because significantly higher numbers of blood vessels were observed in COX-2^{-/-} mice receiving dmPGE2 compared to COX-2^{-/-} receiving vehicle (4 vs. 2 vessels/mm², respectively, $P=0.03$, Figure 10.7B). Similarly, anastomotic tissue mRNA levels of VEGF were higher in wildtype mice compared to COX-2^{-/-} mice ($P=0.02$), and in COX-2^{-/-} mice receiving dmPGE2 compared to COX-2^{-/-} mice receiving vehicle ($P=0.03$).

Figure 10.7 COX-2^{-/-} mice exhibit less vascularization in colonic anastomotic tissue

A, Immunohistochemical staining of CD31 on sections of colonic anastomotic tissue. Upper panel: wildtype; lower panel: COX-2^{-/-}. Magnification 40x, inlay 100x. **B**, Quantification of MPO staining of wildtype and COX-2^{-/-} mice, and COX-2^{-/-} mice receiving PGE2. Data are presented as mean with SEM. CD31 expression was significantly lower in COX-2^{-/-} mice compared to wildtype mice and compared to COX-2^{-/-} mice receiving PGE2. **C**, Relative expression of VEGF mRNA in colonic anastomotic tissue of wildtype and COX-2^{-/-} mice, and COX-2^{-/-} mice receiving PGE2. Data are presented as mean with SEM. Relative VEGF mRNA expression was significantly lower in COX-2^{-/-} mice compared to wildtype mice and compared to COX-2^{-/-} mice receiving PGE2.

Humans with genetically impaired COX-2 expression show high anastomotic leakage incidence

Of 148 patients undergoing colorectal resection for malignancy, 7 were homozygous for the PTGS2-765G>C polymorphism. Three of 7 (43%) developed anastomotic leakage compared with 16 of 141 (11%) patients with the -765GC or -765GG genotype ($P=0.02$, Figure 10.8).

Figure 10.8 Effect of human genotype (a common promoter variant in the COX-2 gene, -765G>C) on anastomotic leakage incidence
-765CC (n=7) and -765GC pooled with -765GG (n=157) are depicted.

Discussion

This study shows that COX-2 is of critical importance in preventing anastomotic leakage after colonic surgery in a mouse experimental colonic anastomosis model using both a pharmacological and a genetic approach. Mice lacking COX-2 showed increased rates of anastomotic leakage and mortality, which could be partly counteracted by administration of PGE₂, supposedly the most important product of COX-2 in preserving gut homeostasis. Intriguingly, PTGS2-765G>C polymorphism in humans, associated with reduced COX-2 expression, was associated with higher anastomotic leakage rates.

Impaired angiogenesis in mice lacking COX-2 seems to be at least part of the explanation for increased anastomotic leakage rates and mortality. A correlation between the use of COX-2 blocking NSAIDs and impaired anastomotic healing after colorectal surgery has already been established in patients^{9,11,12}. However, these data are of retrospective nature and a fundamental role of COX-2 in gut healing after colorectal surgery has never been established. Manieri et al showed that COX-2 is critically involved in mucosal repair after intestinal biopsies⁵. Furthermore, abundant evidence exists showing that COX-2 and its derived prostaglandins stimulate intestinal cancer progression through enhanced angiogenesis and proliferation and by decreasing apoptosis²³⁻²⁵. Although these mechanisms should be counteracted to reduce cancer progression, they are indispensable in adequate wound healing. As shown by Binion et al, COX-2 and PGE₂ stimulate angiogenesis through VEGF production (by endothelial cells)⁸. Indeed, our study showed that mice lacking COX-2 have significantly lower amounts of CD31+ vessels in anastomotic tissue that was accompanied by lower levels of VEGF mRNA levels and was at least partly restored by PGE₂ administration.

The current study has several important implications. As suggested earlier by retrospective human data, the perioperative use of NSAIDs should be avoided in patients undergoing surgery with a bowel anastomosis. Furthermore, patients with mutations in the COX-2 gene may be particularly vulnerable to impaired intestinal wound healing and the subsequent risk of complications after colorectal surgery or other therapeutic hits impairing gut homeostasis. This was underlined in the current study by the increased anastomotic leakage risk in patients carrying 2 alleles of the PTGS2-765G>C polymorphism. Although the prevalence of the homozygous -765C gene is reported to be around 3%²⁶, selection and personalized treatment of these patients should be investigated, for example, the administration of prostaglandins such as PGE₂ in phases when adequate intestinal healing is crucial.

A number of methodologic remarks must be made when interpreting these observations. As with nearly all animal anastomotic leakage models, in the current model a mechanically insufficient anastomosis was created. However, these models provide an excellent and unique opportunity to study the mechanisms of intestinal wound healing,

while also providing clinical readouts, such as anastomotic leakage and mortality. Another limitation was the lack of a random computer-generated multiple-area analysis of our IHC data, which may have biased our observations. Furthermore, there could be differences between inflammatory mediators that were missed. Future studies should address this.

A discrepancy between previous human reports and our used model is that all mice receiving diclofenac developed anastomotic leakage. Although this underscores the importance of this pathway in anastomotic healing, such high anastomotic leakage rates were not observed in previous animal studies ^{27,28}. Blockage of both COX-2 and COX-1 by diclofenac, which is constitutively expressed in colonic tissue ²⁹, may be the reason for this effect.

The survival of mice with anastomotic leakage in the current study was remarkably low, with most mice dying within several days. For example, higher survival rates are reported for most cecal puncture and ligation protocols, although cecal puncture and ligation protocols inducing high-grade sepsis are reported to have similar survival rates ³⁰. This might indicate a high degree of necrosis; however, we did not find this on tissue sections. PGE2 administration did not fully reverse the incidence of anastomotic leakage in COX-2 knockout mice to wildtype levels. This could be explained by the fact that we only used PGE2 to counteract the effects of genetic lack of COX-2. However, other prostaglandins are converted from arachidonic acid by COX-2, as well. In particular, prostacyclin (PGI₂) is expressed in colonic tissue and may therefore play a role in anastomotic healing ⁵. Another explanation could be that the dose or frequency of PGE2 administration should be increased for optimal effects.

In conclusion, we show that COX-2 inhibition is deleterious for anastomotic healing after colonic surgery, which is mediated at least in part by PGE2 production. Mice lacking COX-2 show higher rates of anastomotic leakage and increased mortality. In addition, angiogenesis is significantly impaired in the absence of COX 2 and PGE2, providing clues for the mechanism by which COX-2 influences anastomotic healing. Importantly, we show that patients with a polymorphism that is associated with decreased COX-2 levels were more likely to develop anastomotic leakage, which further supports our conclusions. We suggest that until proven otherwise, the perioperative use of COX-2 inhibitors in colonic surgery should be replaced by alternative analgesics, and future studies should assess the potential of PGE2 administration prior to colonic surgery to reduce anastomotic leakage rates in a selection of patients.

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Chapter 11

The role of digestive enzymes in the pathophysiology of human intestinal ischemia-reperfusion: a pilot study

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Submitted

Abstract

Objective: To study the role of digestive enzymes in the inflammatory response associated with human intestinal ischemia-reperfusion (IR) injury.

Background: Intestinal IR is a potentially fatal abdominal emergency. Better insight in its pathophysiology is needed to improve therapeutic strategies and decrease mortality. Intraluminal pancreatic digestive enzymes may play a major role in this pathology. During intestinal IR, impaired epithelial barrier function is likely to allow pancreatic proteases to leak across the intestinal mucosal barrier initiating autodigestion and inflammation, resulting in systemic inflammatory response syndrome (SIRS) leading to shock and death.

Methods: A unique human experimental jejunal IR model is used, allowing to study 30 and 60 minutes ischemia followed by reperfusion, enabling to collect plasma and tissue samples at different time points. Damage to the epithelial lining (Hematoxylin and eosin) and mucus layer integrity (Periodic Acid Schiff/Alcian Blue) is assessed using histochemistry and quantified with ELISA (I-FABP). Neutrophil influx (myeloperoxidase) and quantitative polymerase chain reaction for TNF- α , IL-1 β , IL-6 and IL-10 were performed to measure the inflammatory response to IR. Proteases localization and activity in intestinal IR is measured using in situ tissue zymography and digestive enzyme activity assays.

Results: Short (30 minutes) periods of intestinal IR lead to a quick repair of the epithelial lining, resulting in an intact intestinal barrier. Prolonged (60 minutes) ischemia followed by reperfusion causes irreversible tissue damage to the epithelial lining with an impaired intestinal barrier. The intestinal barrier integrity loss as a result of IR-induced damage initiates a significant inflammatory response, whereas inflammation is limited after short intestinal IR. Next, damage to the intestinal barrier is observed simultaneously with increased digestive enzymatic activity in the wall of the intestine and is followed by increased appearance of activated protease levels in the circulation.

Conclusion: Pancreatic proteases penetrate the epithelial barrier/bowel wall following long jejunal IR and may trigger inflammation.

Introduction

Intestinal ischemia-reperfusion (IR) is a frequently observed phenomenon. It can occur as part of normal physiology when blood is redistributed from the intestines to muscle tissue during exercise ¹. Furthermore, intestinal IR is considered as a common pathway in several pathologies, where temporary flow reduction results from occlusive vascular disease (thrombosis, embolism), surgical states (aortic surgery, cardiopulmonary bypass) and other pathologies causing splanchnic hypoperfusion, including necrotizing enterocolitis, pancreatitis, septic or hemorrhagic shock ^{2,3}. Moreover, splanchnic hypoperfusion is thought to be an important player involved in the etiology and perpetuation of inflammatory bowel disease (IBD) ⁴.

Intestinal IR results in loss of physical (mucus-layer, epithelial lining) and immunological barrier function ^{5,6}. The intestinal barrier plays an essential role protecting the host from the external environment, including proteolytic degradation, while at the same time allowing the absorption of nutrients and water ⁷. Disruption of this delicate balance is the major event during prolonged intestinal IR, leading to translocation of intraluminal content into the bowel wall and systemic circulation, triggering inflammation ^{8,9}. This leads to systemic inflammatory response syndrome (SIRS) with multiple organ failure (MOF) ¹⁰, resulting in high mortality rates ranging from 60% to 90% ^{2,11}. It remains unclear what factor is the key player in the onset of systemic inflammation and MOF. The role of the microbiome has been studied extensively, however no interventions against bacteria have been successful in clinical trials ¹². A novel theory is that intestinal wall penetrating digestive enzymes present in the small intestine play a detrimental role ¹³. Recent animal studies indicate that these pancreatic enzymes are important factors in acute inflammatory processes resulting from intestinal IR, hemorrhagic and endotoxic shock ¹⁴⁻¹⁶. Serine proteases (e.g. trypsin, chymotrypsin, elastase) are the most abundant pancreatic digestive enzymes ¹⁷. To prevent auto-digestion, trypsin enters the intestinal lumen as the inactive zymogen trypsinogen, and is activated by duodenal brushborder enterokinases ¹⁸. Once activated, trypsin activates more trypsinogen and chymotrypsinogen. Under normal physiological conditions proteases are compartmentalized in the intestinal lumen by the intestinal barrier (mucus-layer and epithelial lining) to prevent digestion of the intestinal tissue itself ¹⁴. Furthermore, enzymes-inhibitors are abundantly present in the circulation to avoid uncontrolled proteases activity ¹².

The pathophysiological role of pancreatic digestive enzymes was discovered after adding protease-inhibitor intraluminally in rats, showing abrogation of the usual sequelae of intestinal IR, inflammation and shock ¹⁹. These results gave rise to the "autodigestion" hypothesis suggesting that proteases leak across the compromised intestinal mucosal barrier during IR, initiating self-digestion of the intestinal wall.

Digestive enzymes may even be able to penetrate into deeper muscle tissue layers of the intestinal wall and enter the systemic circulation ^{15,16}.

The role of pancreatic enzymes as initiator of inflammation upon small intestinal IR is solely studied in animal experimental models. However, a new human small intestinal IR model has allowed a better understanding of pathophysiology of jejunal IR in man ²⁰. These studies revealed, that 30 minutes of ischemia followed by 30 minutes of reperfusion (30R) results only in minor epithelial damage and inflammation, which is completely reversible after 120 minutes of reperfusion (120R) ^{21,22}. Prolonged ischemic periods of 60 minutes followed by 30R and 120R lead to irreversible damage to both the physical and immunological mucosal barrier, causing inflammation ⁹.

In search for new interventional targets to improve therapies and decrease mortality, it is crucial to comprehend the underlying mechanisms associated with human intestinal IR. As animal studies suggest that inhibition of proteases is beneficial, we aim to investigate the role of pancreatic digestive enzymes following both reversible and irreversible human intestinal IR.

Materials and Methods

Ethics

The study was approved by the Medical Ethical Committee of Maastricht University Medical Center (ref. NL12407.068.06 / METC 06-3-044) and was conducted according to the revised version of the Declaration of Helsinki (October 2013). All patients were informed about the experimental procedures and written informed consent of all patients was obtained prior to the surgical procedure.

Patients and Surgical Procedures

Patients

To investigate the role of digestive enzymes during intestinal IR 10 patients (4 female : 6 male, age: 66.2 range 49 to 78 years) undergoing pancreatico-duodenectomy for benign or malignant pancreatic or papillary disease were included in this study. Participants had no history of any underlying intestinal disease. The previously developed human intestinal IR model was applied ²⁰.

Human intestinal IR protocol

The experimental protocol was performed as previously described ²⁰. In short, during pancreatico-duodenectomy, a variable length of jejunum is routinely resected in continuity with the head of the pancreas and duodenum as part of the standard surgical procedure. The terminal 6 cm of this jejunal segment was isolated by transection using a linear cutting stapler (GIATM, Covidien/Medtronic, Eindhoven, the Netherlands). Next, the isolated segment was subjected to either 30 (30I, n=5) or 60 minutes (60I, n=5) of ischemia by placing two atraumatic vascular clamps across the mesentery containing the arteriole and venule draining the segment. Meanwhile, the surgical procedure proceeded as planned. After ischemia, one third (2 cm) of the isolated ischemic jejunum was resected using a linear cutting stapler. Next, clamps were removed to allow reperfusion, as confirmed by retrieval of normal pink color and restoration of gut motility. Another segment of the isolated jejunum (2 cm) was resected similarly after 30 minutes of reperfusion (30R), and 120 minutes of reperfusion (120R). Simultaneously, 2 cm of jejunum, which remained untreated during surgery, was resected and served as internal control tissue. This segment underwent similar surgical handling as the isolated part of the jejunum but was not exposed to IR. Arterial blood was sampled from the radial artery before ischemia, immediately after ischemia, and at 30R and 120R. Simultaneous with each respective arterial blood sample, blood was drawn from the venule draining the isolated jejunal segment to assess concentration gradients across the isolated jejunal segment.

Tissue and blood collection

Tissue samples, obtained at four different time points during the experimental protocol (control, after ischemia, after 30R and after 120R) were immediately formalin-fixed for histology and immunohistochemistry or rinsed in pre-chilled Phosphate Buffered Saline (PBS) and snap-frozen for quantitative polymerase chain reaction (qPCR) and immunofluorescence.

Blood samples were transferred to a pre-chilled EDTA or heparin vacuum tube (BD vacutainer; Becton Dickinson and Company, Breda, Netherlands), kept on ice and centrifuged twice at 3500 rpm, 4°C for 15 minutes to obtain plasma and immediately stored in aliquots at -80°C until further analysis.

Histology and Immunohistochemistry

Tissue samples were incubated overnight at room temperature in 4% formaldehyde fixative (Unifix, Klinipath, Duiven, the Netherlands). Subsequently tissue samples were embedded in paraffin and 4 µm sections were cut.

For histological analysis, sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water and stained with hematoxylin and eosin (H/E) and with Periodic Acid Schiff (PAS)/Alcian Blue (AB) to assess mucus layer integrity.

For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Endogenous peroxidase activity was blocked using 0.6% hydrogen peroxide in methanol for 15 minutes. Non-specific antibody binding was blocked and sections were incubated with specific primary antibody rabbit anti-human myeloperoxidase (MPO staining; DakoCytomation, Glostrup, Denmark) to visualize neutrophil influx. After repeated washing, biotin-conjugated swine anti-rabbit IgG (DakoCytomation) secondary antibodies were used, followed by incubation with the Avidin-Biotine system (Vector laboratories, Burlingame, CA). Binding of primary antibody was visualized with 3-amino-9-ethylcarbazole (AEC; Sigma, St.Louis, MO) and sections were counterstained with hematoxylin. No staining was detected in slides incubated without primary antibody. A Nikon eclipse E800 microscope photographed stained sections with a Zeiss Digital Camera AxioCam MRc5 (100x magnification). H&E staining and Alcian Blue PAS staining were analyzed qualitatively. Neutrophil influx was analyzed semi-quantitatively by two independent observers in a blinded way by counting MPO-positive cells in five representative microscopic fields (200x magnification). Interobserver variability was not statistically significant.

In-situ Tissue Zymography

To assess leakage of active proteases in the bowel wall during intestinal IR, cryostat sections (4µm) were fixed with 96% cold ethanol for 15 minutes and protease were (re)activated by incubation of the slides for 1 hour at 37°C in deionized water. Protease activity on tissue slides was detected via proteolytic cleavage of fluorescent BODIPY TR-

X casein (EnzChek™; Invitrogen, Carlsbad, CA) during 1 hour incubation at 37°C. Subsequently, sections were fixed with 4% formaldehyde fixative (Unifix) for 15 minutes and washed repeatedly in PBS. Finally, sections were counterstained using 4',6-diamidinol-2-phenylindole (DAPI) and mounted with fluorescence mounting solution (DakoCytomation). The tissue sections were evaluated with an immunofluorescence Nikon eclipse E800 microscope with a Zeiss Digital Camera AxioCam MRc5 (100x magnification).

Enzyme-Linked Immunosorbent Assay

To quantify the degree of IR-damage to the epithelial lining, plasma Intestinal Fatty Acid Binding Protein (I-FABP) concentrations were measured by means of an in-house Enzyme-Linked Immunosorbent Assay (ELISA). This ELISA was developed to measure I-FABP in human plasma samples with rabbit polyclonal antibodies, using purified human I-FABP as standard. The assay is capable of measuring I-FABP in the range of 12.5 to 800 pg/mL. Samples with a value of 800 pg/mL or more were diluted properly to determine the I-FABP level.

Plasma IL-6 levels were measured using ELISA to obtain insight in systemic inflammatory response. The IL-6 ELISA was performed as previously described. Monoclonal 5E1 (mouse anti-human IL-6) antibody was used as coating. Human recombinant IL-6 was used for standard titration curves. Biotinylated in-house polyclonal antihuman IL-6 was used as detection antibody. The detection limits for the assay were 15.625 pg/mL to 1000 pg/mL. Samples with a value of 1000 pg/mL or more were diluted properly to determine the IL-6 level.

All samples were run in duplicate and a variability of 5% between sample duplicates was accepted. I-FABP and IL-6 was determined in both arterial plasma and plasma derived from the vein directly draining the isolated jejunal segment, to allow for calculation of arteriovenous (V-A) concentration differences.

Plasma Protease Activity Assay

To measure possible systemic (serine) protease transport, plasma protease activity was determined in the samples taken from venule draining the ischemic jejunal segments using fluorescent BODIPY TR-X casein substrate (EnzChek™). Because of the high plasma concentrations of enzyme inhibitors, it was assumed that only very little, if any, active (unbound) protease would be recovered. Therefore exogenous trypsin from bovine pancreas (Sigma-Aldrich, St.Louis, MO) was dissolved in 1.0mM hydrogencchloride (HCl) and titrated into the venous plasma samples to create calibration curves for each sample. By subsequent addition of a fluorescent casein substrate (10 µg/mL, EnzChek™) and incubation at for 60 minutes at 37°C, a decrease in circulating protease inhibitor buffer capacity was measured as an index of the amount of leaked protease in plasma. Samples were measured at steady-state (excitation 590 nm, emission 625 nm). In every

experiment a negative control was taken by adding casein substrate to the Tris-HCl buffer alone, as well as a positive control consisting of a high level trypsin solution. Results were corrected for plasma autofluorescence (fluorescence of plasma without addition of trypsin) and blank values were subtracted. The left shifted points on each curve correspond to the concentration of added trypsin required to overcome endogenous plasma protease inhibitor buffer.

RNA isolation and quantitative PCR

RNA was isolated from snap-frozen tissue samples using AllPrep DNA/RNA/Protein kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In short, jejunum samples were crushed with a pestle and mortar in liquid nitrogen. Disruption and homogenization of the tissue was achieved using an Ultra Turrax Homogenizer (IKA Labortechnik, Staufen, Germany) in lysis buffer containing β -mercaptoethanol (Promega Corporation, Madison, WI). RNeasy spin columns were used to bind RNA. Columns were washed and RNA was eluted in RNase-free water. To analyze gene expression of tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β) interleukin-10 (IL-10 and interleukin-6 (IL-6), quantitative PCR (qPCR) was performed. RNA samples were treated with DNase (Promega Corporation) to ensure removal of contaminating genomic DNA. RNA quantity was measured using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, MA). Total cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCR reactions were performed in a volume of 20 μ l containing 10 ng of cDNA, 1x Sensimix SYBR Green (Bioline, London, UK) and 300 nM of gene-specific forward and reverse primers. The sequence of primers is provided Table 11.1. cDNA was amplified using a three-step program (40 cycles of 10s at 95°C, 20s at 60°C and 20s at 70°C) with a MyiQ system (Bio-Rad). Specificity of amplification was verified by melt curve analysis. Gene expression levels were determined using iQ5 software and a Δ Ct relative quantification model. The geometric mean of the expression of two internal control genes, RPLP0 and CYPA, was calculated and used as a normalization factor.

Statistics

Statistical analysis was performed using GraphPad Prism 5 for Windows (GraphPad Software Inc., San Diego, CA). Normality of all data was verified by the Kolmogorov-Smirnov test. None of the parameters showed a normal distribution. Dunn multiple comparison test was used (after significant 1-way analysis of variance) to compare values in time in each model. For between-group comparisons, the Mann-Whitney U test was used. All data are presented as mean $\pm$ standard error (SEM). A *P* value below 0.05 was considered statistically significant.

Table 11.1 Human Oligonucleotide Primer Sequences Used for qPCR Analysis

Gene Symbol	Forward Primer Sequence	Reverse Primer Sequence
<i>CYPA</i>	5'-CTCGAATAAGTTTGACTTGTGTTT-3'	5'-CTAGGCATGGGAGGGAACA-3'
<i>RPLP0</i>	5'-GCAATGTTGCCAGTGTCTG-3'	5'-GCCTTGACCTTTTCAGCAA-3'
<i>TNFA</i>	5'-TCAATCGGCCCCGACTATCTC-3'	5'-CAGGGCAATGATCCCAAAGT-3'
<i>IL-1B</i>	5'- CTGAGCTCGCCAGTGAAATG-3'	5'- TTTAGGGCCATCAGCTTCAAA-3'
<i>IL6</i>	5'- TCCAGGAGCCCAGCTATGAA-3'	5'- GAGCAGCCCCAGGGAGAA-3'
<i>IL10</i>	5'- GGCCTGTCATCGATTCTT-3'	5'- TGGAGCTTATTAAAGGCATTCTCA-3'

CYPA indicates Cyclophilin A; *RPLP0*, acidic ribosomal phosphoprotein P0; *TNFA*, tumor necrosis factor alpha; *IL-1*, interleukin 1; *IL-6*, interleukin 6; *IL-10*, interleukin 10

Results

Small intestinal tissue exposed to prolonged periods of IR shows extensive damage to the epithelial lining

Normal villi with an intact epithelial lining were observed in control tissue of both IR groups (Figure 11.1A,E). After 30I, jejunal tissue exhibited subepithelial spaces (Figure 11.1B). Damaged cells were pinched off and shedded toward the lumen after 30I30R (Figure 11.1C). The epithelial lining is fully restored after 30I120R (Figure 11.1D). In contrast, disruption of the epithelial lining was observed at villus tips after 60I (Figure 11.1F). Following 60I30R pronounced injury was seen with complete degradation of the villus tips as a result of abundant epithelial shedding of IR-damaged enterocytes (Figure 11.1G). As depicted in figure 1H, 60I120R led to fully denuded villi, exposing the lamina propria to the harmful intraluminal content. Current morphological perturbations are similar to previous histological observations after human jejunal ischemia ²³.

To quantify the breakdown of the epithelial barrier following intestinal IR, plasma I-FABP levels were measured. I-FABP is a small, highly abundant, cytosolic protein present in mature enterocytes at the tips of the villi. It is release into the circulation upon epithelial damage. I-FABP V-A concentration differences increased from 1.32 (± 0.98) ng/mL in controls to 119.5 (± 61.1) ng/mL directly after 30I ($P < 0.05$, Figure 11.2). After a subsequent 30-minute period of reperfusion, I-FABP V-A concentration differences declined to 29.8 (± 20.6) ng/mL, which was still increased ($P < 0.05$) when compared to control (Figure 11.2). At 120R, I-FABP V-A levels progressively decreased to 4.80 (± 3.27) ng/mL. Directly after 60I, I-FABP V-A differences increased from 1.16 (± 0.40) ng/mL before ischemia to 715.4 (± 255.7) ng/mL directly after ischemia ($P < 0.05$, Figure 11.2). After 30R, I-FABP V-A differences remained elevated at 119.0 (± 32.9) ng/mL ($P < 0.05$, Figure 11.2). Even at 120R, I-FABP V-A differences were 17.7 (± 6.21) ng/mL and still elevated compared to control ($P < 0.05$). Higher I-FABP plasma concentrations were observed in jejunum exposed to 60 minutes of ischemia when compared to 30 minutes ($P = 0.03$) and at 60I30R versus 30I30R. These results indicate that the epithelial lining is severely damaged after prolonged periods of ischemia.

IR leads to disruption of the intestinal mucus layer

Periodic Acid Schiff/Alcian Blue (PAS/ AB) staining shows an intact mucus layer on the apical side of the villi and abundant presence of mucus within the goblet cells in control tissue (Figure 11.3A,E). 30 minutes of ischemia did not appreciably alter the mucus layer (Figure 11.3B).

Break down of the mucus layer became apparent after 30I30R (Figure 11.3C). This process continues at 30I120R (Figure 11.3D), which results in mucus-depleted goblet cells at the tips of the villi. Disruption of the mucus layer, however, is clearly visualized after 60

Figure 11.1 Hematoxylin and eosin staining of jejunal sections exposed to short (30 min) and prolonged (60 min) periods of ischemia followed by reperfusion

A,E, Control jejunal sections show a normal epithelial lining with an intact villus structure. **B**, 30I results in retraction of the basement membrane and the appearance of subepithelial spaces while the epithelial lining remains intact. **C**, Prior to shedding of apoptotic enterocytes into the lumen, opposing mature epithelial cells that have lost contact with the basement membrane are pulled together like a zipper at 30R (arrowheads). **D**, At 120R the epithelial lining is restored and shed enterocytes are pinched off into the lumen. **F**, 60 minutes of ischemia results in appearance of large subepithelial spaces and, in contrast to 30I, disruption of the epithelial lining (arrowhead). **G**, At 60I30R, damaged villus tips are shed into the lumen causing further disintegration of the epithelial barrier (arrowheads). **H**, resulting in a denuded epithelial lining at 60I120.

Figure 11.2 Breakdown of the mucosal barrier reflected by plasma I-FABP levels

Mean I-FABP V-A concentration differences in patients submitted to 30 and 60 minutes of ischemia followed by 30 and 120 minutes of reperfusion.

Significant compared to control (30I). *Significant compared to control (60I).

Figure 11.3 Disruption of the intestinal mucus during ischemia and reperfusion

A,E, Periodic Acid Schiff/Alcian Blue staining shows an intact mucus layer in control tissue. **B**, Directly after 30I the mucus layer is still intact. **F**, Breakdown of the mucus layer becomes visible after 60I. **C**, During reperfusion further depletion of the mucus layer can be observed after both 30I30R (arrowheads) and **G**, 60I30R (arrowheads). **D**, 30I120R results in a less dense mucus layer. **H**, However after 60I120R the mucus layer is completely destroyed with denuded villi exposed to the luminal content.

Figure 11.4 Increased neutrophil influx after 60 minutes of ischemia when compared to 30 minutes of ischemia followed by reperfusion

A,E, Neutrophil influx was visualized by MPO staining after IR. Control tissue showed no neutrophil influx. **B,F**, At 30I and 60I, little influx of neutrophils was observed (arrowheads). **C,G**, At 30R, neutrophil influx was only increased after 60I (arrowheads). **D**, After 30I120R, the intestine was recovered from the IR-induced damage and neutrophil influx was normalized. **H**, However, after 60I120R the presence of neutrophils in the denuded villus tips persisted.

minutes of ischemia with expulsion of goblet cell mucus into the lumen (Figure 11.3F). This is more pronounced after 60I30R minutes of ischemia with massive destruction of the mucosal wall (Figure 11.3G). 60I120R resulted in near obliteration of the mucus layer, with complete denudation of the villi (Figure 11.3H) and compromise of the intestinal barrier. Breakdown of this first line of defense might allow proteases to degrade and enter the intestinal barrier.

Prolonged intestinal ischemia is accompanied by significant neutrophil influx and excessive inflammatory responses

As expected, neutrophils were relatively sparingly present in the lamina propria in jejunal control tissue (Figure 11.4A,E respectively). MPO staining shows rather stable numbers of neutrophils in tissue exposed to 30 minutes of ischemia (Figure 11.4B) and also following 30R or 120R (Figure 11.4C,D, respectively). In contrast to short periods of ischemia, neutrophil influx becomes apparent when the duration of ischemia was prolonged to 60 minutes of ischemia (Figure 11.4F). The neutrophil migration into the lamina propria of IR-damaged villus tips continued to rise during 30R (Figure 11.4G) and 120R (Figure 11.4H). Quantification of MPO-positive cells showed no significant increase in neutrophils being present in the lamina propria of jejunal tissue subjected to short (30 minutes) periods of ischemia followed by reperfusion (Figure 11.5A). Prolonged (60 minutes) periods of ischemia followed by 30R or 120R however resulted in a significantly increased of neutrophil influx compared with control (Figure 11.5A, $P < 0.05$). Direct comparison between the 30IR group and 60 IR group showed a significantly higher presence of neutrophils in jejunal tissue after 60I alone and after 60I followed by 30R or 120R (Figure 11.5A, $P < 0.01$).

To shed further light on the potentially protease-induced inflammatory response during intestinal IR, the production of several cytokines downstream of the NF- κ B-pathway were monitored. In line with the neutrophil influx, mRNA expression of TNF- α , IL-1 β , IL-6 and IL-10 did not change significantly over the course of short (30I) periods of intestinal IR (Figure 11.5B-E). Interestingly, in jejunum exposed to prolonged (60I) periods of ischemia, tissue mRNA expression of TNF- α , IL-1 β , IL-6 and IL-10 significantly increased during 30 and 120 minutes of reperfusion when compared to control tissue (Figure 11.5B-E, $P < 0.01$ for all cytokines). TNF- α mRNA levels were upregulated both at 60I30R and 60I120R when compared to the 30I group at the same time points (2.2 (± 0.46) vs. 0.90 (± 0.16) and (2.3 (± 0.40) vs. 0.95 (± 0.23), both $P < 0.05$, Figure 11.5B). Next, significant differences were observed for IL-1 β expression between the 30I and 60I groups during 30 and 120 minutes of reperfusion (2.8 (± 0.55) vs. 1.4 (± 0.23) and 3.4 (± 0.59) vs. 1.4 (± 0.29), both $P < 0.05$, Figure 11.5C). IL-10 expression was also increased during reperfusion in the 60I group when compared to the 30I group (6.2 (± 1.3) vs. 1.5 (± 0.41) and 8.4 (± 2.6) vs. 1.7 (± 0.49), $P < 0.05$ and $P < 0.01$, respectively, Figure 11.5D). Furthermore, higher expressed IL-6 mRNA levels were observed between 30I and 60I

Figure 11.5 Increased inflammatory response of small intestine exposed to 60 minutes of ischemia followed by reperfusion

A, Quantification of neutrophil influx shows significant increase in MPO positive cells in the 60I group when compared to control and the 30I group. **B**, TNF-α mRNA expression did not change significantly in the 30I group after exposure to IR. In the 60I group, a 2.4-fold increase in TNF-α expression was observed after 30R, compared with control, which remained elevated after 120 minutes reperfusion. Both were significantly elevated when compared to 30I30R and 30I120R. **C**, IL-1βC is significantly increased after 60I30R and 60I120R. **D**, a significant increase in IL-10 expression was observed after 60I30R compared with control and 30I30R, which remained elevated after 60I120R. **E**, IL-6 mRNA expression did not significantly increase after 30IR. After 60I an increase in IL-6 mRNA expression was observed after 60I30R and 60I120R. **F**, IL-6 plasma levels were significantly increased after both 30I30R and 60I30R and 60I120R reperfusion, when compared with their respective controls. Each column represents the mean ± SEM. # $P < 0.05$, compared with control (30I), * $P < 0.01$, compared with control (60I).

Figure 11.6 Detection of protease activity (in red) in the small intestinal wall during IR

A,E, Control tissue of both 60I and 30I show no evidence of protease activity in the intestinal wall. **B**, No proteolytic activity became present during 30I, **C**, 30I30R and **D**, 30I120R. **F**, In the 60I group activity was present even in the absence of reperfusion **G**, and bowel fluorescence was greatly increased after 60I30R **H**, and 60I120R revealing extensive fluorescence deep into all the intestinal layers.

during 30 and 120 minutes of reperfusion ($1.0 (\pm 0.18)$ vs. $0.19 (\pm 0.02)$ and $1.6 (\pm 0.27)$ vs. $0.57 (\pm 0.21)$, $P < 0.01$ and $P < 0.05$, respectively, Figure 11.5E). In line with the mRNA expression IL-6 V-A protein concentrations differences in plasma were increased at 60I30R and 60I120R compared to control ($97.1 (\pm 15.0)$ pg/mL and $410.9 (\pm 55.9)$ pg/mL vs. $0.1 (\pm 0.04)$ pg/mL, both $P < 0.01$, respectively, Figure 11.5F). After 30I, plasma IL-6 levels were only elevated at 120R (82.2 ± 28.1 pg/mL vs. $0.38 (\pm 0.26)$ pg/mL, $P < 0.05$, Figure 11.5F). Again, IL-6 plasma concentrations were significantly higher after 30 and 120 minutes of reperfusion in the 60I group compared to the 30I group ($P < 0.05$). These data are indicative for a higher inflammatory response after intestinal barrier integrity loss as a consequents of prolonged intestinal IR.

Prolonged intestinal IR results in leakage of serine proteases into the intestinal wall

In order to study whether epithelial barrier loss during intestinal IR is accompanied by leakage of pancreatic digestive enzymes into the intestinal wall, in situ zymography of casein substrate specific for serine proteases was used. Control tissue showed fluorescence at the luminal site of the villi, suggesting the presence of residual pancreatic enzyme in the lumen, even after washout of the intestinal contents (Figure 11.6A,E). No increased pancreatic enzyme activity in the mucosal and submucosal layers was observed in tissue subjected to 30I alone or at 30I30R and 30I120R versus control (Figure 11.6C,D). With extensive intestinal barrier loss, there was concomitant increase in pancreatic enzyme activity during 60I in all layers of the small intestine (Figure 11.6F). Furthermore, extensive enzyme activity was greatly increased at 60I30R and 60I120R

(Figure 11.6G,H) and resulted in extensive fluorescence deep into the mucosal and submucosal layers.

Serine protease levels in plasma

Protease buffer capacity of plasma derived from jejunal veins draining the isolated jejunal segments was assessed in the 30I (Figure 11.7A) and 60I (Figure 11.7B) group at all four time points. Results are presented as calibration curves of added exogenous trypsin to protease activity measured in fluorescence units (RFUs). The inflection points on each sigmoidal curve correspond to the concentrations of added trypsin required to overcome the ability of endogenous plasma protease inhibitors to inactivate the cleaving process of the casein substrate. Plasma samples showed trivial protease activity at low concentrations of added trypsin at all time points, indicating little to no free plasma enzymes. When concentrations of added trypsin gradually increase, more or less simultaneously occurring inflection points can be observed in the plasma samples taken at 30I, 30I30R and 30I120R compared to their own control plasma. However, when trypsin concentrations gradually increase in the plasma samples of the 60I group, a

Figure 11.7 Protease buffer capacity of plasma after 30 minutes and 60 minutes of ischemia followed by 30 and 120 minutes of reperfusion

Measuring of proteolytic activity in plasma after **A**, 30 respectively **B**, 60 minutes of ischemia followed by 30 and 120 minutes of reperfusion by adding increasing exogenous trypsin concentrations to samples and measuring protease activity by fluorescence of a nonspecific fluorescently labeled casein substrate. Results are shown in RFUs. Initial levels in all groups are close to zero, indicating little to no active plasma proteolytic activity at baseline. After 30 minutes of ischemia followed by 30 and 120 minutes of reperfusion no obvious shifting of the calibration curve was noted, indicating no decrease in antiprotease buffer capacity. However, 60I30R and 60I120R displays a significantly decreased antiprotease buffer capacity compared with control and 60I. (arrow, showing an essential shifting of the calibration curve to the left), indirectly reflecting an increased amount of active digestive protease that entered the systemic circulation.

significantly increase in enzyme activity can be observed in the plasma samples taken at 60I30R as well as 60I120R compared to control ($P < 0.05$ and $P < 0.01$ respectively), indicating a decrease in circulating protease inhibitor buffer capacity (Figure 11.7B, arrow). This suggests that occupation of plasma protease inhibitors occurred in a greater extent because of presence of endogenous proteases in plasma and therefore implicates an increased concentration of endogenous proteases in the 60 minute ischemia group in comparison with control samples.

Discussion

We used our unique human experimental IR model to explore the role of pancreatic digestive enzymes in the pathogenesis of intestinal inflammation during IR in man. First, this study demonstrates that short (30 minutes) periods of intestinal IR leads to a quick repair of the epithelial lining, resulting in an intact intestinal barrier. However, prolonged (60 minutes) ischemia followed by reperfusion causes irreversible tissue damage to the epithelial lining with an impaired intestinal barrier. Second, we show that intestinal barrier integrity loss as a result of IR-induced damage initiates a significant inflammatory response, whereas inflammation is limited after short intestinal IR. Third, penetration of proteases into the intestinal wall in relation to the length of ischemia was found. Results reveal that damage to the intestinal barrier is observed simultaneously with increased digestive enzymatic activity in the wall of the intestine and is followed by increased appearance of protease levels in the systemic circulation. This study implicates disruption of the intestinal barrier in conjunction with leakage of digestive enzymes as being a potential major mechanism responsible for the activation of the inflammatory response during intestinal IR.

The morphological observations in this study are in accordance with previous work from our group, showing that the human small intestine is capable to rapidly restore the intestinal continuity after short periods of ischemia (≤ 30 minutes) followed by reperfusion using “purse-string” contraction of non-muscle myosin fibers at the basal side of opposing viable enterocytes, pinching off IR-damaged enterocytes into the lumen and restoring the epithelial lining ²¹. Prolonged periods of ischemia (≥ 45 minutes) led to permanent physical and immunological intestinal barrier loss causing severe inflammatory responses ^{6,9}.

The homeostasis of the epithelial barrier function is well recognized in the prevention and development of intestinal and systemic inflammatory diseases ²⁴. Maintenance of the epithelial barrier is therefore of major importance, since loss of this barrier facilitates translocation of potentially harmful intestinal luminal contents towards the circulation, causing a severe inflammatory response. Two important pathways are responsible for the activation of the inflammatory responses during intestinal IR. First, intracellular components leaking from injured cells and tissue (damage associated molecular patterns, DAMPs) can trigger innate immune responses. Second, innate immune cells, including macrophages, dendritic cells and neutrophils, express pattern-recognition receptors (PRRs) that detect infiltrating microbiota and their products which are potential effectors of the inflammatory response (pathogen associated molecular patterns, PAMPs). Both DAMPs and PAMPs interact with transmembrane and intracellular pattern recognition receptors, resulting in pro-inflammatory signaling ²⁵. These danger signals were long thought to consist of bacteria or their products. However, interventions

against bacteria that are aimed at reducing mortality in patients with sepsis have been unsuccessful in clinical trials ¹². This underlines that intestinal bacteria are not solely responsible for the destructive and vigorous inflammation in patients with intestinal ischemia. Further knowledge on danger signals from the lumen causing intestinal damage and inflammation upon IR ultimately leading to transition into systemic inflammation is needed. In recent years the role of powerful digestive enzymes in intestinal IR have become apparent from experimental animal studies. The consequence of pancreatic digestive enzymes leaking from the intestinal lumen into the intestinal wall is self-digestion of the intestinal tissue. Protease activity in the intestinal wall was accompanied by mucus disruption, tight-junction loss and epithelial cell disruption after intestinal IR. We demonstrated this leakage of pancreatic proteases into the intestinal wall for the first time in human intestinal tissue subjected to intestinal IR. These data are in line with previous animal studies revealing cleavage of trypsin specific substrate inside the villi and across the full thickness of the intestinal wall ^{14,26}. Interestingly, colon ischemia has a more favorable outcome, although the high colonic microbiota density is potentially more toxic than small intestine intraluminal content ²⁷. This may be explained by differences in mucus organization ²⁸. Another explanation is the detrimental activity of intestinal wall penetrating digestive enzymes present in the proximal small intestine and not in the colon.

Proteases activate the inflammation cascade by cleaving and activating a family of G protein-coupled Protease-Activated Receptors (PARs). Mainly PAR2 is activated by serine proteases induced cleavage of their amino-terminus, which acts as a tethered ligand, binding to the receptor to initiate multiple proinflammatory signaling cascades ²⁹. PAR2-activating peptides stimulate inflammatory responses via the NF- κ B-pathway, which is responsible for transcriptional induction of a large number of inflammatory genes, including those encoding TNF- α , IL-1 β and IL-6. Furthermore, the NF- κ B-pathway regulates the recruitment of neutrophils ³⁰. Our data show a significant upregulation of these NF- κ B-pathway related inflammatory genes and protein levels after intestinal barrier integrity loss as a consequents of prolonged intestinal IR. Rodent studies also suggest an important role for PAR-2 in inflammation following intestinal IR with upregulated PAR-2 mRNA and protein expression in intestinal mucosa after IR ³¹. In line, IBD patients have increased levels of PAR-2 activating proteases in lumen and colonic tissue ²⁹.

This study focused on the relation between IR-induced intestinal barrier loss, tissue penetration of proteases and local inflammation potentially reflecting PAR-2 activation. The results of this study will add to a better understanding of the pathophysiology of intestinal ischemia. However the role of digestive enzymes and its receptor, PAR-2, need to be studied in depth. This will open new opportunities to study therapeutic interventions directed at the inhibition of PAR activation by pancreatic enzymes. Blocking

of pancreatic proteases was successfully applied in animals undergoing intestinal ischemia ¹² and recently a report showed promising results that intraluminally administered protease-inhibitors decreased shock and sepsis in a patient with Fournier's gangrene ³².

A limitation in the current study is the small sample size, which is inherent to a pilot-study.. Because of the promising results, a further in depth study using our human IR model will be performed to explore proteases localization and PAR-2 activation in intestinal IR and correlate these with epithelial barrier damage and inflammation markers. To definitely explore the autodigestive hypothesis in man, protease-inhibitors will be administered intraluminally in the IR jejunal experimental model. Last, the current assays only measure nonspecific serine protease activity. They are not capable to specifically detect which pancreatic enzymes are responsible for infiltrating into the intestinal wall and the elevated systemic proteolytic activity in plasma.

In conclusion, the current results demonstrate that IR-induced epithelial barrier loss allow pancreatic digestive proteases to enter the intestinal wall. Protease leakage in the intestinal wall was accompanied by increased inflammatory responses and elevated protease activity in the systemic circulation. Data presented in this study confirm the evidence that pancreatic digestive proteases may play a major role in the pathogenesis of the destructive and vigorous inflammation in patients suffering from intestinal ischemia. These results will serve as a basis for new therapeutic strategies to minimize the systemic inflammatory responses during the early stages of intestinal ischemia.

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Summary and Discussion

Intestinal ischemia-reperfusion (IR) is a frequently observed and critical abdominal emergency. There exist a large variety of underlying pathologies that can lead to interruption of the splanchnic circulation. It is the consequence of an acute occlusion of the mesenteric blood supply due to vascular disease (arterial thromboembolism, venous thrombosis) or the result of non-occlusive mesenteric ischemia secondary to diminished blood flow caused by intestinal strangulation, major surgery, shock, trauma and sepsis ¹⁻³. Furthermore, intestinal IR is considered as common pathway in several pathologies, including necrotizing enterocolitis, pancreatitis and as phenomenon involved in aetiology or perpetuation of inflammatory bowel disease (IBD) ⁴. Mortality in patients suffering from intestinal IR ranges from 60% to 80% ^{1,5,6}. These high mortality rates are associated with the difficulty to diagnose intestinal IR at an early stage ^{7,8}. In part A of this thesis, several potential markers capable of detecting early intestinal IR injury and determining the gravity of the illness were studied.

The high mortality of intestinal IR is also due to the unknown pathophysiology underlying this phenomenon. Therefore, in part B of this thesis the pathophysiological consequences of intestinal IR and its sequelae were studied.

The intestinal epithelial lining is responsible for absorption of water and nutrients from the lumen but also forms an effective barrier for the separation of potentially toxic luminal content (external environment) from the host (internal environment) ⁹. This barrier is essential to permanently protect the body against invasion and systemic dissemination of bacteria, toxins and antigens. Disruption of this delicate balance at the mucosal-luminal interface is considered to play a key role during intestinal IR ¹⁰. Intestinal IR results in local tissue damage to the epithelial lining with intestinal barrier function loss facilitating bacterial translocation, triggering systemic inflammation and multiple organ failure (MOF) leading to sepsis, shock and eventually death ^{5,11,12}. In search for new interventional targets to improve patient outcome, it is crucial to comprehend the underlying molecular mechanisms associated with human intestinal IR related intestinal damage. In part B of this thesis, these factors were studied using our human intestinal IR model. Moreover, the safety of this human in vivo intestinal IR model was investigated.

Human experimental models: a safe and feasible way to study in vivo intestinal IR

For long time only animal studies were used to study intestinal IR. Therefore, a human model was developed, enabling our group to clarify the sequelae of jejunal IR ¹³. Previous research of our group using this newly developed human intestinal IR model showed the importance of several physical and immunological barriers, preventing invasion of foreign or endogenous threats from intestinal lumen to sterile interior milieu upon 30 minutes of human jejunal ischemia ¹³⁻¹⁵. We also showed that prolonged (>45 minutes) ischemia followed by reperfusion led to irreversible damage, resulting in bacterial translocation and systemic inflammation ^{15,16}. Furthermore, using a newly

developed human experimental colon-IR model, colonic epithelium was shown more resistant to IR-induced damage than jejunum, which was mainly attributable to goblet cell compound exocytosis, flushing bacteria from crypts leading to mucus-layer recovery¹⁷. In addition, these models also made it possible to evaluate new biomarkers to facilitate the earlier diagnosis of acute mesenteric ischemia (AMI).

Although adverse events in patients subjected to the experimental IR model were accurately reported, no structural evaluation was yet provided of complications following our human in vivo intestinal IR. Moreover, we wanted to evaluate whether patients undergoing experimental intestinal IR were non-inferior compared with patients who underwent the same type of surgery in the same hospital over the same time period as the IR-patients, but were not exposed to the experimental intestinal IR regarding peri-operative complication rates. Therefore, the **first aim** of this study was to evaluate the safety of our two human in vivo intestinal IR models.

In **Chapter 2**, it is described that it is safe to study in vivo human intestinal IR by applying intestinal ischemia to six cm of isolated jejunum or colon during surgery. Using patients undergoing 60 minutes of jejunal or colonic intestinal IR, we observed a similar percentage of complications after in vivo IR when compared to well matched control-groups subjected to the similar surgical procedure without the IR protocol.

The overall complication rate classified by the Clavien-Dindo (CD) system¹⁸ in our pancreatic surgery patients with and without in vivo jejunal IR was 63.3%. The frequency and severity of postoperative complications were equivalent for patients undergoing surgery with experimental IR (70%) and without experimental IR (60%). Minor (CD 1 and 2) complications were not different for both groups (47% vs. 45%). Also the number of major (CD $\geq$ 3) complications were similar between patients with and without in vivo jejunal IR (52% vs. 55%). These data are in line with the current literature and considered acceptable due to the complexity of a pancreaticoduodenectomy^{19,20}. Furthermore, operation time, amount of blood loss, length of hospital stay or mortality did not differ between both groups or from previously reported data²¹.

No significant difference in complication rate was found in our patients undergoing colorectal surgery with (50%) and without (65%) experimental in vivo colonic IR. However, the overall complication rate of 60% in both groups is fairly higher than complication rates shown in previous published studies^{22,23}. This difference is most likely due to the meticulous documentation of the complications in our patients. Furthermore, the majority of complications are graded as Clavien-Dindo 1 or 2, signifying minor, common surgical complications such as urinary tract infection and wound infection. From all complications after colorectal surgery, anastomotic leakage is the most feared. Importantly, 13.3% of our included patients developed an anastomotic leakage which did not differ significantly between the IR and no IR group. These rates are similar as reported in recent literature²⁴.

These findings indicate that it is safe to study in vivo human intestinal IR with both our jejunal and colonic model during surgery.

Plasma biomarkers reflecting activation of intravascular coagulation and intestinal mucosal damage are of potential value in the diagnosis of acute mesenteric ischemia

A critical factor contributing to the high mortality rates of AMI is the delay in diagnosis and treatment ⁶. Presenting clinical signs are often vague or variable, and there is a lack of specific laboratory tests to detect intestinal ischemia ^{2,3,25,26}. Therefore all patients suspected to have acute intestinal ischemia should undergo an intravenous contrast enhanced CT scan (CTA) of the abdomen. However, CTA is a time-consuming technique and interpretation by an expert radiologist is warranted. Also, obvious radiological findings, including pneumatosis and portal venous gas are not specific for either infarction or ischemia ³. Rapid diagnosis and (surgical) intervention are paramount to limiting mortality ⁸. Earlier diagnosis using routine laboratory tests available around the clock would be of great help.

To generate more clarity on the diagnostic value of the currently known circulating biomarkers, the **second aim** of this thesis was to systemically review the literature regarding human plasma biomarkers for AMI reported during the last ten years.

One of the main outcomes of **Chapter 3** is that none of the classically used markers for intestinal ischemia (lactate, LDH, CK, leukocyte count, base excess) had good diagnostic accuracy. This has been demonstrated before, but these markers are still used in daily clinical practice. Furthermore, the usage of these markers is discouraged by the recommendations in the current guidelines on AMI, which state that routine laboratory tests should not be used for diagnostic purposes ^{2,3}.

Numerous potential new plasma markers have been studied, such as procalcitonin, ischemia-modified albumin, D-dimer, α -glutathione S-transferase and I-FABP. In short, both procalcitonin and ischemia-modified albumin are only used in a few pilot experiments and should be better investigated in larger diagnostic studies ^{27,28}. Based on the results from our systemic review D-dimer, I-FABP and α -GST are the most potential markers for intestinal ischemia with I-FABP being the most promising. D-dimer is already available in daily clinical practice and has a high sensitivity of > 95%, making it a useful marker to exclude patients suffering from AMI in patients with acute abdominal complaints ^{2,29}. However many other conditions are associated with high D-dimer levels and it does not differentiate between patients with reversible and irreversible intestinal ischemia.

Ideally, a potential biomarker in the diagnosis for AMI would be an endogenous cytosolic enterocyte protein that is released into the circulation upon intestinal damage, which is metabolic stable from intestine to peripheral blood, reflecting actual damage and be specific for ischemia and have high sensitivity so it can rule out the disease ^{30,31}.

Of all new plasma markers I-FABP is studied most in depth. It showed good diagnostic accuracy in distinguishing patients with and without intestinal ischemia, as it is tissue specific and released into the circulation upon enterocytes damage ^{32,33}.

I-FABP is a small (14-15 kDa), cytosolic, water-soluble protein specifically and abundantly expressed in mature enterocytes of the small and large intestine ³². I-FABP is present in very small amounts in the plasma of healthy individuals, probably representing the normal turnover of enterocytes, but is rapidly released after episodes of acute intestinal damage and inflammation ^{13,34}. It passes through the glomerular filter (with a fractional renal excretion of 28%; half life time 11 minutes) and can readily be detected in the plasma or urine ^{35,36}.

Gollin showed for the first time that I-FABP was released into the peripheral circulation after intestinal ischemic injury in rats and had potential as a diagnostic marker for the early detection of AMI ³⁷. Next, elevated I-FABP levels were found after intestinal ischemia in man compared to healthy volunteers ^{34,38}.

Increased urinary and plasma I-FABP levels have been observed in patients with splanchnic hypoperfusion as a result of major vascular ³⁹ or non-abdominal surgery ⁴⁰, following major trauma ⁴¹ or sepsis ⁴², and in neonates with necrotizing enterocolitis ^{43,44}. Furthermore, our group showed that I-FABP is a useful biomarker to identify patients with intestinal ischemia following aortic aneurysm repair ⁴⁵. Subsequently, we and others showed that I-FABP has a good diagnostic accuracy in distinguishing patients with and without intestinal ischemia from other acute abdominal complaints in patients suspected of intestinal ischemia ^{33,36,46}.

However, one should bear in mind that several aspects of the performance of the I-FABP measurement need to be addressed before conducting multicenter studies or worldwide use in clinical practice. That is why phase 4 diagnostic studies are needed. Therefore, the currently available tests need to be improved as they take at least three hours to conduct. Also, various I-FABP assays exist reporting different cut-off values with variable detection limits, ranging from picograms to nanograms. The creation of a rapid I-FABP assay with good diagnostic performance and worldwide accepted clinical cut-off values that could be used in a phase 4 diagnostic study in patients suspected for AMI would be a major step forward.

Phase 4 studies address the question; Do patients who undergo the diagnostic test fare better (in their ultimate health outcomes) than similar patients who do not ⁴⁷. These questions have to be answered by randomizing patients to undergo the test of interest or some other (or no) test. Randomized control trials (RCTs), however, are not often applied in diagnostic research, for reasons such as complex logistics, the need for large sample sizes to show statistically significant differences and high costs. Designing an RCT to assess a new diagnostic test is also difficult because of ethical considerations. Ethical issues will arise when a diagnostic test will be withheld in half of the patients and

different treatment strategies following the tests are applied. An alternative design for assessing phase 4 questions is a prospective cohort study, moving the point of randomization from the decision point whether or not to test, to what to do with the test result ⁴⁸. These studies have a pragmatic character. Clinicians will include patients based on the standard diagnostic work-up. All patients will undergo the diagnostic test and clinical practice will be as usual, which allows an evaluation of the diagnostic test in its clinical context. In addition, this will minimize the workload of clinicians and high inclusion rates ⁴⁸.

Better insight into the clinical usefulness of I-FABP as diagnostic marker to detect intestinal ischemia at an early stage

The results of our systematic review revealed that I-FABP has the most potential as early marker for intestinal ischemia. However, more insight into the characteristics of I-FABP release is needed before this test can be implemented into daily clinical practice such as the relation between plasma I-FABP levels and the severity of ischemic mucosal injury, and define a cut-off point for systemic I-FABP to estimate the extent of ischemia-induced enterocyte damage.

The **third aim** of this thesis was therefore to provide more insight into the clinical usefulness of circulating I-FABP as early diagnostic marker to detect intestinal ischemia. Using our well-controlled human intestinal IR model, in **Chapter 4** the relation between I-FABP levels and the severity of ischemic mucosal injury was investigated and the clinical usefulness of systemic I-FABP following ischemia was further defined.

The results of this thesis showed that in the human intestine short ischemic periods (15 and 30 minutes) are associated with considerably less I-FABP release into the circulation than long periods (60 minutes) of ischemia ⁴⁹. The results of this study show that the amplitude of increased arteriovenous (V-A) I-FABP concentration differences significantly correlate with the extent of epithelial damage (measured by the loss of villus height) of jejunum exposed to IR in our standardized human experimental model.

In addition to this relation between plasma I-FABP and the extent of epithelial intestinal damage, it is interesting that I-FABP was also found to be a very sensitive marker for intestinal epithelial damage. I-FABP was found to be a tenfold elevated in V-A plasma concentration differences after only 15 minutes of ischemia, whereas morphological changes were hardly noticeable at this time-point.

We also obtained more knowledge on the dynamics in I-FABP levels during human intestinal IR. First, following a short exposure to ischemia (ie 15 and 30 minutes), I-FABP levels rapidly decreased over the course of a period of 120 minutes reperfusion, demonstrating rapid and full recovery of epithelial membrane integrity. In contrast, I-FABP V-A plasma concentrations remained significantly elevated during reperfusion of tissue exposed to longer periods of ischemia (60 minutes). These results provide more insight into the characteristics of I-FABP release during human intestinal IR. For daily

clinical practice however, it is more important to investigate the diagnostic value of systemic I-FABP levels, rather than I-FABP V-A concentration differences from a 6 cm isolated part of jejunum. Indeed, we showed that systemic I-FABP levels are helpful to discriminate mild, reversible jejunal ischemia from more extensive, irreversible ischemia with an AUC of 0.97 (95% CI 0.89-1.03). This indicates that the levels of systemic circulating I-FABP are related to the duration of ischemia and the extent of small intestinal tissue damage.

The results in **Chapter 4** again indicate the great potential of plasma I-FABP as marker in the early diagnosis of AMI. However, prospective multicenter studies are still needed to assess whether I-FABP can be used in the diagnostic work-up in patients with suspected intestinal ischemia.

SM22 is a useful new marker for the detection of transmural intestinal ischemic damage

The main risk of intestinal ischemia is transmural infarction, leading to intestinal wall perforation, sepsis and death ^{1,5}. Early detection of transmural damage is imperative because it mandates emergency surgery ^{2,3}. Therefore, it would be interesting to find an additional biomarker to distinguish patients with irreversible transmural intestinal ischemic injury from patients suffering from reversible mucosal intestinal damage at an early stage.

Because of its abundant expression in smooth muscles cells in the outer layer of the intestine, the small, water-soluble Smooth Muscle protein of 22-kDa (SM22) is of interest ^{50,51}.

The **fourth aim** of this thesis was to discover a new marker for transmural intestinal ischemic damage by developing a new quantitative ELISA for smooth muscle protein of 22 kDa (SM22) and to evaluate the diagnostic potential of SM22 as plasma biomarker for the detection of transmural intestinal ischemia.

In **Chapter 5** an experiment was performed to develop and validate a new sandwich enzyme-linked immunosorbent assay (ELISA) to quantify SM22. Using mouse monoclonal antibodies I build an accurate SM22 ELISA with values for inter-assay and intra-assay precision and accuracy within current guidance limits of 20% for method acceptance ⁵².

Next, we improved the insight into the characteristics of SM22 release during transmural intestinal damage. It was found that SM22 is rapidly released in plasma upon the onset of ischemia with peak concentrations at six hours. AV-concentration gradients across the intestines, liver and kidneys show that SM22 was specifically released from the intestine and that kidneys are fully responsible for its clearance without involvement of the liver. Moreover, the results reported a short half-life time of 23 minutes in man.

After these results we analyzed the diagnostic potential of SM22 for the detection of transmural intestinal ischemia in man. SM22 levels were investigated in 50 healthy

volunteers, 22 patients with histologically proven intestinal ischemia (of which 13 had transmural ischemia (TI) and 9 suffered from mucosal damage (MI)). In patients with TI, SM22 plasma levels were significantly higher compared with patients with only MI or healthy controls ($P < 0.001$). Next, we measured the plasma concentrations of I-FABP. Mean plasma I-FABP levels were significantly increased in both patients with only MI and patients with TI when compared to healthy controls ($P < 0.05$, for both; **Chapter 5**). Second, to investigate the clinical potential diagnostic value of SM22 we measured SM22 plasma levels of 40 patients with other acute abdominal complaints (OAAC). We showed that SM22 was found to be significantly increased in 13 patients with proven TI compared to 40 patients with OAAC ($P < 0.001$). Combined with the data from the MI patients and healthy controls, this yielded an overall accuracy of the test, summarized as area under the receiver operating curve (AUC), of 0.97. This is considered to be clinically relevant. Interestingly we observed no differences in plasma I-FABP concentrations between the MI patients and TI patients ($P = 0.77$). As the intestinal mucosa is most vulnerable to ischemia, transmural ischemia is always associated with mucosal ischemia. As I-FABP is solely expressed by mature enterocytes, it was not surprising that there was no difference in I-FABP concentrations between mucosal and transmural ischemia. In addition, none of the classically used laboratory markers for intestinal ischemia (plasma lactate levels, WBC count, and arterial pH) could discriminate patients with TI from patients with MI damage.

A phase 3 diagnostic study answers the question if, among patients in whom it is clinically sensible to suspect the target disorder, the test result distinguishes those with and without the target disorder⁴⁷. This is the next step that needs to be conducted before SM22 can enter the clinic.

In conclusion, increased SM22 plasma levels were specific for the patients with transmural intestinal ischemia in contrast to the intestinal mucosal damage marker I-FABP and the classical markers lactate, WBC count, and arterial pH. Furthermore, plasma levels of SM22 could also differentiate between patients with transmural intestinal ischemia and other causes of acute abdominal complaints, including acute appendicitis, diverticulitis, gynecological/urological pathology, gastroenteritis and nonspecific abdominal pain.

Next, we need to study whether the diagnostic accuracy of SM22 for transmural intestinal ischemia remains high when analyzed in a phase 3 diagnostic study. Eventually a combination of markers, reflecting different types and stages of intestinal ischemia, is thought to be the key approach in improving the diagnostic accuracy in patients suffering from intestinal IR (**Chapter 6**).

Better insight into the pathophysiology of intestinal ischemia-reperfusion

To improve therapeutic strategies in patients suffering from intestinal IR, it is crucial to understand the molecular mechanisms leading to intestinal barrier loss and

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inflammation. Although there is a growing knowledge about the pathophysiology of intestinal ischemia, there are still several issues that remain unclear. In part B of this thesis we further elucidate the pathophysiology of human intestinal ischemia-reperfusion.

Figure 12.1 Reaction of the epithelial lining to intestinal ischemia-reperfusion

(A) Healthy small intestine with a normal epithelial lining and an intact villus structure. (B) Ischemia results in retraction of the basement membrane and the appearance of subepithelial spaces while the epithelial lining remains intact. (C) During reperfusion, opposing epithelial cells that have lost contact with the basement membrane are pulled together like a zipper, bringing these cells together. (D) This mechanism results in a restored epithelial lining with damaged enterocytes pinched off from the tips of the villi and shed into the lumen. This limits the exposure of lamina propria to the pro-inflammatory intraluminal content, which is hypothesized to be a key factor in the prevention of the development of inflammation. (E) In contrast, prolonged IR leads to irreversible damage to the epithelial lining, thereby exposing the lamina propria to the hostile luminal content. This leads to a severe inflammatory response, characterized by neutrophil influx and upregulation of pro-inflammatory cytokines.

The intestinal barrier is capable of morphological and functional recovery after short episodes of ischemia-reperfusion

In Chapter 7, the fifth aim of this thesis, i.e. the relation between histological and functional intestinal epithelial barrier recovery during human small intestinal IR was further explored.

Previous research of our group revealed the striking ability of the human gut to reduce and restore IR-induced intestinal damage^{13,14}. In these studies it was observed that after 30 minutes of ischemia, villous sloughing with epithelial lining disruption was rapidly followed by complete histological recovery during reperfusion (Figure 12.1). The

important remaining question however is whether the observed histological perturbations also resulted in a functional barrier restoration.

In **Chapter 7**, we set out to study the relation between histological and functional barrier recovery using a human experimental small intestinal IR model.

This study demonstrates that after a short, 30 minute period, of ischemic injury the intestinal epithelial lining is efficiently recovered during 120 minutes of reperfusion by villus contraction, enterocytes recovery and tight junction (TJ) restoration. To test if the intestinal barrier is indeed functionally repaired, it is necessary to be able to actively and reliably measure intestinal barrier function. However, this has been a major challenge in clinical practice. Using lanthanum nitrate as an electrondense marker for TJ function loss together with the currently used gold standard for the measurement of intestinal permeability in clinical practice, the dual-sugar absorption tests (DST) ⁵³ the functional intestinal barrier loss was evaluated. DST are based on the differential absorption of intraluminal sugars of the larger disaccharide lactulose and the smaller monosaccharide L-rhamnose molecules across the intestinal barrier. An increased intestinal absorption of sugars indirectly measures intestinal barrier loss, but there is a lack of knowledge on how these DST correlate to epithelial cell damage and tight junction loss in vivo. Using our in vivo human model, we were able to demonstrate the direct relationship between morphological barrier loss (epithelial cells and TJ proteins) and functional intestinal barrier loss (DST and leakage of lanthanum nitrate through defective TJ complexes as visualized by electron microscopy). Damage to the epithelial lining was quantified using plasma levels of I-FABP. Plasma I-FABP V-A differences significantly correlated with the permeability changes in the small intestine. Our results showed that the DST data closely reflect the morphological findings during intestinal IR. This is of importance as it supports the usefulness of DST ⁵⁴⁻⁵⁶.

Next, accurate measurement of viable enterocytes mass and function, following small bowel diseases, is challenging ⁵⁷. Citrulline has been used for this purpose as it is an amino acid mainly produced by small intestinal enterocytes by the conversion of glutamine through the glutamate to ornithine pathway, without incorporation into proteins ⁵⁸. Plasma concentrations of citrulline has been previously purposed as a marker for functional enterocyte mass ⁵⁹. In **Chapter 7** further support for the use of citrulline as a marker for enterocyte function was provided. We showed that the difference in arterial-venous concentrations of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln A) reflect enterocyte functional capacity. Several studies showed that there is a link between low plasma citrulline concentrations and an impaired intestinal barrier ⁶⁰⁻⁶². Next, citrulline performed better as a marker detecting early impairment of the intestinal epithelial lining and small intestinal barrier integrity loss compared with the DST and indicated recovery more accurate ^{63,64}.

Our findings reflect the ability of the intestine to withstand short episodes of ischemia, with morphological and functional recovery of the intestinal barrier within 120 minutes of reperfusion. Loss of intestinal barrier function is associated intestinal inflammation, bacterial translocation, the development of sepsis and multiple organ failure^{12,65,66}. In addition, loss of intestinal barrier function plays a role in the pathophysiology of various intestinal diseases including IBD and celiac disease^{67,68}. The results in **Chapter 7** may explain why there are often no signs of inflammation or bacterial translocation after short periods of intestinal ischemia. Part of the mechanism of intestinal barrier repair during intestinal IR is previously described. This is a highly regulated event involving villus contraction, epithelial restitution and closure of the paracellular space leading to a remarkably rapid closure of mucosal wounds in the mammalian intestinal epithelial lining to prevent the onset of sepsis⁶⁹. Further exploration of the mechanisms responsible for this rapid morphological and functional recovery will improve treatment of intestinal diseases associated with barrier recovery loss.

The data from the DST and Cit V-A/Gln A ratio's closely reflect the histological perturbations during intestinal IR and can be of potential diagnostic value the follow-up of patients with intestinal disease associated with intestinal barrier loss.

Female small intestine is more resistant to IR-induced tissue damage compared to males

The **sixth aim** of this thesis was to find out whether there is a sex-difference in response to intestinal ischemic hit and to investigate the mechanisms involved in the differences in human intestinal mucosal responses to IR between males and females. Sex disparities regarding pathogenic mechanisms, age of onset, progression and therapeutic response are common in human disease⁷⁰. There is increasing evidence, mainly from animal studies, for sex differences in responses to IR in the intestine, in favour of the female gender^{71,72}. Although the results of IR-induced tissue damage in patients participating in our studies were consistent, we observed that some patients had considerably less intestinal IR-induced damage than others. The results of **Chapter 8** show a distinct sexual difference in human small intestinal mucosal responses to IR, with females be more resistant to IR injury.

The female jejunum showed less morphologic epithelial damage using HE staining during intestinal IR. We quantified this epithelial injury using plasma I-FABP levels, revealing significantly higher I-FABP concentrations across the isolated jejunal segment in males than of females exposed to IR.

Intestinal IR leads to an inflammatory response, characterized by influx of neutrophils into the damaged villus tips¹⁶. Neutrophil influx was significantly more abundant in male intestinal villi compared to female intestinal villi after exposure to IR. Second, the inflammatory response is accompanied by increased expression of pro-inflammatory cytokines like IL-1, IL-6, IL-10 and TNF- α . Our data demonstrate significant higher levels

of these pro-inflammatory mediators at 30 and 120 minutes of reperfusion in males when compared to females, indicative for a more activated inflammatory response in males.

Another hallmark of intestinal IR is endoplasmic reticulum (ER) stress. ER stress arises from environmental conditions that reduces the protein folding capacity of the ER, including inflammation, exposure to microbiota and hypoxia. This results in the accumulation of unfolded proteins, leading to the activation of the unfolded protein response (UPR) and subsequent X-box binding protein 1 (XBP1) splicing⁷³. The UPR is initially directed at cell survival, by inducing a translational block to reduce protein load to the ER and activating programs that increase protein folding capacity of the cell to cope with ER stress. If ER stress is too severe, the cells become irreversibly damaged and the signalling pathways switches from pro-survival to pro-apoptotic with the transcription of C/EBP homologous protein (CHOP), promoting apoptotic cell death.

The UPR is activated via three ER stress sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE-1), and activating transcription factor 6 (ATF6). Under normal conditions Binding protein (BiP, also known as Grp78) is attached to the ER stress sensors, deactivating these factors. During ER stress unfolded proteins accumulate in the ER lumen. BiP binds to these proteins, thereby dissociates itself from the ER stress sensors, activating several transcriptional programs. The latter leads, along with other reactions, to XBP1 splicing allowing the cell to cope with ER stress⁷³.

The XBP1 splicing was shown to be strongly correlated with Paneth cell apoptosis, which can be explained by the high secretory activity of these cells and the subsequent susceptibility to ER stress^{15,74}. In line with these results, our data demonstrate presence of ER stress and Paneth cell apoptosis in both males and females, especially after short reperfusion. Interestingly, male small intestine displayed a higher amount of ER stress with significantly more XBP1 splicing and a higher number of apoptotic Paneth cells per crypt than female small intestine, rendering them more prone to decreased production of antimicrobial proteins and subsequent bacterial translocation and systemic inflammation.

The exact mechanisms accounting for the differential response to intestinal IR in men and women remain to be determined. Sex differences have mainly been attributed to the effect of sex hormones. The deleterious effects of testosterone and the beneficial effects of estrogen on IR-induced injury in mice have previously been demonstrated. Oestrogen has been shown to have anti-apoptotic and anti-inflammatory properties and strong anti-oxidative actions, thereby protecting tissues from oxidative damage. Testosterone was demonstrated to have a pro-apoptotic effect. Nonetheless, other mechanisms responsible for the feminine intestinal resistance to IR could certainly be involved.

In conclusion, recognition of this sex differences and elucidation of the underlying pathophysiological mechanisms is important as it is expected to be beneficial for treating both males and females with different therapeutic strategies in an attempt to reduce IR-associated morbidity and mortality.

COX-2 is of importance in restorative capacity of the epithelial lining of the human intestine

Next, the **seventh aim** of the thesis was to study the role of Cyclooxygenase-2 (COX-2) during intestinal epithelial damage and recovery.

COX-2 plays a key function in intestinal wound healing and several animal studies suggest an important role for COX-2 in intestinal IR ^{75,76}. Fredenburgh et al. showed that COX-2 deficiency led to intestinal barrier dysfunction, increased bacterial translocation and inflammation in a murine model for polymicrobial sepsis, emphasizing the important protective function of COX-2 in the intestine ⁷⁶. Furthermore, several studies have shown that COX inhibition, with the use of NSAIDs, resulted in gut damage in both animal models of IR and models for enterocolitis ⁷⁷⁻⁷⁹.

Our study reports for the first time differences in IR-induced damage and inflammation between wild-type and COX-2 KO mice subjected to either short (30 minutes) or prolonged (60 minutes) periods of ischemia have been investigated. Results in **Chapter 9** indicate that COX-2 deficiency aggravates intestinal damage after a short period (30 minutes) of ischemia. Following reperfusion, a lack of COX-2 causes more intestinal injury than was seen in wild-type mice. At this time-point, the amplified damage was accompanied by increased neutrophil infiltration in COX-2 KO mice compared to wild-type mice. Remarkably, although prolonged periods (60 minutes) of ischemia led to markedly more intestinal damage and neutrophil influx, no difference were observed between wild-type and COX-2 KO mice. These results support the hypothesis that COX-2 is of importance in restorative capacity of the gut after short periods of small intestinal ischemia followed by reperfusion and that this restorative mechanism fails during prolonged periods of ischemia. The role of COX-2 is controversial with various studies demonstrating a protective function for COX-2 during intestinal IR, as well in models of sepsis and colitis ^{76,80-82}. In other studies of intestinal IR, COX-2 inhibition, however, led to attenuation or no differences on intestinal damage and inflammation ⁷⁵. One reason for this different outcomes is the complexity of the COX pathway. Knockout of one of the COX enzymes may result in compensatory upregulation of the other COX isoforms. Because anastomotic healing requires adequate perfusion of the anastomosis and anastomotic healing seems to be affected by the use of NSAIDs, we also set out to investigate the role of COX-2 in the development of anastomotic leakage in colonic surgery. In **Chapter 10** we provide evidence that COX-2 is essential for neovascularization of the colonic anastomosis and thereby plays a crucial role in colonic anastomotic wound

healing. Both pharmacological and genetic disruption of COX-2 function, by use of NSAIDs or

COX-2^{-/-} mice respectively, increased colonic anastomotic leakage significantly after colonic surgery in mice, which was associated with severely decreased survival. Additionally, humans with decreased COX-2 expression due to a COX-2 gene polymorphism show increased risk for developing anastomotic leakage. These results indicate that COX-2 is essential in anastomotic healing.

Our and previous other studies suggest that the role of COX-2 in intestinal recovery from ischemic injury lies in the COX-elaborated prostanoids as prostaglandin E2 (PGE2). Interestingly, exogenous PGE2 administration reduced the severity of injury to baseline levels, indicating COX-2 derived PGE2 is protective against IR injury of the small intestine⁸². This could be a starting point in the development of new medical therapies that can limit intestinal IR damage.

Novel insight into role of digestive enzymes in the pathophysiology of human intestinal ischemia-reperfusion

The final aim of this thesis was to investigate the role of pancreatic digestive enzymes in intestinal inflammation associated with human intestinal IR.

Intestinal IR results in loss of physical (mucus-layer, epithelial lining) and immunological barrier function^{10,15}. This facilitates translocation of intraluminal content into the bowel wall and systemic circulation, triggering inflammation^{16,65}. This leads to SIRS with MOF causing the high morbidity and mortality rates associated with intestinal IR⁵. It still remains unclear what factor is the key player in the onset of the overwhelming systemic inflammation reaction as a result of intestinal IR. The role of the microbiome has been studied extensively, however no interventions against bacteria have been successful in clinical trials⁸³. Additionally, results for colonic IR revealed that the human colon ischemia has a more favourable outcome after IR^{84,85}, although the high colonic microbiota density is potentially more toxic than small intestine intraluminal content¹. This was explained by differences in mucus organisation and to goblet cell compound exocytosis, flushing bacteria from crypts leading to mucus-layer recovery^{86,87}. Another explanation is the detrimental activity of intestinal wall penetrating digestive enzymes present in the proximal small intestine. Recent rat studies undergoing intestinal IR indicate that pancreatic enzymes play a major role in acute inflammatory processes resulting from intestinal ischemia, hemorrhagic and endotoxic shock⁸⁸⁻⁹⁰. Serine proteases (e.g. trypsin, chymotrypsin, elastase) are the most abundant pancreatic digestive enzymes, which are stored in secretory membrane-bound vesicles as zymogens⁹¹. The trypsin zymogen is activated by duodenal brushborder enterokinases, while activated trypsin activates more trypsinogen and chymotrypsinogen⁹². Under normal physiological conditions, proteases are compartmentalized in the intestinal lumen by the mucosal barrier (mucus-layer and epithelial lining) to prevent digestion

of the intestinal tissue itself⁸⁸. Also, to avoid uncontrolled proteases activity, enzymes-inhibitors are abundantly present in the circulation⁸³. The pathognomonic role of proteases was discovered by adding protease-inhibitors intraluminally in rats, showing abrogation of the usual sequelae of intestinal IR, inflammation and shock⁹³. These results gave rise to the 'autodigestion' hypothesis suggesting that powerful proteases leak across the intestinal mucosal barrier, initiating self-digestion of the intestinal wall and leading to SIRS with MOF. Protease activity in the intestinal wall was accompanied by mucus disruption, tight-junction loss and epithelial cell disruption after intestinal IR in animals^{88,94}. In search for new interventional targets to improve therapies and decrease mortality, it is crucial to comprehend the underlying mechanisms associated with human intestinal IR. As animal studies suggest that inhibition of proteases is beneficial, we aimed to investigate the role of pancreatic digestive enzymes following both reversible and irreversible human intestinal IR.

Figure 12.2 Schematic illustration of autodigestion by digestive enzymes during intestinal IR

(A) Digestive enzymes are normally compartmentalized in the lumen by the epithelial barrier. **(B)** Intestinal IR compromises the epithelial barrier, leading to the escape of digestive enzymes into the wall of the intestine and starting the autodigestion process. **(C)** Entry of the digestive enzymes into the intestinal wall causes further destruction of the intestinal wall. Digestive enzyme leakage is accompanied by a massive inflammatory response and elevated digestive enzyme activity in the systemic circulation, leading to systemic inflammation, shock and multi organ failure.

In **Chapter 11** it is demonstrated for the first time in human intestinal tissue subjected to IR that IR-induced epithelial barrier loss allows for pancreatic digestive proteases to enter the intestinal wall. Protease leakage in the intestinal wall was accompanied by

increased inflammatory responses and elevated protease activity in the systemic circulation. Data presented in this study confirm the evidence that pancreatic digestive proteases may play a major role in the pathogenesis of the destructive and vigorous inflammation in patients suffering from intestinal ischemia (Figure 12.2).

The results of **Chapter 10** will add to a better understanding of the pathophysiology of intestinal ischemia. This will open new opportunities to study therapeutic interventions directed at the inhibition of pancreatic enzymes. Blocking of pancreatic proteases was already successfully applied in animals undergoing intestinal ischemia ⁸³. Recently, a report showed that intraluminally administered protease-inhibitor gabexate mesilate decreased shock and sepsis in a patient with Fournier's gangrene ⁹⁵.

The promising results of this pilot-study will serve as a basis for new therapeutic strategies to minimize the systemic inflammatory responses during the early stages of small intestinal ischemia. However, a further in depth study using our human IR model need to be performed to explore proteases localization in intestinal IR and correlate these with epithelial barrier damage and inflammation markers. To definitely explore the autodigestive hypothesis in man, protease-inhibitors will be administered intraluminally in the IR jejunal experimental model.

After more than a decade of research to elucidate pathophysiological consequences of intestinal IR and its sequelae, it seems the time has come to set up new therapeutic approaches to improve the morbidity and mortality in patients suffering from intestinal ischemia.

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Summary and discussion

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Inleiding

Darmischemie is een vaak voorkomende en zeer ernstige aandoening. De bloedstroom naar de darm is dan beperkt of gestopt. Bij een ernstige doorbloedingsstoornis krijgen de darmcellen te weinig zuurstof (ischemie), waardoor deze kunnen beschadigen, minder gaan functioneren of zelfs afsterven. Er zijn veel verschillende oorzaken van deze verminderde doorbloeding. Darmischemie wordt gezien bij acuut zieke patiënten met een bloedstolsel in de (slag)aders die de darm van bloed voorzien. Deze zogenaamde acute mesenteriale ischemie (AMI) is vergelijkbaar met een hart- of herseninfarct en gaat gepaard met een hoog sterftepercentage van 60 tot 80%. Daarnaast wordt darmischemie gezien bij patiënten die veel bloed verliezen door een (verkeers)ongeval of tijdens grote operaties. Het lichaam kiest er dan voor om het overgebleven bloed naar de belangrijkste organen te laten stromen, zoals het hart, longen en hersenen en dat gaat ten koste van de doorbloeding van de buikorganen. Hetzelfde mechanisme vindt ook plaats bij ernstig zieke patiënten als gevolg van bloedvergiftiging (sepsis), bij alvleesklierontsteking (pancreatitis) en inflammatoire darmziekte (M. Crohn, colitis ulcerosa). Daarnaast kunnen onder andere verklevingen, buikwandbreuken en draaiing van de darm (volvulus) een afsluiting van de bloedstroom veroorzaken.

Darmischemie is een ziekte waaraan nog altijd veel mensen overlijden. Goede diagnostiek en de correcte behandeling kunnen in een vroeg stadium levensreddend zijn. Herkenning van darmischemie is in een vroeg stadium echter moeilijk. Dit komt omdat de buikklachten zeer specifiek zijn, het lichamelijk onderzoek opvallend normaal lijkt en er geen specifieke laboratoriumonderzoeken voor deze ziekte zijn. Er is dus behoefte aan betere laboratoriumtesten die duidelijk maken of de darm beschadigd is. Dit proefschrift gaat ten eerste over het verbeteren van de diagnostiek waarbij een aantal potentiële nieuwe testen zijn bestudeerd.

Het hoge sterftecijfer is ook het gevolg van onvoldoende kennis over de ontstaanswijze van darmischemie en de daaropvolgende ernstige complicaties die optreden bij patiënten die lijden aan darmischemie. De veronderstelling is dat het falen van de darmbarrièrefunctie een belangrijke rol speelt bij het ontstaan van de uit de hand gelopen ontstekingsreactie en ernstige ziekte bij patiënten met darmischemie. De darm is verantwoordelijk voor de opname van water en voedingsstoffen uit het lumen. De inname van voedsel zorgt daarbij voor blootstelling van de darm aan potentieel schadelijke lichaamsvreemde stoffen. Ook is er kans op het binnendringen van verschillende soorten bacteriën, virussen en schimmels. Daarom vormt de darm een effectieve barrière tegen deze potentieel schadelijke stoffen ter bescherming van de

gastheer. Deze barrière is van belang om het lichaam voortdurend te beschermen tegen het binnendringen van bacteriën, virussen en schimmels met hun antigenen en toxines. De darmwand is opgebouwd uit verschillende lagen om zijn barrièrefunctie goed uit te voeren. De eerste laag, de mucosa, bestaat uit een aaneengeschakelde rij van epitheelcellen (de epitheellaag). Om te voorkomen dat er iets tussen de epitheelcellen doorlekt, zit tussen de epitheelcellen een eiwitcomplex die de epitheellaag stevig bij elkaar houdt: de tight junctions. Daarnaast maken slijmbekercellen (gobletcellen) een slijmlaag (mucus) die over de epitheelcellen heen ligt. Naast deze fysieke barrière bestaat de laatste verdedigingslinie uit speciale cellen in de epitheellaag die antimicrobiële eiwitten aanmaken: de Panethcellen. De eiwitten uit deze Panethcellen doden potentieel schadelijke bacteriën voordat zij de darmbarrière kunnen passeren. Verstoring van dit delicate evenwicht wordt geacht een sleutelrol te spelen tijdens darmischemie. Zuurstoftekort (ischemie) al dan niet gevolgd door herintroductie van zuurstofrijk bloed (reperfusie) resulteert in lokale weefselschade aan de darmcellen, wat gepaard gaat met functieverlies van de darmbarrière. Dit leidt tot passage van schadelijke stoffen en bacteriën met hun toxinen door de darmwand heen de bloedbaan in. Hierdoor ontstaat een ernstige systemische ontstekingsreactie met meervoudig orgaanfalen (MOF). Meervoudig orgaanfalen leidt tot bloedvergiftiging (sepsis), shock en uiteindelijk de dood. Op zoek naar nieuwe behandelmogelijkheden om de uitkomst van patiënten met darmischemie te verbeteren, is het cruciaal om de onderliggende moleculaire mechanismen die geassocieerd zijn met darmischemie-reperfusie (IR) gerelateerde darmbeschadiging te begrijpen. In het tweede deel van dit proefschrift zijn deze factoren met behulp van ons humaan darmischemie-model bestudeerd, evenals de veiligheid van dit model.

Al eerder is door onze onderzoeksgroep een uniek humaan dunne darm-IR model en een dikke darm-IR model ontwikkeld. Hierdoor is meer inzicht ontstaan in belangrijke cellulaire processen die een rol spelen bij ontwikkeling en herstel van schade aan darmcellen bij darm-IR. Hoewel de patiënten die deelgenomen hebben aan het IR-model nauwkeurig zijn opgevolgd, zijn de mogelijke complicaties na uitvoering van ons humane darm-IR model niet structureel geëvalueerd. Bovendien moeten we onderzoeken of patiënten die de experimentele darm-IR modellen ondergaan geen schade ondervinden als gevolg van het model in vergelijking met patiënten niet blootgesteld aan het darm-IR model die dezelfde operatie in hetzelfde ziekenhuis ondergaan in de overeenkomstige periode. Daarom is gestart met het evalueren van onze darm-IR modellen. **Hoofdstuk 2** beschrijft dat het veilig is om darm-IR te bestuderen in onze humane in vivo modellen. Wij vinden vergelijkbare complicatiepercentages bij patiënten blootgesteld aan het darm-IR protocol in vergelijking met controlepatiënten zonder blootstelling aan het darm-IR protocol.

Tegelijk komen de hoeveelheid complicaties overeen met de huidige internationale literatuur en deze aantallen worden als acceptabel beschouwd. Ook de operatietijd, hoeveelheid bloedverlies, opnameduur of mortaliteit verschillen niet tussen de groepen.

Nieuwe markers voor verbetering van de diagnostiek en bij darmischemie

Om stappen vooruit te zetten in de diagnostiek bij darmischemie is het van belang de diagnostische waarde van bestaande laboratoriumtesten bij AMI inzichtelijk te maken. Daarom geeft **hoofdstuk 3** een systematisch overzicht van alle bekende humane plasma biomarkers voor AMI beschreven in de afgelopen 10 jaar. Een uitkomst van deze studie is dat de huidige laboratoriumtesten, zoals lactaat, LDH, CK, leukocytose en base excess, niet bruikbaar zijn voor de diagnose darmischemie. Het gebruik van deze testen wordt ook ontraden in de huidige richtlijnen. Eveneens concludeert deze studie dat D-dimeren, Alpha Glutathione S-Transferase (a-GST) en Intestinal Fatty Acid Binding Protein (I-FABP) veelbelovend zijn in de diagnostiek bij darmischemie, waarbij I-FABP de meeste potentie heeft. I-FABP is een klein eiwit (14-15 kDa) dat specifiek voorkomt in epitheelcellen van de darm en water oplosbaar is. Deze eigenschappen zorgen ervoor dat I-FABP gemakkelijk vrijkomt uit IR beschadigde darm en terechtkomt in de bloedsomloop. Onze onderzoeksgroep en andere wetenschappers hebben al aangetoond dat I-FABP een goede biomarker voor darmschade is. Niettemin is er meer onderzoek nodig naar de diagnostische kenmerken van I-FABP voordat deze biomarker kan worden gebruikt in de dagelijkse patiëntenzorg. Immers, er moet een directie relatie bestaan tussen de hoeveelheid vrijgekomen I-FABP gemeten in het bloed en de ernst van IR-schade aan de darmcellen. De resultaten uit **hoofdstuk 4** laten zien dat I-FABP een zeer gevoelige biomarker is voor het detecteren van darmischemie in een vroeg stadium van het ziekteproces. Daarnaast toont deze studie aan dat de hoeveelheid I-FABP in het bloed uitstekend overeenkomt met de hoeveelheid schade aan darmcellen blootgesteld aan IR in ons humane darm-IR model. Hieruit kan worden geconcludeerd dat I-FABP een waardevolle marker is in de vroegdiagnostiek bij darmischemie. Toekomstige prospectieve studies zullen uitsluitsel geven of I-FABP inderdaad gebruikt kan worden in de dagelijkse diagnostiek bij patiënten verdacht voor darmischemie.

Het meest gevreesde risico van darmischemie is het ontstaan van zogenaamde transmurale schade. Bij transmurale schade raakt niet alleen de binnenbekleding (epitheellaag/mucosa) van de darm beschadigd maar worden de dieper gelegen spierlagen ook getroffen waardoor er een gat ontstaat in de darmwand. Door dit gat komt de darminhoud met alle schadelijke stoffen en bacteriën in de vrije buikholt terecht. Dit leidt tot een zeer ernstige systemische ontstekingsreactie met een buikvliesontsteking en heeft hoge sterftcijfers tot gevolg. Vroege opsporing van transmurale schade is daarom essentieel als de patiënt een kans wil maken op overleving, omdat behandeling een spoedoperatie vereist. Daarom zou het interessant

zijn om een extra biomarker te ontdekken die patiënten met onomkeerbare transmurale darmschade kan onderscheiden van patiënten die lijden aan omkeerbare, oppervlakkige darmschade.

Vanwege zijn veelvuldige voorkomen in gladde spiercellen in de buitenste laag van de darm, is het kleine, in water oplosbare Smooth Muscle eiwit van 22 kDa (SM22) een goede kandidaat. Deze eigenschappen zorgen ervoor dat SM22 de gladde spiercel makkelijk verlaat bij celschade en terecht komt in de bloedsomloop. **Hoofdstuk 5** beschrijft de ontwikkeling en evaluatie van SM22 als diagnostische biomarker voor het aantonen van transmurale darmischemie.

Een nieuwe en bovenal betrouwbare Enzyme-Linked Immuno Sorbent Assay (ELISA) voor SM22 is door onze onderzoeksgroep ontwikkeld en veelvuldig getest. Daarna zijn de diagnostische eigenschappen van SM22 in een experimenteel darm-IR model in ratten bestudeerd. De resultaten van deze studie laten zien dat SM22 inderdaad vrijkomt in de bloedsomloop bij transmurale schade aan de darm. Transorgaan-metingen bevestigen dat SM22 specifiek vrijkomt uit de darm en door de nier wordt geklaard. Om de klaringsdynamiek van SM22 in de mens verder te onderzoeken is bij patiënten die een operatie ondergaan bloed verzameld uit de aan- en afvoerende vaten van darmen, lever en nieren. Analyse van dit bloed stelt vast dat SM22 ook bij de mens door de nier geklaard wordt met een plasma halfwaardetijd van 23 minuten. Vervolgens is de klinische toepasbaarheid voor het vaststellen van darmischemie van SM22 samen met I-FABP voor het eerst getoetst in de mens. Dit is gedaan door de plasma-concentraties van SM22 en I-FABP te meten in patiënten met bewezen darmischemie, patiënten met andere acute buikklachten en gezonde vrijwilligers. De belangrijkste bevindingen zijn dat patiënten met oppervlakkige, mucosale, darmischemie en patiënten met diepere, transmurale, darmischemie hogere I-FABP waarden in het bloed hebben dan patiënten met andere acute buikklachten en de gezonde vrijwilligers. SM22 waarden zijn alleen verhoogd bij patiënten die lijden aan transmurale darmischemie. Derhalve maakt SM22 de opsporing van transmurale darmischemie mogelijk. Concluderend, zal enerzijds een biomarker voor het aantonen van oppervlakkige, mucosale darmschade (I-FABP) en anderzijds een biomarker voor het aantonen van diepe, transmurale darmschade (SM22) ons in de dagelijkse patiëntenzorg helpen een beeld te vormen over het niveau van de darmischemie en zodoende een betere behandelstrategie te kiezen. Toekomstige studies moeten uitwijzen of SM22 gebruikt kan worden in de diagnostiek bij patiënten verdacht voor darmischemie. Verwacht wordt dat een combinatie van biomarkers, die de verschillende stadia van darmischemie weerspiegelen, de sleutel tot het diagnostische probleem zal zijn (hoofdstuk 6).

Nieuwe inzichten in de pathofysiologie van darmischemie

Waar het eerste deel van dit proefschrift gaat over de verbetering van de diagnostiek bij patiënten met darmischemie, heeft het tweede deel van dit proefschrift tot doel meer inzicht te geven in de ontstaanswijze van darm-IR.

Eerdere onderzoeken van onze groep hebben aangetoond dat de humane darm over een uniek herstelmechanisme beschikt. De darm herstelt zichzelf na een kortdurende periode van 30 minuten zuurstoftekort. Dit mechanisme lijkt bij te dragen aan het behoud van de darmbarrière. **Hoofdstuk 7** onderzoekt of het zichtbare weefselherstel ook leidt tot functioneel herstel van de darmbarrière. Dit is gedaan door gebruik te maken van het humane dunne darm-IR model. Tijdens IR zijn de histologische veranderingen van de darmbarrière en gelijktijdig de barrièrefunctie gemeten. De darmbarrièrefunctie kan worden bepaald door de doorlaatbaarheid (permeabiliteit) van de darm te meten. Deze permeabiliteitstesten werken op basis van een kleine suiker (rhamnose) en grote suiker (lactulose). Na het toedienen van deze suikers kunnen de concentraties van deze teststoffen in het bloed worden bepaald. De ratio tussen de grote en kleine suiker weerspiegelt de mate van doorlaatbaarheid van de darmbarrière. Darmischemie leidt ook tot verlies van tight junctions. Tight junctions voorkomen dat er iets tussen de epitheelcellen van de darmen doorlekt en houden de epitheellaag stevig bij elkaar. Het verlies van tight junctions verlies is gevisualiseerd met behulp van een elektronenmicroscop. De elektronenmicroscop brengt lekkage van de contrastvloeistof, lanthaannitraat, tussen twee epitheelcellen in beeld. De lekkage van de contrastvloeistof is een maat voor verlies van tight junctions in dit onderzoek. Schade aan de epitheellaag wordt gekwantificeerd met behulp van plasmaspiegels van I-FABP. Daarnaast is de concentratie citrulline, een aminozuur dat alleen gemaakt wordt door goed functionerende darmepitheelcellen, gemeten. De resultaten tonen aan dat na een korte periode van 30 minuten ischemie, de darmepitheellaag efficiënt herstelt gedurende 120 minuten reperfusie. Dit gebeurt door insnoering van de beschadigde villustoppen met herstel van de epitheelcellen en de tussenliggende tight junctions. Het darm-IR model onderbouwt de directe relatie tussen fysiek barrièreverlies (van epitheelcellen en tight junctions) en verlies van functionele darmbarrière (met behulp van de suikertest en lekkage van lanthaannitraat door defecte tight junction complexen). Plasma suikerconcentraties zijn significant verhoogd na ischemie. Deze concentraties normaliseren weer na 120 minuten reperfusie. Analyse met de elektronenmicroscop demonstreert dat de tight junction complexen na 30 minuten ischemie lanthaannitraat tussen de cellen laten lekken. Ook deze lekkage herstelt zich na 120 minuten reperfusie. Plasmaconcentraties van I-FABP correleren met de permeabiliteitsveranderingen in de dunne darm. De resultaten van de functionele testen reflecteren nauw de zichtbare bevindingen van het darmweefsel tijdens darm-IR. In **hoofdstuk 7** wordt ook onderbouwd hoe citrulline als marker voor functie van darmepitheel kan worden

gebruikt. We tonen aan dat de plasmaconcentratie citrulline de functionele capaciteit van de darmepitheelcel goed weergeeft en vrijwel parallel loopt met de verstoringen van het darmweefsel tijdens darm-IR. De bevindingen weerspiegelen het vermogen van de darm om korte episodes van ischemie te weerstaan, met histologisch en functioneel herstel van de darmbarrière binnen 120 minuten reperfusie. De resultaten in **hoofdstuk 7** kunnen verklaren waarom vaak geen tekenen van ontsteking of bacteriële translocatie optreden na korte perioden van darmischemie. Verder inzicht in de mechanismen die verantwoordelijk zijn voor dit snelle histologische en functioneel herstel zal de behandeling van darmaandoeningen die gepaard gaan met het verlies van darmbarrière-verlies kunnen verbeteren.

Gebruikmaken van het unieke humane darm-IR model is een ander doel van dit proefschrift om erachter te komen of er een sekseverschil is in de reactie op darmischemie en om meer inzicht te krijgen in de mechanismen die betrokken zijn bij het verschil in reactie op darm-IR tussen mannen en vrouwen. Verschillen tussen mannen en vrouwen betreffende de ontstaanswijze van ziekten, ziekteprogressie en therapeutische respons worden vaak beschreven. Er is steeds meer bewijs, voornamelijk uit dierstudies, die sekseverschillen aantonen in respons op darm-IR. Dit lijkt in het voordeel te zijn van het vrouwelijk geslacht. De resultaten in **hoofdstuk 8** ondersteunen dat vrouwen beter bestand lijken tegen IR-geïnduceerde darmschade. De vrouwelijke dunne darm toont minder weefselschade aan het darmepitheel. Daarnaast blijken de I-FABP-concentraties, een goede maatstaf voor de ernst van de darmschade, significant hoger bij mannen dan bij vrouwen wanneer blootgesteld aan darm-IR. Darm-IR leidt tot een ernstige ontstekingsreactie. Een aantal ontstekingsmediatoren tijdens darm-IR waren ook hoger bij mannen in vergelijking met vrouwen, een indicatie dat de ontstekingsreactie onstuimiger en actiever lijkt te verlopen bij mannen.

Een ander kenmerk van darm-IR is endoplasmatische reticulum (ER) stress. De functie van het ER is het vouwen van eiwitten om ze zo hun juiste vorm te geven waardoor de eiwitten werkzaam zijn. Dit is een belangrijk mechanisme want verkeerd gevouwen eiwitten zijn schadelijk voor de cel. ER stress ontstaat door omgevingsfactoren die de vouwcapaciteit van het ER verminderen waaronder ontsteking, blootstelling aan bacteriën en ischemie. Als de ER stress te ernstig is, worden de cellen onomkeerbaar beschadigd en gaan ze in geprogrammeerde celdood (apoptose). Een cel die veel eiwitten produceert en daardoor erg kwetsbaar voor ER stress is de Panethcel. Onderzoek toont aan dat Panethcellen belangrijk zijn voor de instandhouding van de darmbarrière. Darm-IR zorgt voor ER stress en leidt tot sterfte van deze Panethcellen. Het verlies van Panethcellen heeft een verstoring van de darmbarrière tot gevolg met bacteriële translocatie en ernstige ontstekingsreacties. In overeenstemming met deze gegevens toont **hoofdstuk 8** de aanwezigheid van ER stress en Panethcel apoptose aan

bij zowel mannen als vrouwen. Het is interessant dat de mannelijke dunne darm een hogere hoeveelheid ER stress vertoont. Er is sprake van een hoger aantal Panethcellen in apoptose in de mannelijke dunne darm in vergelijking met de vrouwelijk dunne darm. De dunne darm van mannen lijkt hierdoor vatbaarder voor de verstoring van de darmbarrière. Verder onderzoek is noodzakelijk om de exacte mechanismen die verantwoordelijk zijn voor het verschil in respons op darm-IR tussen de mannen en vrouwen verder in kaart te brengen.

Sekseverschillen worden vaak toegeschreven aan de effecten van geslachtshormonen. Oestrogenen hebben ontstekingsremmende en sterke antioxidatieve eigenschappen om de geprogrammeerde celdood tegen te gaan. Weefsels worden door deze eigenschappen beschermd tegen oxidatieve schade. Testosteron daarentegen lijkt juist een stimulerend effect te hebben op de geprogrammeerde celdood. Dit zou kunnen verklaren waarom vrouwen beter bestand zijn tegen darm-IR. Desalniettemin zouden andere mechanismen zeker betrokken kunnen zijn voor de betere weerstand tegen darm-IR bij de vrouw. Concluderend is (h)erkenning van deze sekseverschillen en opheldering van de onderliggende mechanismen belangrijk omdat verwacht wordt dat dit gunstig is voor de behandeling van zowel mannen als vrouwen door verschillende therapeutische strategieën toe te passen in een poging om darm-IR geassocieerd lijden te verminderen.

In **hoofdstuk 9 en 10** wordt onderzoek gedaan naar de rol van het enzym cyclooxygenase-2 (COX-2) bij herstel van darmschade. COX-2 speelt een sleutelfunctie bij de wondgenezing. Verschillende proefdierstudies suggereren dat een belangrijke rol voor COX-2 is weggelegd in het herstel na darm-IR. Eerder is aangetoond dat een tekort aan COX-2 leidt tot disfunctie van de darmbarrière. Daarnaast is er bewijs dat een tekort aan COX-2 door het gebruik van NSAID's (afkorting van non-steroidal anti-inflammatory drugs, niet-steroïde anti-inflammatoire geneesmiddelen) resulteert in darmschade. NSAID's zijn een bekende en veel gebruikte pijnstillende geneesmiddelengroep. De werking van deze pijnstillers, waar diclofenac en aspirine bekende voorbeelden van zijn, berust op de remming van COX-2. De rol van COX-2 is controversieel tijdens darm-IR. Enerzijds zijn er studies die de beschermende functie van COX-2 aantonen, terwijl in andere onderzoeken naar darm-IR, COX-2-remming juist leidt tot afname van darmbeschadiging en ontstekingsreactie. Door gebruik te maken van muizen waarbij het gen voor COX-2 is uitgeschakeld – zogenaamde COX-2 knock-out muizen – en muizen met het natuurlijk voorkomende genotype – ook wel wildtype genoemd – onderzoeken we de rol van COX-2 tijdens darm-IR. **Hoofdstuk 9** laat zien dat een tekort aan COX-2 in muizen darmschade verergert na een periode van darmischemie. Daarnaast zorgt een gebrek aan COX-2 eveneens voor meer darmschade tijdens de reperfusiefase, dan werd waargenomen bij wildtype muizen. Deze resultaten

ondersteunen de hypothese dat COX-2 van belang is voor het herstellend vermogen van de darmen na perioden van ischemie in de dunne darm gevolgd door reperfusie. Een van de meest gevreesde complicaties in de darmchirurgie, als gevolg van een gestoorde darmgenezing, is naadlekkage. Wanneer een stuk (dikke) darm chirurgisch is verwijderd, worden de twee uiteinden van de darm weer aan elkaar gehecht. Naadlekkage treedt op als deze nieuwe verbinding, ook wel de darmnaad genoemd, niet geneest. Hierbij kan de darminhoud weglekken in de buik. Deze lekkage leidt tot buikvliesontsteking, ernstige bloedvergiftiging (sepsis) en zelfs overlijden. De resultaten in **hoofdstuk 10** van dit proefschrift laten zien dat een gebrek aan COX-2 zorgt voor een slechtere genezing van de darmnaad na dikke darmchirurgie in muizen. Door wederom gebruik te maken van COX-2 knock-out muizen werd een deel van het mechanisme waarom COX-2 belangrijk is in de genezing van darmweefsel ontrafeld. Muizen die dikke darmchirurgie ondergaan en geen COX-2 kunnen aanmaken hebben significant meer naadlekkages. Deze gestoorde wondgenezing van de darmnaad wordt hoogstwaarschijnlijk veroorzaakt door een verminderde aanmaak van nieuwe bloedvaatjes. Als deze muizen prostaglandine E2 (PGE2) krijgen, een eiwit wat het COX-2 enzym normaliter maakt in de darm, verbetert de genezing van de darmnaad weer. COX-2 speelt dus een grote rol in de genezing van darmweefsel na IR geïnduceerde schade (**hoofdstuk 9**) en is essentieel voor een goede wondgenezing van de darmnaad na darmchirurgie (**hoofdstuk 10**). NSAID's zouden dus vermeden moeten worden in patiënten die lijden aan een vorm van darmischemie of darmchirurgie ondergaan. Daarnaast zou toediening van PGE2 een bijdrage kunnen leveren in het herstel van deze patiënten. Dit is onderwerp van verder toekomstig onderzoek.

Het laatste doel van dit proefschrift was te onderzoeken welke rol verteringsenzymen spelen in de pathofysiologie van humane darmischemie-reperfusie. Het is nog altijd onduidelijk waarom darmischemie bij patiënten zulke ernstige gevolgen heeft. In recente proefdierstudies zijn aanwijzingen gevonden dat verteringsenzymen uit de alvleesklier (proteasen) een essentiële rol spelen in het ontstaan van de extreem hevige ontstekingsreactie en ernstige ziekte bij patiënten die lijden aan darmischemie. Proteasen zijn heel agressief en horen in het darmlumen thuis, waar ze zorgen voor de voedselvertering. Eerdere proefdierstudies laten zien dat proteasen door de darmwand lekken na darmischemie wat leidt tot ernstige darmschade. Door wederom gebruik te maken van het dunne darm-IR model wordt in **hoofdstuk 11** voor het eerst aangetoond dat proteasen ook bij de mens door de darmwand lekken en in de bloedbaan terechtkomen na schade aan de darmbarrière veroorzaakt door darm-IR. Dit leidt tot een grotere toename van de heftige lokale ontstekingsreactie dan wanneer deze proteasen niet aantoonbaar zijn in de darmwand en het bloed.

De resultaten in **hoofdstuk 11** bevestigen de veronderstelling dat proteasen een belangrijke rol spelen bij de pathofysiologie van de destructieve en heftige ontsteking bij patiënten die lijden aan darmischemie. De uitkomsten zullen bijdragen aan een beter begrip van de pathofysiologie van darmischemie. Dit opent nieuwe mogelijkheden om therapeutische interventies gericht op het blokkeren van pancreasenzymen te bestuderen. Het blokkeren van pancreasproteasen is al met succes toegepast bij proefdieren met darmischemie. Een onlangs verschenen studie laat tevens zien dat het direct in de darm toedienen van een proteaseremmer, shock en sepsis vermindert bij een ernstig zieke patiënt met een vleesetende bacterie.

De veelbelovende resultaten van **hoofdstuk 11** zullen dienen als basis voor nieuwe therapeutische strategieën om de systemische ontstekingsreacties tijdens de vroege stadia van dunne darmischemie te minimaliseren. Er moeten echter verdere diepgaande studies worden uitgevoerd met behulp van ons humaan darm-IR model om de exacte rol van proteasen bij darm-IR te onderzoeken en deze te correleren met darmbarrière-beschadiging en ontstekingsmarkers. Om de protease-hypothese bij de mens definitief te onderzoeken, zullen uiteindelijk ook proteaseremmers in de darm moeten worden toegediend in het darm-IR model.

Na meer dan een decennium van onderzoek om de pathofysiologische gevolgen van darm-IR en de gevolgen daarvan te ontrafelen, lijkt nu de tijd te zijn gekomen om nieuwe therapeutische benaderingen op te zetten om de overleving bij patiënten die lijden aan darmischemie te verbeteren.

Chapter 1	Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9	Chapter 10	Chapter 11	Chapter 12
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Valorisation addendum

The aim of this chapter is to audit this doctoral thesis in terms of scientific, social and economic value that can lead to societal benefits. It is a critical look upon the return of investment that the world community may receive from the knowledge gathered during the studies in this dissertation ¹.

Scientific relevance

Acute intestinal ischemia is a disease caused by occlusion (e.g. thrombosis) of the intestinal blood flow which often leads to death (60-80%), because it is difficult to diagnose intestinal ischemia at an early stage and adequate treatment lacks. Furthermore, intestinal ischemia is often responsible for a complicated course after major (cardio-) vascular or abdominal surgery, trauma and in patients with systemic inflammatory response syndrome (SIRS) and/or sepsis in the intensive care unit ². Results of this thesis will add to a better understanding of the pathophysiology of intestinal ischemia-reperfusion (IR). The knowledge gained in this thesis on biomarkers for detecting intestinal ischemia and differentiating between mucosal and transmural intestinal damage are essential in the development of effective diagnostic and therapeutic strategies. This will aid in the early detection of intestinal ischemia and its serious consequences. It was found that plasma levels of smooth muscle protein of 22 kDa (SM22) are potentially useful for the detection of transmural intestinal damage. SM22 in combination with intestinal fatty acid binding protein (I-FABP), as marker for the early diagnosis of mucosal intestinal ischemia, may help us in daily clinical practice to gain a more complete picture of the level of intestinal injury. This will identify patients with transmural ischemia in need of emergency surgery, from patients with only mucosal ischemia which can be treated using endovascular revascularization techniques and a more intestinal preserving strategy.

Knowledge on intestinal IR is of importance for surgeons, anesthetists, intensive care physicians, cardiologists, pulmonologists, gastroenterologists and pediatricians. Furthermore, all researchers, pharmaceutical companies and physicians involved in intestinal transplantation or dealing with patients with intestinal ischemia-related injuries will benefit from results of this thesis. The knowledge obtained in the search of new biomarkers to detect intestinal ischemia will also support the search of biomarkers to diagnose other ischemic organ diseases like acute ischemic heart disease, ischemic kidney injury or ischemic cerebrovascular disease.

The new insights in the pathophysiology of intestinal ischemia will be the start of a new research line aiming to develop treatment strategies improving the outcome of patients suffering from intestinal IR. Using the present unique model to study the consequences of intestinal ischemia without any risk for patients during major surgery, the present group will try to further elucidate why patients with intestinal ischemia develop such

severe inflammation and illness. The hypothesis is that measurement of digestive enzymes (proteases) in plasma enables clinicians to early detect whether the intestine is functionally damaged. Inhibition of these proteases will potentially lead to a new treatment, approaching the cause of the disease instead of its symptoms ³. This hopefully will result in enhanced (postoperative) recovery and less admissions in the intensive care unit. In line, society costs for hospital stay and intensive treatment will decrease.

Economical relevance of the results

Although acute mesenteric ischemia accounts for less than 1% of the patients seen at the emergency department with acute abdominal complaints, this number increases exponentially with age resulting in an incidence of 10% in patients with acute abdominal pain aged over 70 years ⁴. Next, intestinal ischemia or splanchnic hypoperfusion is a frequently occurring phenomenon during major surgery, trauma and shock. It often leads to systemic inflammatory response syndrome (SIRS), sepsis and multiple organ failure (MOF), important causes of death in critically ill patients. Moreover, patients suffering from chronic heart failure and chronic obstructive pulmonary disease (COPD) are known to experience intestinal ischemic episodes during daily activities, resulting in bacterial translocation, which can trigger SIRS ⁵. This underlines the economical issues related to intestinal ischemia, since many (older) patients have cardiovascular diseases, COPD, IBD, pancreatitis or undergo major surgery or trauma, which increasingly leads to intensive care admissions for SIRS, sepsis and MOF. Given the aging population, the number of patients suffering from intestinal ischemia will increase with time. This eventually leads to increased (healthcare) costs.

As described in this thesis, intestinal ischemia is notorious difficult to diagnose. An inaccurate or delayed diagnosis leads to an impaired therapeutic management causing catastrophic complications.

When diagnosed in an early state, mortality rates are between 0-40% and intestinal ischemia can mostly be treated by restoring the mesenteric circulation with minimally invasive endovascular techniques, before irreversible intestinal injury develops ⁵. After this intervention the patients will be admitted to the ICU for observation. This therapy plan will cost approximately €12,000. When the intestinal ischemia is diagnosed 12-24 h after the onset of the clinical symptoms, mortality steps up to 60-70% and transmural infarction has already occurred. In these patients intestinal revascularization still should be performed to reduce the extent of damage and facilitate healing of the potential intestinal anastomoses, followed by an exploratory laparotomy to resect unsalvageable parts of the intestine ⁵. After the bowel resection is done, the decision has to be made whether the patient is stable enough to create primary bowel reconstructions and finish the initial operation or the patient needs to be stabilized in the ICU with open abdomen and the anastomoses are performed later as a planned second-look operation. This therapeutic strategy is accompanied with increased costs up to €30,000. Delayed

diagnoses not only leads to dramatically increasing mortality rates, but it will also lead to increased healthcare costs. Using the proposed new biomarkers the diagnosis for intestinal ischemia could be made in an earlier state, potentially resulting in less patients with transmural intestinal damage and thereby reducing healthcare costs in the short-term and costs for society on the long-term.

In addition, the current new biomarkers could contribute in monitoring intestinal graft viability in patients suffering from intestinal failure. Small bowel transplantation is the ultimate life-saving therapy for patients with intestinal failure. The main complication in intestinal transplantation is graft rejection. Around 50-75% patients undergoing small bowel transplantation experience acute rejection leading to serious morbidity and mortality rates ⁶. Monitoring the graft viability is crucial in these patients. Unlike other types of transplantation, there is no non-invasive or reliable marker to predict rejection in small bowel transplantation. The rejection reaction must be detected at a stage when immunologic therapy could salvage the intestinal graft. The current diagnosis of rejection is made with protocol biopsies taken by invasive endoscopy. Besides the complications of taking biopsies, it is time-consuming and expensive. This delay in diagnosis results in loss of the intestinal graft ⁷. Biomarkers, like I-FABP, detecting graft rejection early in the process would allow for the timely diagnostic detection when pharmacologic therapy can still save the intestinal graft, leading to increased graft survival. Together with the reduced costs to perform these diagnostic tests, these biomarkers will also result in lower healthcare costs in patients who undergo small intestinal transplantation because of better graft survival.

Societal relevance

Elucidation of the mechanisms involved in the transition of mild inflammation towards severe inflammation following human intestinal IR, is of major importance since intestinal IR causes critical illness in multiple pathologies. Moreover, new strategies to treat intestinal IR and its sequelae would be of importance to many clinical fields. Patients suffering from intestinal ischemia are admitted in hospital for long time and their mortality rate is high. This leads to diminished quality of life (QoL). Part of the patients with intestinal ischemia in need of surgical exploration will end up with an ileostomy or colostomy after resection of nonviable bowel. The decision for an ostomy versus primary anastomosis depends upon the patient's general condition and assessment of the transected ends of the intestine. Potential reversal of the ostomy should be postponed for about six months ⁸. Given a majority of the patients with intestinal ischemia is older and likely more frail with higher risk for subsequent surgery, a substantial part will end up with an ostomy. Up to two thirds of the above mentioned patients never proceed to reversal of the ostomy because of comorbid conditions ⁹. These patients are also exposed to common ostomy complications including poor ostomy location, high output, skin irritation, retraction and prolapse. Due to this major change in physical appearance

and bodily function, an ostomy has a considerable impact on a patient's QoL afterwards. Patients with an ostomy have a poorer QoL caused by fear of leakage and they also have to deal with a certain stigma that deeply impacts them psychologically. Furthermore, their difficulties also extend to an altered body image and sexual malfunctions ¹⁰. Next, extensive bowel resections as a result of intestinal ischemia are still the leading cause of short-bowel syndrome ¹¹. This again has a major impact on patient's QoL as short-bowel syndrome is accompanied with high mortality and morbidity rates. Therefore, modern treatment of intestinal ischemia is focused on intestinal preserving management. The goal of treatment for patients with acute intestinal ischemia is to restore intestinal blood flow as quickly as possible instead of an early surgical laparotomy. Accurate diagnosis is essential in this regard to avoid the potential for intestinal infarction and to differentiate between patients eligible for revascularisation and those in need for acute surgical exploration. Using the knowledge of the diagnostic biomarkers presented in this thesis, treatment options and strategies can be more personalized and improve the chance of survival. Next, resection of bowel should ideally be delayed until endovascular mesenteric arterial revascularization can be performed to salvage as much bowel as possible. This approach remains somewhat controversial as direct visualization of the bowel is not an option during percutaneous intervention but it leads to a lower frequency of bowel resection and death rates ¹². Using plasma values of I-FABP and SM22 to monitor the intestinal viability, the above mentioned treatment strategy can be improved with better results in patient's mortality and morbidity.

Last, for long time only animal studies were reported on intestinal IR. These animal models are indispensable for the understanding of the different mechanisms contributing to the development of IR-induced organ damage. The use of animal models however, is also considered to be one of the main reasons that no effective clinical treatment for intestinal IR was developed and the associated high morbidity and mortality rates are unchanged the last 70 years ¹³. With the development of the current human experimental IR model, of which the safety evaluation is presented in this thesis, a basis is formed to obtain the knowledge essential to develop new effective therapeutic means. Secondly, the place of the animal models in modern societies is often debated, particularly the right to use animals to benefit human purposes, with the possibility that animals are harmed. The present human IR model will reduce the number of animals used for scientific purposes. This is in line with the statement of the European commission aiming at replacing animal methods as much as possible.

Potential knowledge utilization

In life sciences and medicine knowledge utilization be characterized as effective translation of the newly obtained knowledge by basic science research into state-of-the-art approaches for the prevention, diagnosis, and treatment of disease. This has the

potential to transform research from “bench-to-bedside”, delivering new medical options for patients. For this area of research, the end point is the production of a new diagnostic tool or a promising new treatment that can be used clinically or commercialized.

Before the proposed biomarkers can enter the daily clinical practice a phase four diagnostic study needs to be preformed, assessing the effectiveness of these new markers in large surveillance cohort studies. Therefore the creation of a rapid test for the proposed biomarkers with good diagnostic performance (low detection limit, clinical relevant cut-off points) is imperative. However, the limited availability of financial resources, scientific staff and laboratory capabilities to efficiently develop such tests and to perform these kind of studies are major obstacles for public research institutes and universities.

Public-private partnerships for innovation are an important part of the answer to such challenges. One of the most important weaknesses of the current innovation system is an inadequate interaction between science and industry. Scientists must continue to build partnerships with the industry to combine the strengths of both parties. Together, a unifying goal should be embraced: to act on behalf of patients and provide solutions to unsolved clinical problems. To aid in this field between science and biomedical companies the Dutch government and European Union (EU) face a couple of challenges. First, the incentives and the institutional frameworks for co-operation between public and private actors of innovation need to be improved. Second, the public support schemes for innovation and the complex innovation governance systems need to be streamlined lowering the administration burden. Last, the interdepartmental co-ordination needs to be improved causing research to work together side-by-side instead of against each other back-to-back ¹⁴.

To strengthen the position of public labs the government and EU stimulates the financing mechanisms of university research by increasing the available funding for public-private partnerships. Also more performance-based criteria (including applicability of research for innovation) in the allocation of institutional funding were introduced, stimulation future innovations. The exchange of researchers between universities and business enterprises is another way to enhance knowledge utilization. It is still rare for university researchers to spend time at the research and development departments of industrial partners companies. This is a lost opportunity for academic researchers to get better acquainted with industrial needs and market-oriented research, and may reflect the fact that university researchers do not feel a sense of competition for translating research into the daily clinical practice. It is noticed that academic investigators lack the knowledge assessing issues affecting the potential success of their translational research in the daily clinical practice. This shortcoming needs to be resolved by additional training

¹⁴.

The presented studies will also open new opportunities to develop therapeutic interventions directed at key aspects of the pathogenomic process. Especially new therapies directed at the inhibition of the pancreatic enzymes or the subsequent PAR-receptor activation by these proteases, which were successfully applied in animals undergoing intestinal ischemia and recently in a septic patient with Fournier's gangrene

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List of publications

1. Schellekens DHSM, Hulsewe KW, van Acker BA, van Bijnen AA, de Jaegere TM, Sastrowijoto SH, Buurman WA, Derikx JP. Evaluation of the diagnostic accuracy of plasma markers for early diagnosis in patients suspected for acute appendicitis. *Acad Emerg Med*. 2013;20(7):703-710.
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9. Hundscheid IHR, Schellekens DHSM, Grootjans J, Derikx JPM, Buurman WA, Dejong CHC, Lenaerts K. Females Are More Resistant to Ischemia-Reperfusion-induced Intestinal Injury Than Males: A Human Study. *Ann Surg*. 2018.
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11. Schellekens DHSM, Reisinger KW, Lenaerts K, Hadfoune M, Olde Damink SW, Buurman WA, Dejong CHC, Derikx JPM. SM22 a Plasma Biomarker for Human Transmural Intestinal Ischemia. *Ann Surg*. 2018;268(1):120-126.
12. Leenarts CA, Grootjans J, Hundscheid IH, Schellekens DHSM, Lenaerts K, Buurman WA, Dejong CH, Derikx JP. Histopathology of human small intestinal and colonic

- ischemia-reperfusion: Experiences from human IR-models. *Histol Histopathol.* 2019;34(7):711-722.
13. Schellekens DHSM, Hundscheid IHR, Grootjans J, Leenarts CA, Beets GL, den Dulk M, van Dam RM, Buurman WA, Lenaerts K, Derikx JPM, Dejong CHC. Evaluating the safety and complications of two human experimental intestinal ischemia reperfusion models: a retrospective observational study. *Submitted*
 14. Schellekens DHSM, Leenarts CA, Lenaerts K, Buurman WA, den Dulk M, Dejong CHC, Derikx JPM. The role of digestive enzymes in the pathophysiology of human intestinal ischemia-reperfusion: a pilot study. *In preparation*
 15. Schellekens DHSM, Bosmans JW, Reisinger KW, Grootjans J, Lenaerts K, Buurman WA, Dejong CHC, Derikx JPM. The role of cyclooxygenase-2 in the development of ischemia-reperfusion induced small intestinal damage. *In preparation*

Grants and awards

- 2011 Oral Free Paper Prize in the category "Intestinal ischemia: From basic mechanisms to diagnostic tests", United European Gastroenterology Week (UEGW), Stockholm, Sweden
- 2011 Travel Grant United European Gastroenterology Week (UEGW), Stockholm, Sweden
- 2013 Gastrostart Grant: Smooth muscle protein 22 (SM22) as potential biomark for transmural ischemic intestinal damage. Dutch Gastroenterology Federation (NVGE)
- 2013 2nd prize in Best Abstract Session United European Gastroenterology Basic science course 2013, London, UK
- 2014 1st prize in the category "most promising studies", AAV/MUMC science day, Maastricht, the Netherlands
- 2014 Travel Grant United European Gastroenterology Week (UEGW), Vienna, Sweden

Abbreviations

AB	Alcian blue
AEC	3-amino-9-ethylcarbazole
α -GST	α -glutathione S-transferase
AJ	Adherens junctions
AMI	Acute mesenteric ischemia
ATF6	Activating transcription factor 6
AUC	Area Under the Curve
BIP	Binding Protein
CHOP	C/EBP homologous protein
CI	Confidence interval
Citr	Citrulline
CK	Creatine kinase
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
DAMP	Damage-Associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DST	Dual-sugar absorption tests
ED	Emergency department
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ET-1	Endothelin-1
GADD34	Growth arrest and DNA damage-inducible protein 34
GALT	Gut associated lymphoid tissue
GLN	Glutamine
HE	Hematoxylin and eosin
IBD	Inflammatory bowel disease
ICU	Intensive care unit
I-FABP	Intestinal fatty acid binding protein
IL	Interleukin
IMA	Inferior mesenteric artery
IMV	Inferior mesenteric vein
IR	Ischemia-reperfusion
IRE-1	Inositol-requiring enzyme-1
KO	Knockout
L	Lactulose
LDH	Lactate dehydrogenase

LR	Likelihood ratios
mAbs	Monoclonal antibodies
MI	Mucosal infarction
MOF	Multiple organ failure
MPO	Myeloperoxidase
NEC	Necrotizing enterocolitis
NF- κ B	Nuclear factor kappa B
NLR	Nucleotide-binding and oligomerization domain (NOD)-like receptors
NOMI	Non-occlusive mesenteric ischemia
NSAID	Non-steroidal anti-inflammatory drug
OAAC	Other acute abdominal complaints
PAMP	Pathogen-associated molecular patterns
PAS	Periodic Acid Schiff
PAR	Protease-Activated Receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCT	Procalcitonin
PERK	PKR-like ER kinase
PGE2	Prostaglandin E2
PRR	Pattern-Recognition Receptors
QoL	Quality of life
R	Rhamnose
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of mean
SIRS	Systemic inflammatory response syndrome
SMA	Superior mesenteric artery
SMV	Superior mesenteric vein
SM22	Smooth muscle protein of 22 kDa
TI	Transmural ischemia
TJ	Tight junctions
TLR	Toll-like receptors
TNF	Tumor necrosis factor
UPR	Unfolded protein response
WBC	White blood cell
WT	Wild type
XBP1	X-box binding protein 1
ZO	Zonula occludin

Curriculum vitae

Dirk Schellekens was born in Baarle-Nassau, the Netherlands on August 11th 1986. After graduating from Mill Hill College (high school) in Goirle, he continued his education in 2004 to study Medicine at the faculty of Health, Medicine and Life Sciences at the Maastricht University. After receiving his medical degree in 2010, he started working as a PhD candidate at the Department of General surgery and the School of Nutrition and Translational Research in Metabolism (NUTRIM) of the Maastricht University Medical Center (MUMC+), under the supervision of Prof. Dr. W.A. Buurman, Prof. Dr. C.H.C. Dejong and Dr. J.P.M. Derikx, which resulted in the present thesis.

From October 2014 until December 2015, he worked as a surgical resident not in training at the Orbis Medical Center in Sittard-Geleen (supervision Dr. A.G.M. Hoofwijk). He started his surgical training in January 2016 at the Maxima Medical Center in Veldhoven (supervision Dr. R.H.M. Roumen, Dr. A.J.G. Maaskant), which is part of the educational region of the Maastricht University Medical Center+. (Prof. Dr. L.P.S Stassen). Currently Dirk is finishing his last two years of his surgical training to specialize in traumatology and lung surgery. He currently lives together with his girlfriend Chantal Weijzen in Maastricht.