Histopathology of human small intestinal and colonic ischemia-reperfusion

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Review

Histopathology of human small intestinal and colonic ischemia-reperfusion: Experiences from human IR-models

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Summary. Intestinal ischemia-reperfusion (IR) injury is a frequent, but potentially life-threatening condition.

Although much has been learned about its pathophysiology from animal IR models, the translation to the human setting is imperative for better understanding of its etiology. This could provide us with new insight into development of early detection and potential new therapeutic strategies. Over the past decade, we have studied the pathophysiology of human small intestinal and colonic ischemia-reperfusion (IR) in newly developed human *in vivo* IR models. In this review, we give an overview of new insights on the sequelae of human intestinal IR, with particular attention for the differences in histopathology between small intestinal and colonic IR.

Key words: Human, Intestine, Ischemia-reperfusion, Histology

Introduction

Intestinal ischemia is a condition that occurs when a comprised intestinal blood flow is insufficient to meet the metabolic demands of the visceral organs. Intestinal ischemia can be divided into acute mesenteric ischemia (AMI) and chronic mesenteric ischemia (CMI) based upon the rapidity at which reduced blood flow develops and the degree at which blood flow is comprised. AMI can be a consequence of occlusion of intestinal vasculature due to an arterial embolus or thrombus, a venous thrombus, or segmental strangulation. In addition, also non-occlusive reduction of the intestinal circulation can cause intestinal ischemia. This entity is most frequently caused by splanchnic vasoconstriction and blood flow redistribution associated with a low cardiac output state such as an important share of intensive care unit patients, but can also be caused by hypoperfusion in low flow surgical states, necrotizing enterocolitis, sepsis, pancreatitis, hemorrhagic shock or increased intra-abdominal pressure due to oedema and/or ascites (American-Gastroenterological-Association, 2000; Sertaridou et al., 2015; Tilsed et al., 2016). We have developed a surgical model for AMI which we will explain in the next section. AMI accounts for 0.1% of hospital admissions (Stoney and Cunningham, 1993; Panés and Granger, 2009). The incidence rate is recently rising due to increased awareness among clinicians, an aging population with severe cardiovascular and/or systemic disease as well as the prolonged survival of critically ill patients (Panés and Granger, 2009).

Apart from alleviation of symptoms, a laparotomy to resect necrotic bowel and local vascular desobstructive interventions in case of an occlusive etiology, there is no treatment for intestinal ischemia-reperfusion (IR) (Schmid-Schonbein and Chang, 2014). Mortality rates of AMI exceed 60% and are assumed to be related to the development of transmural damage and intestinal barrier

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function loss (American-Gastroenterological-Association, 2000; Oldenburg et al., 2004; Fink and Delude, 2005; Derikx et al., 2007). This results in exposure of the inner milieu to the intraluminal intestinal content with subsequent translocation of luminal content towards the circulation, triggering systemic inflammation and eventually resulting in multiple organ failure (MOF) (Feinman et al., 2010).

Remarkably, the human colon is more resistant to IR-induced epithelial damage than the human small intestine (Hundscheid et al., 2015). Revelation of the behavior patterns of small intestine and colon in ischemia and reperfusion can provide insight into pathophysiological mechanisms underlying intestinal IR-damage. Knowledge of defense mechanisms is imperative in the quest for strategies to prevent excessive transmural damage in for example low flow states during surgical interventions. This review provides an overview of new insights into the histopathology in human intestinal IR and the differences between small intestinal and colon IR in particular.

Various human intestinal IR models

Over the past decade, we have studied the pathophysiology of acute human small intestinal and colonic IR in newly developed unique human *in vivo* IR models that allow detailed and controlled investigation of acute human intestinal ischemia and reperfusion. The studies were approved by the Medical Ethical Committee of Maastricht University Medical Center and were conducted according to the revised version of the Declaration of Helsinki (October 2013) and the rules of good clinical practice. All patients were informed about the experimental procedures and written informed consent of all patients was obtained prior to the surgical procedure. In the small intestinal IR model, the jejunum is subjected to IR. 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 surgical procedure. In our research model, at the end of this segment, 6 cm jejunum is isolated from the remaining part using a linear cutting stapler. This isolated segment is subjected to either 30 or 60 minutes of ischemia by placing two atraumatic vascular clamps across the mesentery containing the arteriole and venule draining the segment. All collateral blood vessels are ligated to prevent unwanted perfusion. Meanwhile, surgery proceeds as planned. After ischemia, one third (2 cm) of the isolated ischemic jejunum is resected using a linear cutting stapler.

Next, clamps are 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) is resected similarly after 30 minutes of reperfusion, and 120 minutes of reperfusion. Simultaneously, 2 cm of jejunum which remains untreated during surgery is resected and serves as internal control tissue.

Analogous to the model for acute human small intestinal IR as described above (Derikx et al., 2009a), a human *in vivo* colon IR model was developed. (Grootjans et al., 2013a,b) For this experimental protocol, patients undergoing low-anterior resection or abdominoperineal resection for colon or rectal cancer were included. During surgery, the colon segment to be removed for surgical reasons is identified and the proximal 6 cm of this segment is isolated using a cutting stapler. The isolated segment is exposed to 30 or 60 minutes of ischemia by placing 2 atraumatic vascular clamps across the mesentery. Afterwards the reperfusion phase is initiated by removal of the clamps. Tissue collection takes place similarly to the small intestine IR procedure. Because of the duration of surgery, we were only able to reach a maximum of 60 minutes of ischemia followed by 60 minutes of reperfusion in the colon IR model.

The human colon is less susceptible to IR-induced epithelial damage than human jejunum

The first event in small intestinal IR in animal models using rodents, cats, dogs and pigs is the appearance of characteristic subepithelial spaces, immediately followed by loss of enterocytes in the lumen of the small intestine (Chiu et al., 1970; Park et al., 1990). When subjecting human jejunum to a limited period of ischemia (i.e. 30 minutes) similar subepithelial spaces appear (Fig. 1B) as shown by a hematoxylin and eosin staining in Fig. 1 (Derikx et al., 2008a; Grootjans et al., 2011a). These spaces of Gruenhagen (Chiu et al., 1970; Park et al., 1990) are the result of detachment of the epithelial cells in the villi tips from the retracting basal membrane. Exposure of distal colon shows similar appearance of subepithelial spaces although their presence is more subtle (Fig. 1F). There are no obvious epithelial defects visible in either jejunum or colon at this point. During early reperfusion (30 minutes) of jejunum, opposing mature epithelial cells that have lost contact with the basement membrane are pulled together like a zipper. Through this phenomenon the exposure of the lamina propria to intraluminal potentially harmful content is profoundly limited and homeostasis is maintained (Fig. 1C) (Derikx et al., 2008a; Grootjans et al., 2011a). A similar short period of reperfusion of the colon results in the shedding of damaged epithelial cells into the lumen. (Fig. 1G). A comparable process is noticeable in the small intestine after a prolonged period of reperfusion (i.e. 120 minutes) resulting in a reduced villus height (Fig. 1D). Jejunum as well as distal colon consist of a morphologically restored epithelial lining within 120 minutes respectively 60 minutes of reperfusion, reflecting their capability of withstanding 30I without significant consequences for the epithelial barrier which prevents a vigorous inflammatory response. (Fig. 1D,H). Interestingly, this is in contrast with animal studies, in which IR of the small intestine has been shown to elicit an inflammatory response after

a short period (up to 30 min) of ischemia (Panes and Granger, 1998; Chen et al., 2003; Hart et al., 2005; Blikslager et al., 2007).

Prolonged ischemia (i.e. more than 45 minutes) of human jejunum does not only result in the appearance of subepithelial spaces, a clear disruption of the epithelial lining occurs as well, as can be observed in the H&E staining of jejunum exposed to 60 minutes ischemia in Fig. 2B. A subsequent period of 30 minutes reperfusion results in the shedding of damaged villus tips into the lumen causing obvious further disintegration of the epithelial barrier (Fig. 2C) which is not restored even after 120R (Fig. 2D). The restorative mechanisms functional in jejunum exposed to a limited period of

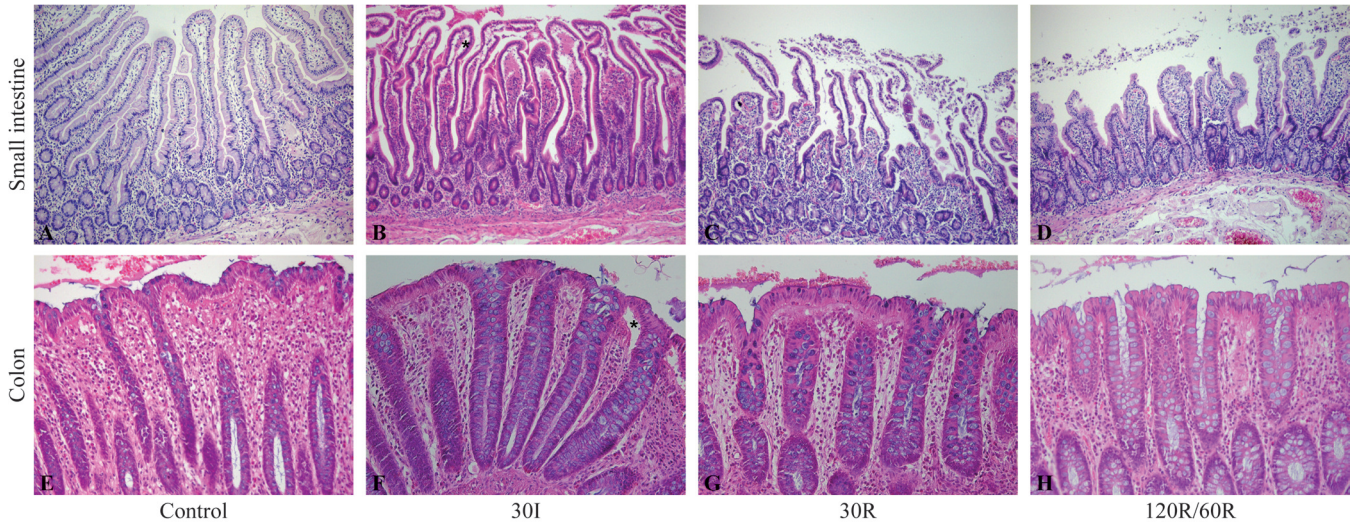

Fig. 1. Hematoxylin and eosin staining of jejunum and colon sections after 30I at 100x or 200x magnification. Control sections show a normal epithelial lining **(A, E)** of both jejunum and distal colon. 30I results in retraction of the basement membrane and the appearance of (small) subepithelial spaces (asterisk) while the epithelial lining remains intact in both colon and jejunum **(B, F)**. At 30R opposing mature jejunal epithelial cells that have lost contact with the basement membrane are pulled together like a zipper **(C)** whilst damaged colonocytes are shedded into the lumen **(G)**. At 120R and 60R respectively there is a morphologically intact epithelial lining in both jejunum and distal colon **(D, H)**.

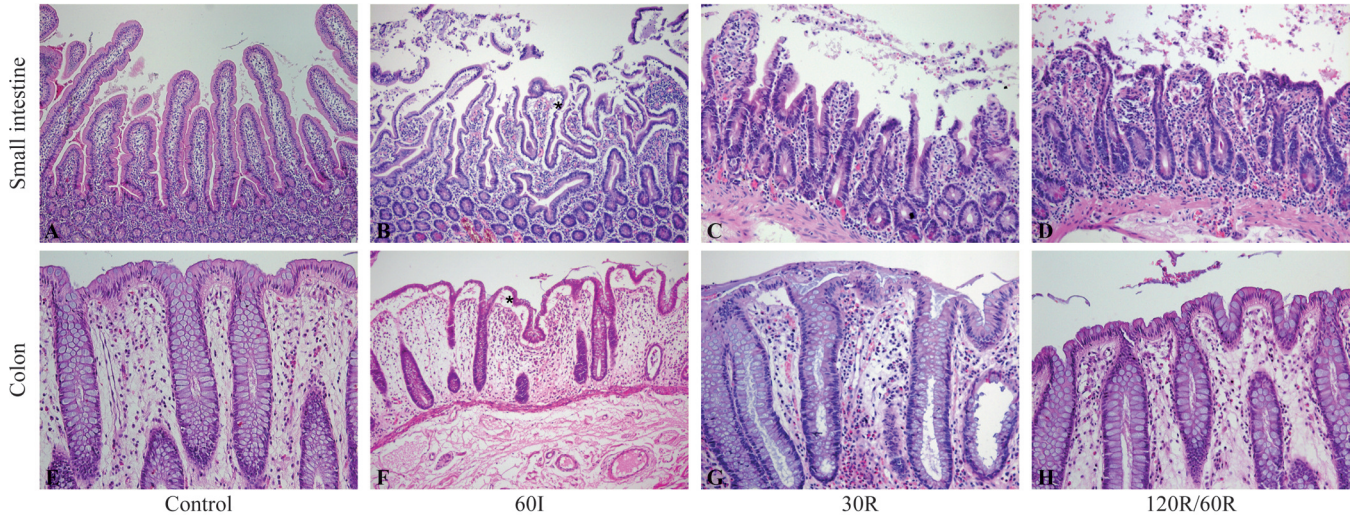

Fig. 2. Hematoxylin and eosin staining of jejunum and distal colon sections after 60I at 100x or 200x magnification. 60I results in the appearance of subepithelial spaces in jejunum as well as colon (asterisk) **(B,F).** However a disruption of the epithelial lining can be observed solely in jejunum **(B).** At 30R, damaged villus tips are shed into the lumen causing further disintegration of the epithelial barrier in the small intestine **(C)** that is still present at 120R **(D).** At 30R **(G)** and 60R **(H)** the epithelium of the colon appears somewhat irregular, however no significant gaps in the epithelial lining are observed.

ischemia, fail to prevent exposure of the lamina propria to intraluminal content after a substantial period of ischemia in the jejunum (Grootjans et al., 2010) While the reperfusion phase has a healing effect after a limited period of ischemia, it deteriorates histological damage after a substantial period of ischemia in humans. Multiple (animal) studies have demonstrated that the degree of injury attributed to reperfusion following mesenteric vascular occlusion is variable. The degree of reperfusion injury appears to depend on the animal species, the type of ischemia (complete versus incomplete vascular occlusion), the duration of preceding ischemia and the segment of affected intestine (Parks et al., 1982; Linfert et al., 2009).

In the human distal colon, a similar ischemic insult of 60 minutes results solely in the appearance of subepithelial spaces quite similar to the ones visible after a short ischemic insult. Although the epithelium might appear slightly irregular, the epithelial lining remains intact (Fig. 2F) even after 30 minutes reperfusion (Fig. 2G) when damaged cells are shed into the lumen. While the epithelial lining of the colon is still intact at 60 minutes of reperfusion (Fig. 2H), a period of even 120 minutes reperfusion does not result in restoration of the jejunal epithelial lining (Fig. 2D). Similarly, rat colon was shown less susceptible to ischemic injury than rat small intestine (Linfert et al., 2009).

Which protective mechanisms are present in the human colon and lacking in the small intestine that make the colon less susceptible to IR-induced epithelial damage? It can be hypothesized that this resistance of the colon is due to ischemic preconditioning mechanisms such as a relatively limited blood flow in the colon under physiological circumstances (Geber, 1960; Parks and Granger, 1985). Differences between jejunum and colon might also be explained by the functional organization of the intestinal mucus system. Mucus is the first layer of the bowel mucosal barrier and has a function in the absorption of important nutrients while simultaneously excluding pathogens (Godl et al., 2002; Chang et al., 2012a). The human small intestine, in analogy with mouse small intestine, consists of a single unattached mucus layer that may allow easy penetration of nutrients but consequently forms a less distinctive physical barrier (Atuma et al., 2001; Schmid-Schonbein, 2009; Chang et al., 2012b; Kistler et al., 2012; Ermund et al., 2013; Grootjans et al., 2013b; Johansson et al., 2013; Johansson, 2014). In contrast, the human colon consists of a two-layered mucus system, similar to the mouse colonic mucus. (Johansson et al., 2013) The compact and firm inner mucus layer is attached to the epithelium and is impervious to bacteria under physiological conditions, creating a defensive barrier for the immense (commensal) bacterial load in the colon (Vadlamudi et al., 2012; Johansson et al., 2013). The outer colonic mucus layer is less dense and hosts the commensal bacteria (Pelaseyed et al., 2014).

Ischemia in human and rat colon results in disruption of the mucus layer, facilitating exposure of the epithelium to intraluminal content and bacterial penetration (Grootjans et al., 2013b). This is rapidly counteracted by increased secretory activity of goblet cells, leading to expulsion of bacteria from the crypts as well as restoration of the mucus barrier limiting inflammation when followed by a substantial period of reperfusion (Grootjans et al., 2013b) Furthermore the lack of oxidant-producing enzymes in the colon has been suggested as a possible explanation for differences in (reperfusion) injury in colon compared to the small intestine in animal studies (Moore et al., 1995; Murthy et al., 1997; Grosche et al., 2011).

A novel theory explaining enhanced sensitivity of the small intestinal epithelium to intestinal ischemia is the autodigestion hypothesis (Schmid-Schonbein, 2009). Recent studies show that IR-induced mucin breakdown makes the small intestinal epithelium accessible to activated pancreatic enzymes, causing autodigestion of the intestinal wall, increased intestinal permeability, and onset of inflammatory responses. In addition, most pancreatic enzymes are degraded before reaching the colon. Interestingly, the protective effects of pancreatic enzyme inactivation by serine protease inhibitors FOY or FUT-175 were demonstrated in mice and rats animal models for small intestinal IR, in which blocking of the pancreatic enzymes reduced the destruction of the mucosal barrier and decreased the level of systemic inflammation (Mitsuoka et al., 2002; Gobbetti et al., 2012). These fundamental differences in the mucus system of the small intestine and colon might explain the sustainability of the mucosal barrier in case of an ischemic insult.

Basal membrane retraction succeeds in protection of the lamina propria from exposure to luminal contents in prolonged distal colonic ischemia but not in prolonged jejunal ischemia

An in-vitro model of chemically-induced injury to guinea pig ileal epithelium has taught that the subepithelial spaces that appear after ischemia result from the retraction of the basement membrane by contraction of myofibroblasts (Powell et al., 1999). To clarify the development of these subepithelial spaces, the network of myofibroblasts underlying the basement membrane within the villus lamina propria can be visualized by immunohistochemistry of smooth muscle actin (SMA), a major constituent of myofibroblasts. (Powell et al., 1999) Control jejunum and colon show SMA positive cells directly beneath the epithelial layer and basal membrane, confirming attachment of the epithelial cells to the basal membrane and underlying myofibroblast layer (Fig. 3A,E). Upon 30 minutes of ischemia a retraction is observed of the SMA positive cells from the basal pole of the epithelial cells at the jejunal villus and colonocytes, causing subepithelial spaces (Fig. 3B,F), although more subtle in colon than jejunum. After a subsequent period of 30 minutes reperfusion the retracted basement membrane is still

observed in small intestine and colon (Fig. 3C,G). However, after persistent reperfusion of 120R and 60R of jejunum respectively distal colon, the myofibroblasts and basement membrane on top are again attached to the epithelial lining (Fig. 3D,H).

In both small intestine and distal colon obvious subepithelial spaces occur after exposure to 60I (Fig. 4B,F). However, in contrast to an intact epithelial lining in the distal colon (Fig. 4F), there is an interrupted epithelial lining in jejunum (Fig. 4B) which is aggravated during a short period of reperfusion (Fig. 4C). After 120 minutes of reperfusion the basal

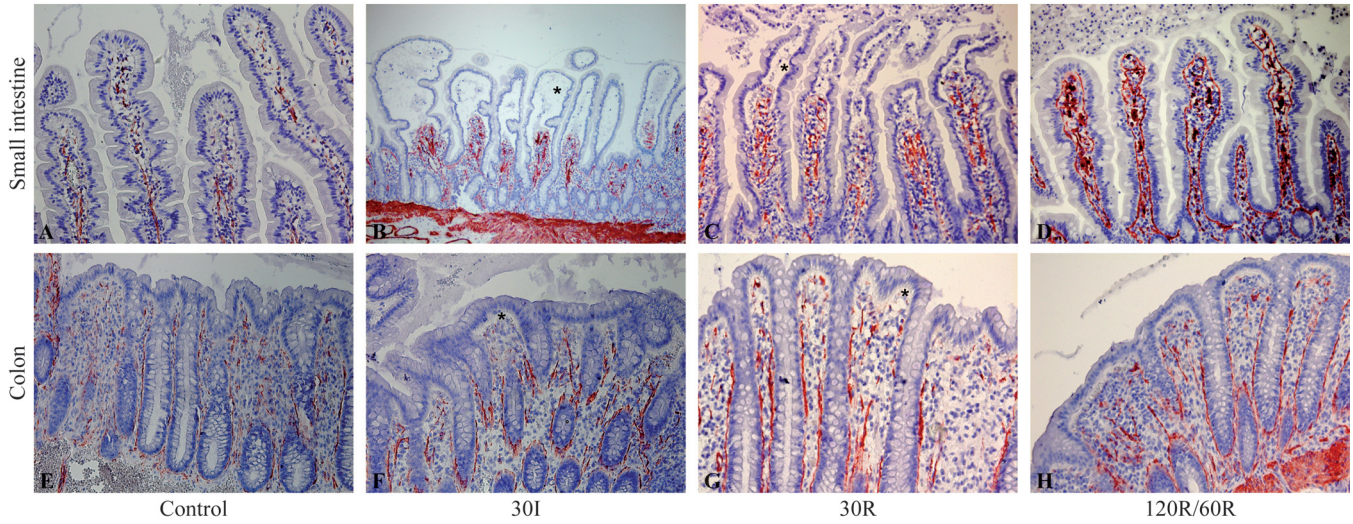

Fig. 3. Characterization of the basement membrane in jejunal and colon sections after 30I with α-SMA staining in red (AEC) at 100x or 200x magnification. Control jejunum and colon show SMA positive cells beneath the epithelial layer and basal membrane **(A, E).** At 30I a retraction is found of the SMA positive cells from the basal pole of the epithelial cells in jejunum and colon, causing subepithelial spaces (asterisk) **(B, F).** At 30R the retracted basement membrane is still observed (asterisk) **(C, G).** However, at 60R (distal colon) and 120R (jejunum) the myofibroblasts and basement membrane on top are again attached to the epithelial lining **(D, H)**.

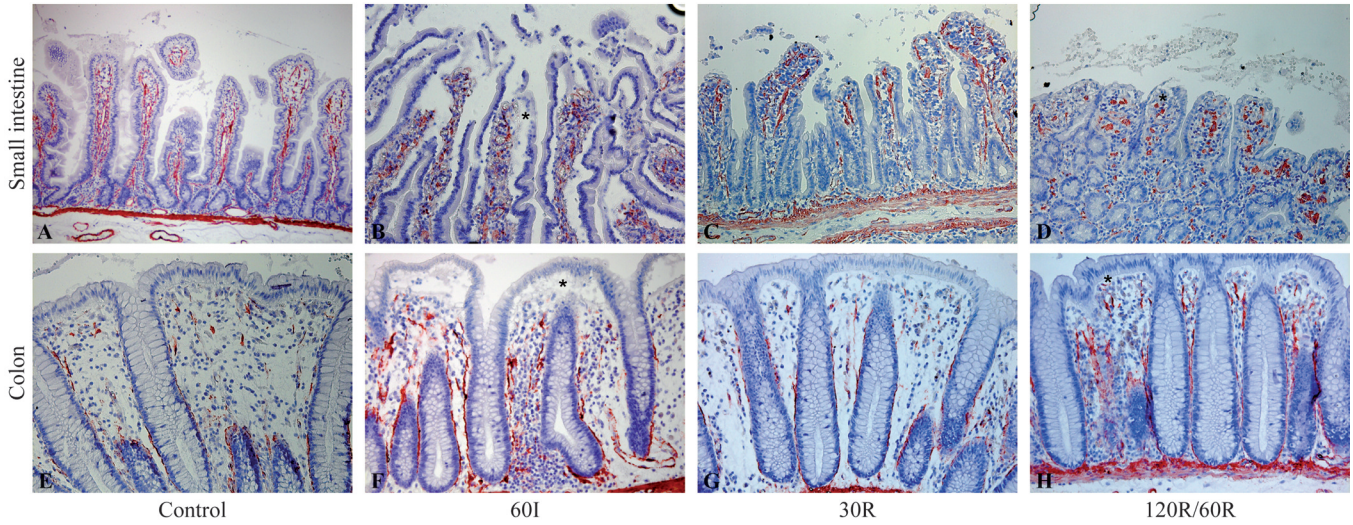

Fig. 4. Characterization of the basement membrane in jejunal and distal colon sections after 60I with α-SMA staining in red (AEC) at 100x or 200x magnification. At 60I an obvious retraction is found of the SMA positive cells in jejunum and distal colon (asterisk). The distal colon still consists of an intact epithelial lining **(F)** in contrast to the small intestine **(B)**, which is aggravated by short reperfusion **(C)**. At 120R in jejunum, there is still no positive α-SMA staining beneath the apical epithelial layer (if present) (asterisk) **(D).** At 30R of the distal colon the retracted basement membrane is still observed **(G)**, however, a 60 minute reperfusion period results in an epithelial lining that is again attached to the basement membrane and underlying myofibroblasts (asterisk) **(H)**.

membrane and myofibroblasts are still not reattached to the epithelium (Fig. 4D) in contrast to a substantial period of reperfusion (i.e. 60 minutes) in the distal colon (Fig. 4H). This process of detachment of epithelial cells from the basal membrane is also well documented in many research animal species (Chiu et al., 1970; Parks et al., 1982; Parks and Granger, 1983; Rosow et al., 2005). Cells closest to the intestinal lumen on the villus tips in the small intestine and inter-crypt surface epithelium in the colon initially lose their attachment to the basement membrane with progressive detachment of cells extending toward the crypt base as the duration of ischemia increases (Chiu et al., 1970; Parks et al., 1982).

L-FABP shows different patterns of jejunal and colonic epithelial cells after exposure to similar periods of ischemia

Although hematoxylin and eosin staining has provided elementary insights in the morphological differences between small intestine and colon exposed to similar periods of ischemia, epithelial cells can be visualized in more detail by immunohistochemical analysis of fatty acid–binding proteins (FABP). Liver fatty acid binding protein (L-FABP) is a small cytosolic protein expressed in liver epithelial cells but also in mature enterocytes of the villus and in lower amounts in the upper half of the colon crypts (Pelsers et al., 2003; Derikx et al., 2009b).

After 30 minutes of ischemia of the jejunum, cytosolic L-FABP staining is decreased in mature enterocytes with abundant staining in the subepithelial spaces (Fig. 5B). In colon sections little staining can be detected in the small subepithelial spaces if present (Fig. 5F). In jejunum as well as colon the epithelial lining remains intact. Upon short reperfusion (30R) a decreased cytosolic staining in jejunum is still observed (Fig. 5C). Some shedded colonocytes in the lumen can be observed in colon tissue at this point (Fig. 5G). However, upon prolonged reperfusion (120 minutes) of the jejunum, L-FABP cytosolic positive cells are part of the resealed epithelial barrier again while shedded L-FABP containing enterocytes are found in the debris in the lumen (Fig. 5D). At 60R the colonic epithelial barrier is completely intact as well (Fig. 5H).

In contrast to 30 minutes ischemia, L-FABP staining reveals leakage of L-FABP positive intracellular components in both the subepithelial spaces and intestinal lumen after exposure of jejunum to 60 minutes of ischemia (Fig. 6B). Such a disruption of the epithelial lining is not be observed in colon sections (Fig. 6F). Subsequent short reperfusion of 30 minutes leads to massive shedding of damaged villus tips into the lumen in jejunum sections. Consequently, a decreased staining of L-FABP is observed in the remaining part of the villi with L-FABP positive enterocytes present in the intraluminal debris (Fig. 6C). At 30R some shedded colonocytes can be found in the lumen (Fig. 6G). The jejunal epithelial lining is not restored after 120 minutes of reperfusion and shortening of the villi can be

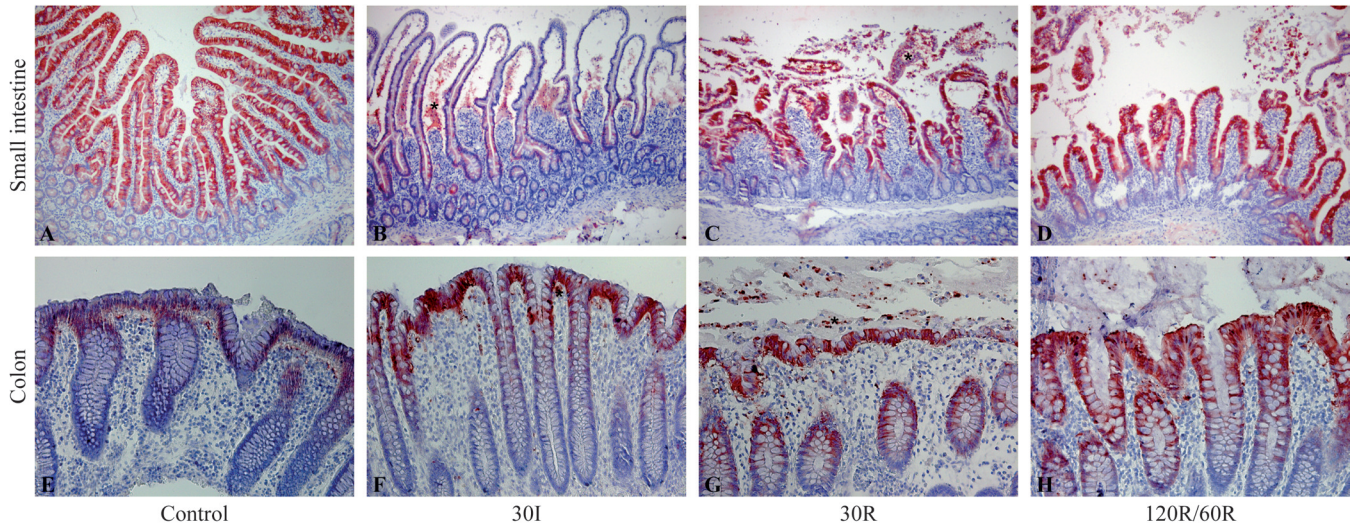

Fig. 5. Micrographs of jejunal and colon sections exposed to 30I stained for L-FABP in red (3-amino-9-ethylcarbazole, AEC) at 100x or 200x magnification. L-FABP staining in the control jejunal and colon tissue demonstrates the abundant cytosolic presence of L-FABP in mature enterocytes of the villi **(A)** and upper half of the crypts in colon tissue **(E)**. At 30I cytosolic L-FABP staining in jejunal sections is decreased in mature enterocytes with abdundant staining in the subepithelial spaces (asterisk) **(B)**. In colon sections little staining can be detected in the small subepithelial spaces if present (asterisk) **(F)**. In jejunum as well as colon the epithelial lining remains intact. At 30R a decreased cytosolic staining with abundant presence of shedded epithelial cells in the lumen is observed in jejunal tissue (asterisk) **(C).** Some shedded colonocytes in the lumen can be observed (asterisk) **(G)**. At 120R L-FABP cytosolic positive cells are part of the resealed jejunal epithelial barrier while shedded L-FABP containing enterocytes are found in the debris in the lumen **(D)**. At 60R the colonic epithelial barrier is completely intact as well **(H)**.

observed (Fig. 6D). The epithelium of the colon after 60R however, appears somewhat irregular, however no significant gaps in the epithelial lining are observed (Fig. 6H).

From a clinical perspective it is important to mention that FABP's are released early into the circulation upon enterocyte membrane integrity loss. Measurement of systemic plasma L-FABP or intestinal fatty acid binding protein (I-FABP) levels can accurately differentiate between experimentally-induced extensive irreversible

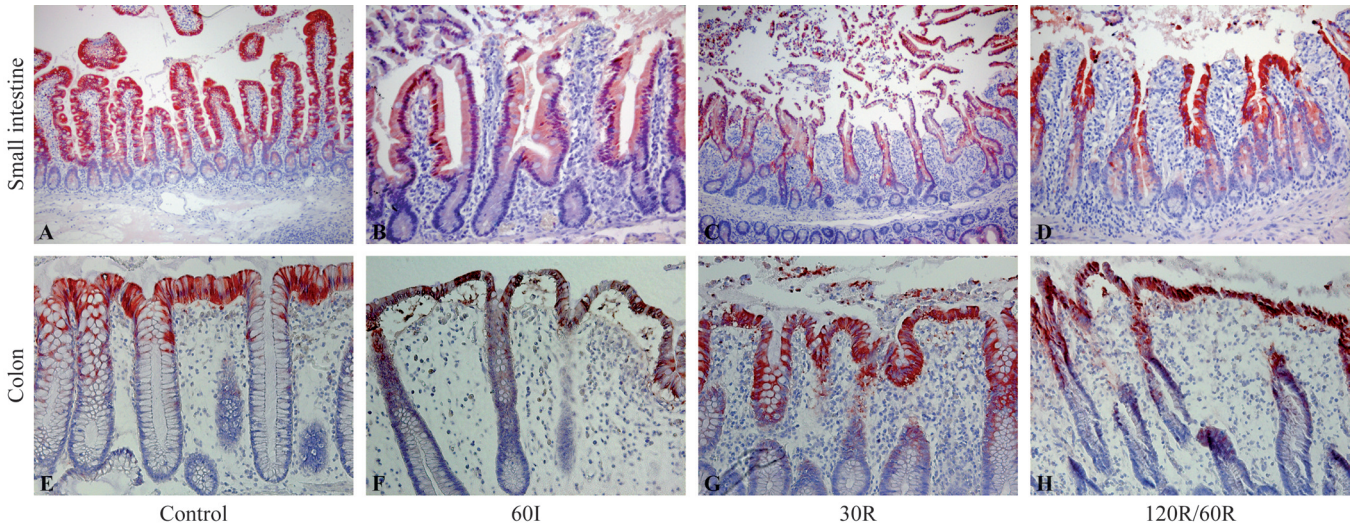

Fig. 6. Micrographs of jejunal and distal colon sections exposed to 60I stained for L-FABP at 100x or 200x magnification. At 60I L-FABP staining of jejunum reveals leakage of L-FABP positive intracellular components in both the subepithelial spaces and intestinal lumen **(B).** However a clear disruption of the colonic epithelial lining cannot be observed **(F).** 30R leads to massive shedding of damaged jejunal villus tips into the lumen. Consequently, a decreased staining of L-FABP is observed in the remaining part of the villi with L-FABP positive enterocytes present in the intraluminal debris **(C).** At 30R some shedded colonocytes can be found in the lumen **(G)**. At 120R the jejunal epithelial lining was not restored with marked shorting of the villi **(D)**. The epithelium of the colon after 60R however, appears somewhat irregular; however no significant gaps in the epithelial lining are observed **(H)**.

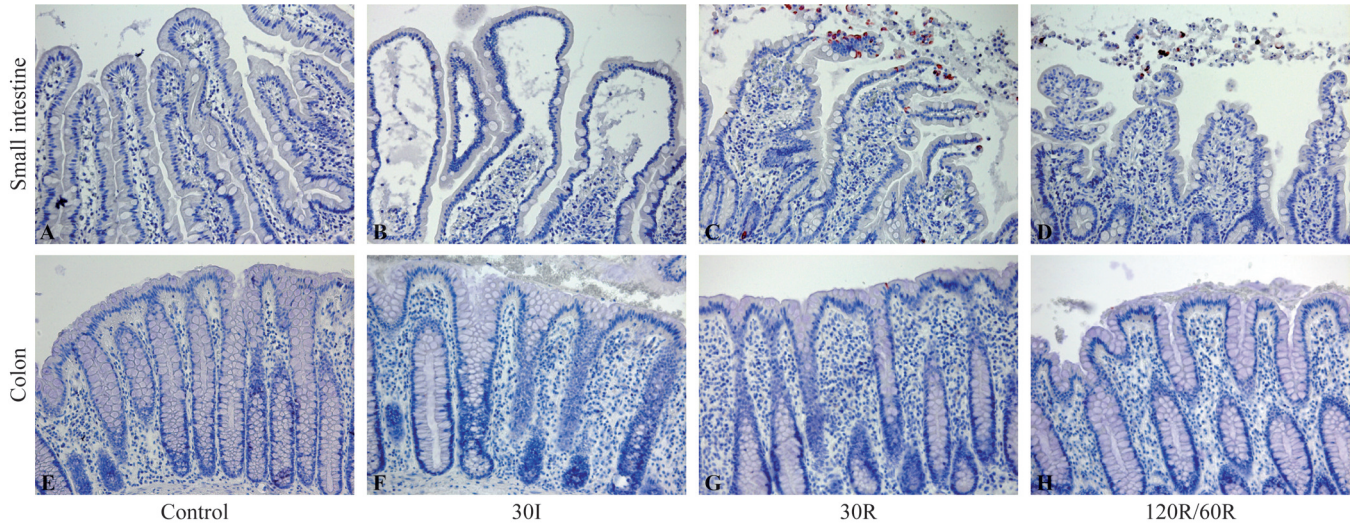

Fig. 7. M30 staining of jejunal and colon sections after 30I at 200x magnification. M30 staining is generally absent in villi of control jejunum and colon **(A,E).** At 30I no obvious M30 positive staining is found in jejunum nor colon **(B, F).** While at 30R in jejunum intense M30 immunostaining is observed at the villus tips near the shedding of mature epithelial cells into the lumen **(C),** only some immunostaining becomes visible at the surface epithelial cells that are to be shed into the lumen of colon sections (asterisk) **(F)**. At 120R respectively 60R, M30 immunoreactivity is no longer detectable in the intact, resealed epithelial barrier **(D,H)** while debris of M30 positive shedded epithelial cells is observed in the lumen of jejunum **(D).**

intestinal IR damage (60I) and mild and reversible intestinal IR without obvious morphological damage (15I) in a short segment of the small bowel (Schellekens et al., 2014). This can be useful in the decision-making whether to surgically intervene or not. However I-FABP and L-FABP have not yet been implemented as routine plasma biomarkers in the diagnosis of acute mesenteric ischemia in daily clinical practice. Before implementation of a new biomarker, different phases of a diagnostic study should be followed. A phase 3 study has yet to be published and a phase 4 study has yet to be performed. (Derikx et al., 2017) Furthermore clinical implementation is still impeded due to the use of multiple tests that measure different concentrations in plasma (Peoc'h et al., 2017).

Apoptosis in jejunal crypt epithelium as opposed to limited apoptosis of colonic surface epithelial cells after prolonged ischemia

Since apoptosis is the major mode of cell death in intestinal epithelial cells, the apoptosis marker M30, which detects the Asp396 caspase cleavage site in cytokeratin-18 (Leers et al., 1999), was used to demonstrate cell death in the jejunal and colon sections. M30 staining is generally absent in villi of control jejunum and colon (Fig. 7A,E), although a single positive cell can be observed at the top of the villus, indicating the physiologic pro-apoptotic state of enterocytes, goblet cells and enteroendocrine cells at the end of their lifespan. After 30 minutes of ischemia, M30 staining is generally absent in jejunum as well as colon tissue (Fig. 7B,F). Although after a subsequent limited period of reperfusion (i.e 30 minutes) minimal M30 positive staining might appear at the surface epithelial cells that are to be shed into the colon lumen (Fig. 7G), significant apoptosis can be observed at the jejunal villustips (Fig. 7C). After prolonged reperfusion of 60 respectively 120 minutes, M30 immunoreactivity is no longer detectable in the intact, resealed epithelial barrier of the distal colon respectively jejunum while debris of M30-positive shedded epithelial cells is observed in the lumen (Fig. 7D,H).

After 60 minutes of ischemia, apoptosis can be observed in both jejunal villus tips as well as in jejunal crypts (Fig. 8B). However, apoptosis of colonocytes in the distal colon is limited to epithelial cells in the surface epithelium that have lost contact with the basement membrane (Fig. 8F). During a subsequent short period of reperfusion, this contrast becomes even more obvious. There is abundant apoptosis throughout the entire epithelial layer of the jejunal villus (Fig. 8C), as opposed to apoptosis of colonocytes that is limited to superficial epithelial cells that are to be shed into the lumen (Fig. 8G) (Grootjans et al., 2010; Hundscheid et al., 2015). Studies in animal models have also shown that with increasing duration of the ischemic periods, progressive cell death occurs from villus to crypt base (Chiu et al., 1970; Parks et al., 1982; Blikslager et al., 1997). Our previous studies have shown that these M30 positive cells in the crypt are the Paneth cells which have an important role in the immunologic barrier function of the gut by producing and secreting antimicrobial products (Grootjans et al., 2011b). Loss of intestinal epithelial barrier and Paneth cells through a period of 60 minutes ischemia followed by 120 minutes reperfusion of the

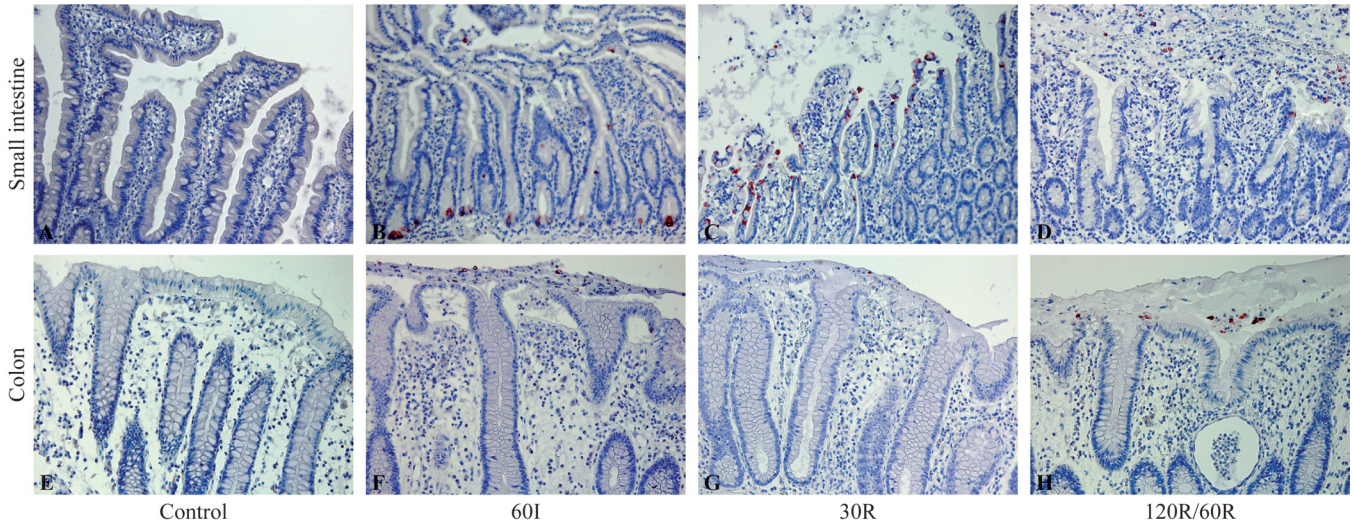

Fig. 8. M30 staining of jejunal and distal colon sections after 60I at 200x magnification. At 60I, apoptosis can be observed in both villus tips as well as in crypts of jejunum sections **(B).** In contrast, apoptosis of colonocytes in the distal colon is limited to epithelial cells in the surface epithelium that have lost contact with the basement membrane **(F).** This contrast is even more pronounced at 30R: there is abundant apoptosis throughout the entire epithelial layer of the jejunal villus, as opposed to apoptosis of colonocytes that is limited to superficial epithelial cells that are to be shed into the lumen **(G).** At 120R in jejunum respectively 60R in distal colon these IR-damaged apoptotic cells are observed in the luminal debris (**D and H,** respectively)**.**

small intestine of rats, results in an increase in bacterial translocation as well as in systemic inflammation (Grootjans et al., 2011b). In contrast, apoptosis of colonocytes in the human distal colon is still limited to the surface epithelium cells that are to be shed into the lumen after 30 minutes of reperfusion. After prolonged reperfusion of 120 minutes in jejunum respectively 60 minutes in distal colon these IR-damaged apoptotic cells are observed in the luminal debris whereas hardly any apoptosis is observed in the epithelial lining (Fig. 8D,H). Considering the immunologic function of Paneth cells, apoptosis might very well contribute to bacterial translocation and systemic inflammation (Vaishnava et al., 2008) in jejunum exposed to a significant ischemic insult. We have previously shown significant human intestinal IR-induced inflammation, which is characterized by complement activation, production and release of cytokines into the circulation, and neutrophil influx into IR-damaged tissue (Grootjans et al., 2010). Interestingly and in line with the higher resistance of the colon to IR-induced epithelial damage, inflammation is limited in IR-exposed colonic tissue despite the presence of a dense bacterial load (Hundscheid et al., 2015).

What can we learn from the human intestinal ischemia-reperfusion models?

Considering the rising incidence, high mortality rates and absence of an adequate causal treatment there is a serious need for improved knowledge about the pathophysiology of human intestinal ischemia reperfusion. Much has been learned about the pathophysiology of intestinal IR from animal IR models. Complete ischemia by temporary or permanent vascular occlusion of the superior mesenteric artery (SMA) in rodent models is currently the most commonly used method of inducing intestinal ischemic injury (Gonzalez et al., 2015). However, multiple *in vivo* and *in vitro* intestinal permeability studies have demonstrated significant differences between man and rodents and greater correlation between humans and pigs, especially concerning the microvascular architecture (Delahunty and Hollander, 1987; Bijlsma et al., 1995; Neu and Walker, 2011). Each model however has distinct disadvantages so that none of them is ideal to represent the onset and course of human disease. The specific research animal species chosen may contribute significantly to the outcome, for example through variations of the mucosal vascular supply between species (Gonzalez et al., 2015). These inter-species diversities may impact the physiological function of the intestinal mucosa and potentially their susceptibility or response to injury. The introduction of the novel human model of ischemia-reperfusion has contributed to effectuation of the Three R's principles for the ethical use of laboratory animals (Russell and Burch, 1959). Moreover it has provided us with more detailed information about the human pathophysiology of small intestinal and colonic ischemia-reperfusion. Parallels between the sequelae in human intestinal IR and *in vitro* and *in vivo* animal studies after various ischemic insults have been identified. The appearance of subepithelial spaces followed by loss of enterocytes in the lumen, progressive loss of epithelial cells extending toward the crypt base with increasing ischemia duration, the higher resistance of colon compared to small intestine to ischemic injury are some similarities. However various animal models have shown to elicit an inflammatory response after early ischemia. This is in contrast with results from the human IR model in which the restored epithelial lining of jejunum as well as distal colon after substantial reperfusion following a 30-minute ischemic insult prevents a vigorous inflammatory response. These discrepancies between human and animal studies in particular, demonstrate that translation to the human setting is imperative to work towards better detection and therapeutic strategies. Time restrictions due to the experimental design of the ischemia reperfusion models are a limitation of the current human *in vivo* studies. Prolongation of ischemia and reperfusion times in order to observe long term effects might be effectuated by the use of intestinal and colonic organoids.

Conclusion and future perspectives

The high mortality rates of mesenteric ischemia are assumed to be related to the development of intestinal barrier function loss with subsequent translocation of luminal content, triggering systemic inflammation and eventually resulting in multiple organ failure (American-Gastroenterological-Association, 2000; Oldenburg et al., 2004; Fink and Delude, 2005; Derikx et al., 2007; Feinman et al., 2010). Remarkably, the human colon is less susceptible to IR-induced epithelial damage than the human small intestine (Hundscheid et al., 2015). Profound apoptosis even of crypt epithelium including local Paneth cells undermines the physical as well as immunological barrier and lamina propria retraction is not able to prevent exposure to intraluminal content in prolonged jejunal ischemia. Apoptosis limited to surface epithelial cells and successful lamina propria retraction in human colon however is able to prevent exposure to luminal contents in prolonged ischemia. Given the wide range of pathologies associated with mesenteric ischemia, improvement in early diagnosis and therapy of mesenteric ischemia is of utter importance (American-Gastroenterological-Association, 2000; Sertaridou et al., 2015; Tilsed et al., 2016). Knowledge of potential defense mechanisms for IR of the human colon compared to jejunum such as mucus integrity and protease-inhibition might provide us with new points of engagement in the quest for therapeutic strategies to prevent excessive transmural damage in case of a mildly decreased splanchnic perfusion, such as in low flow states during major (cardiovascular) surgery (Derikx et al., 2008b). Currently we are focusing on the elucidation of the role of pancreatic enzymes in the pathophysiology of human intestinal IR, measuring protease activity in

intestinal tissue and plasma in correlation to intestinal damage and inflammation. Furthermore we used the previously described small intestinal human ischemia reperfusion model to administrate various protease inhibitors in the bowel and compare this to the administration of a placebo (EudraCT number 2014-002970-36).

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