Point to point reply to the editor and reviewers

We would like to thank the Editor and reviewers for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which helped to improve the quality of this manuscript.

Editorial office comments:

Please provide language a certificate letter from a professional English language editing company.

As prof. C.H.C. Dejong has lived and worked for quite some time in Scotland we would like to consider him as a native speaker of the English language. Furthermore he is a well established member of the editorial board of the British Journal of Surgery. He critically revised the manuscript for language quality which improved the manuscript.

Reviewers' comments:

Reviewer #1:

The manuscript by Schellekens DHSM et al. reports on the ability of the small intestine to rapidly restore the barrier function following a short exposure to ischemia. By subjecting a section of human jejunum of consenting patients undergoing surgery to 30 min of ischemia, they studied barrier loss and recovery during reperfusion periods of 30-120 min. Tissue damage was assessed by microscopy of various markers of epithelial integrity, barrier loss by a dual-sugar absorption test, and plasma citrulline -and glutamine concentrations were determined as a marker for enterocyte function. The authors conclude that the intestine is capable of a rapid morphological and functional recovery following a short exposure to ischemia. Evaluation: Maintenance of the intestinal barrier is an important defense against invasion of luminal pathogens, and assessment of barrier function is relevant in a number of diseases of the gut where the barrier is compromised. The paper presents some interesting in vivo data showing the ability of the jejunal epithelium to recover rapidly following ischemic injury. Overall, the study is well designed and sound. However, the conclusions could be strengthened by addressing the following points:

Page 4, last sentence of abstract: "Data from DST and citrulline closely parallel…" Yet, it is puzzling that the DST is at baseline level at 30I and only increased at 30I30R (Fig. 3C), whereas the Citr/Gln ratio is only decreased at the 30I time point and not during reperfusion. The authors should discuss this apparent discrepancy and maybe adjust their conclusion.

Answer: We thank the reviewer for this valuable comment. We agree with the point raised that "parallel" isn't the accurate word. Throughout the manuscript "parallel" was changed in "reflected". Also the reviewer suggests to discuss the discrepancy between DST and Citr/Gln ratio during intestinal IR. It is indeed 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 of Papadia et al. (Plasma Citrulline Concentration: A Reliable Marker of Small Bowel Absorptive Capacity Independent of Intestinal Inflammation,Am J Gastroentrol 2007) and Lutgens et al. (Monitoring myeloablative therapy-induced small bwel toxicity by serum citrulline concentration: a comparison with sugar permeability tests, Cancer, 2005) where permeability was not correlated with changes in plasma citrulline concentrations. 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. We added this point to the discussion. (please see page 21, line 18-28)

Fig. 3B: The contrast of the image showing the 30I120R time point is rather poor and should be replaced, as it does not reveal the lateral membranes between the cells.

Answer: According to the reviewers suggestion we have changed the image of Figure 3B at the 30I120R time point. The new picture reveals the lateral membranes between the cells better.

Fig. 4: Only ratios of citrulline/glutamine are presented, so is the drop at 30I solely due to a decrease in venous citrullline? For clarification, a comprehensive table showing all the V- and A measurements should be provided.

Answer: We thank the reviewer for this helpful comment. The drop of the Citr/Gln ratio was indeed the result of a solely drop of venous citrulline concentration. We agree that a table showing all venous and arterial measurements would be a valuable addition to the manuscript. Therefore we created supplementary table 1 (please see page 15, line 1-2 and the supplementary data)

The attached ethical approval document dated 08-10-2012: I am not at all familiar with the Dutch system, but to me the document looks like an application for ethical approval. Has approval also been granted?

Answer: Indeed, the approval is also been granted by the Medical Ethics Committee of the Maastricht University Medical Center. In addition to the reviewers comment, the letter of approval, although written in Dutch, and the reference number was provided. (please see page 8, line 5).

Minor point: Unclear sentence on p. 3, line 10: "… function were measured of function enterocytes restoration."

Answer: According to the reviewer's suggestion, this sentence was changed in the revised manuscript. (See page 4, line 10)

Reviewer #2:
The paper is interesting and is well written and generally results support the conclusions

Answer: We thank the reviewer for the positive comments on our manuscript.

Reviewer#3:
The aim of this descriptive human study was to extend the scope of previous works of the research group (Am J Pathol 2010, WJG 2016) and to confirm the presence and consequence of reperfusion injury in the human gut. According to the authors only few human experimental studies exist that directly correlate barrier function to histological appearance. This is true, and in this sense the novelty is certainly questionable, but the study is well-planned and executed and deserves further attention. Nevertheless, there are some problems also which limit the conclusions that can be drawn from this investigation.

1. The study was approved by an ethics committee –please provide exact reference number here.

Answer: As suggested by the reviewer, we have provided the exact reference number of medical ethics committee of the Maastricht University Medical Center. (please see page 8, line 5)

2. Patients undergoing pancreatico-duodenectomy were included to assess mucosal permeability and mucosal structural changes, and the authors concluded that the "study directly correlates histological data with intestinal permeability tests". This statement is misleading. The authors performed immunohistochemistry and electron microscopy to visualize certain changes linked to tight junctions but a direct correlation between these parameters (quantitative data on functional and structural damage of the mucosa) is missing. The results of an appropriate statistical analysis (with probability values) testing the association between permeability data and structural mucosal damage should be provided. In other words, the results of a statistical analysis looking for the association between the degree of structural injury (using a semi-quantitative scoring system like the Chiu grading on HE-stained light microscopy sections - and not only immunohistochemistry for tight junctions and electron microscopy of single cells) and functional data should be shown to reach meaningful conclusion.

Answer: We agree with the reviewer's statement that a statistical analysis looking for the association between the degree of structural injury and functional data would be interesting. However, using semi-quantitative scoring systems on tissue-sections are still a point of discussion. IHC is very useful to visualize the distribution and localization of particular cells and proteins or to identify cellular events. Several scores, including Chiu-grading, have been implemented for grading the degree of IR-induced damage, but these are prone to subjective interpretations because of individual differences in staff (causing selection and observation bias) and technique (staining intensity, duration of fixation, etc). Furthermore: chiu-grading is an intestinal injury score and less of a system for scoring recovery during the reperfusion-phase. Therefore, quantitative diagnostic biomarkers for intestinal damage have been increasingly investigated. Intestinal fatty acid binding protein (I-FABP), a low-molecular-mass cytosolic protein found in mature enterocytes on the tip of the small intestinal villi, is one of the most promising biomarkers for detection of loss of enterocytes and, therefore, structural mucosal barrier injury. To address the reviewer's point, we decided to use plasma I-FABP and measured arterial and venous I-FABP concentrations as assessment for structural damage. By using I-FABP as a quantitative measurement, we were able to investigate the association between enterocytes damage and permeability. We found that plasma levels of I-FABP correlated with plasma L/R ratios 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.
All information concerning the use of I-FABP in this study has been added to the Methods section (page 12, lines 1-13 and page 13, line 20-24) Result section (page 14 line 20-14, page 15, line 21-24 and page 16, lines 15-18 and page 17, lines 6-15) and Discussion section (page 20 lines 22-28).

3. Human clinical data are very important, but it would be equally important to provide a comparison with comparable animal data. In other words, it would be important to correlate these human data with previous data from standardized animal models. Here it should be mentioned that the exact magnitude of ischemia or reperfusion-induced intestinal mucosal damage as a function of the occlusion time was evaluated in standardized animal models of complete segmental arterial ischemia (see e.g. Boros M et al Ischemic time-dependent microvascular changes and reperfusion injury in the rat small intestine. J Surg Res 1995), and besides, the time scale of restoration of barrier function in injured intestinal mucosa is well described as well (e.g. Blikslager AT: Physiol Rev, 87: 545–564, 2007). These important background references should be included and properly discussed.

Answer: According to this reviewer suggestion we have reported and discussed the results of the two papers mentioned above (new references # 26 and 37) 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. 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. (Please see page 19. Line 19-29 and page 20, line 1-4)

4. Legends to figure 1, 2 and 3 are erroneous (panels to B and C are changed).

Answer: We thank the reviewer for pointing out this error. We adjusted the figure legends accordingly in the revised manuscript.

5. Is it possible to generalize these findings to the whole length or other segments of the human GI tract? Isn't there a region-specificity of permeability changes in the human mucosa? Besides, in animals Takeyoshi and coworkers (Transplantation 2001) has shown remarkable differences in the regenerative capacity of the small intestinal sections -regeneration being more pronounced in the jejunum than in the ileum. Again, this possibility should be properly discussed.

Answer: We thank the reviewer for this comment. According to the suggestion we have reported and discussed the results of the papers by Takeyoshi et al (new references 51) and Arrieta et al. in relation to our data. 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, limits their capabilities to detect permeability changes throughout the hole intestinal tract. For example lactulose and rhamnose are destroyed in the caecum and therefore provide only information regarding the small intestinal epithelium. 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 of 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 ileum. This more pronounced recovery effect could have a beneficial effect on our data. Taken together one should be careful generalizing the current findings to the whole length of the human gastro-intestinal tract.
This was added to the discussion of the manuscript (please see page 22, line 19-29 and page 23, line 1-5)

Reviewer#4:

This is a straight-forward paper associating results from lactulose/rhamnose ratios with histology and microscopic observations of the intestine following ischemia reperfusion. Much of what is reported has been demonstrated in animal models, but not before in human tissue. The major issue is the rigor of the microscopy studies. It is unclear if the data presented are reproducible among the additional subjects. Ideally, images should be scored in a blinded fashion.

Answer: We have addressed this issue accordingly to the suggestions of reviewer 3 by using arteriovenous (V-A) concentration differences of I-FABP as a quantitative measurement for structural mucosal injury to the epithelial lining. This was done as semi-quantitative scoring systems on tissue-sections are still a point of discussion. Even scored in a blind fashion, these scoring-systems are prone to subjective interpretations because of individual differences in staff (causing selection and observation bias) and technique (staining intensity, duration of fixation, etc).
Using I-FABP, we found that plasma levels of I-FABP correlated with plasma L/R ratios 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.
All information concerning the use of I-FABP in this study has been added to the Methods section (page 12, lines 1-13 and page 13, line 20-24) Result section (page 14 line 20-14, page 15, line 21-24 and page 16, lines 15-18 and page 17, lines 6-15) and Discussion section (page 20 lines 22-28).

Remove rapidly from the title.

Answer: According to the reviewer's suggestion, we deleted this word in the revised manuscript. (See page 1, line 1)

Remove "unique" from the conclusions in the abstract

Answer: According to the reviewer's suggestion, this was changed in the revised manuscript. (please see page3, line 24).

The term DST needs to be defined.

Answer: We appreciate the reviewer's perspective. We believe that we did not clearly explain the term DST in the original manuscript. This was adjusted in the revised version. Dual sugar absorption tests (DST) 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. (please see page 7, line 14-19).

The description of Patients in the methods section does not make sense.

Answer:
We thank the reviewer for this observation, but with all due respect to the reviewer, we would like to explain why we do not agree with this suggestion. We believe that a study should be fully reproducible. This also means that it is important that all relevant patients characteristics are well described in the methods section. Therefore we would like to keep this part of the manuscript.

We thank you again for considering our manuscript for publishing in the *World Journal of Gastroenterology*.

Sincerely yours,

Dirk Schellekens and Joep Derikx
Corresponding Authors
Email: D.Schellekens@maastrichtuniversity.nl