Author's response to reviews

Title: Validation of intraluminal and intraperitoneal microdialysis in ischemic small intestine

Authors:

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Author's response to reviews: see over
Dear Editor, Dear referees,

Accompanied by this cover letter, we will submit the revised manuscript entitled, “Validation of intraluminal and intraperitoneal microdialysis in ischemic small intestine” for publication BMC Gastroenterology. We would like to thank the referees for the careful and constructive criticism. After our best and time consuming attempt to see to all issues raised by the reviewers we feel that indeed the referees have taken considerable effort to evaluate our manuscript. We are indeed grateful for that. Also, we would like to bring up the general idea of constructive criticism and how important that is in order not to lose the potentially important information in a manuscript. We certainly hope that the very idea of our study was to come up with accepted analyses for sensitivity and specificity for intestinal microdialysis. With that in mind we humbly ask for the reviewers to open-mindedly re-review the manuscript.

Based the comments from the referees, we have modified the manuscript. We feel that the most salient message of the study with its’ limitations is now more readily available to the reader.

**Reply to the evaluation by the first referee**

Erik Solligard pointed out 5 things.

1. Major Compulsory revisions
   The equilibration dialysis part of the paper is interesting. The method is described earlier, and several studies has been performed. But in this paper this section is not well designed, nor well written.
   The principle of the method is not described, the placement of the sacs are not possible to reproduce based on your description, and you do not describe the method of collection of the samples etc. The number of patients is small.
   The study is not designed to answer your questions about the usefulness of this method in clinical monitoring.
   My request is to take out this topic from the paper, redesign the study and publish as a separate paper.

Reply: Grateful for the comment and suggestion: Equilibrium dialysis section is totally removed from this paper.
Page 2: The phrases “In addition, clinical feasibility of small intestinal luminal equilibration dialysis as a postoperative metabolic monitoring was tested.” and “Intraluminal jejunal equilibration dialysis was used for monitoring of 11 patients up to at least 24 hours. “ and “Furthermore, we propose that nasojejunal feeding tube with equilibration dialysis system could be a feasible method for clinical purposes when the metabolism of small intestine needs to monitored.” are removed from the abstract.

Page 5: The phrase “In addition to the feasibility/clinical validation of the method, we describe a pilot material with peri- and postoperative monitoring of intestinal anastomosis region with intraluminal approach with equilibration dialysis as described by Perner (19) and modified by us. “ is removed from the end of the abstract.

Page 8: the paragraph:

“Equilibration dialysis – postoperative feasibility

In the beginning of the surgery a nasogastric tube was inserted to the patients according to a standard procedure. This was replaced by a feeding tube (Freka ® Trelumina CH/FR 16/9, 150 cm) guided to jejunum at the end of the surgery to enable post-operative feeding. The correct placement was confirmed manually. A sack of dialysate membrane (semipermeable cellulose, cut-off 12 kDa; Sigma, St
Louis, MO, USA) was attached to this tube for dialysate sampling twice a day postoperatively. Feeding tube was removed when clinically indicated. Dialysate samples were collected every 12 hours. The samples were frozen \((-20 \, ^\circ C)\) for further analysis. “ is removed.

Page 10: the paragraph:

“**Clinical feasibility of intraluminal equilibration dialysis:**

Nasojejunal feeding tube with the equilibration dialysis sack on the tip was applied to 11 patients. One complication occurred where the distal part of the tube was inadvertently sutured to gut wall. Fiberoscopic release of the suture was applied without further complications. From 5 patients post operative dialysate sampling was succesful only once a day. Individual patients’ intraluminal L/P ratio varied from 0-38 and L/G ratio from 0-20 within the first 24-48 hours and later if the feeding tube was held in place (Fig 2a). Postoperative intraluminal glycerol varied from 0-400 within the first 24 hours (Fig 2b).” is removed.

Page 15: The phrase “Furthermore, we propose that jejunal feeding tube with equilibration dialysis system is a feasible method for clinical purposes when metabolism of small intestine needs to monitored.” is removed from conclusions.

Page 19: The titles “Figure 2.a. Postoperative jejunal microdialysate, lactate to pyruvate and lactate to glucose ratios. Figure 2.b. Postoperative jejunal microdialysate, glycerol.” and the following figures are removed.
2. Minor Essential Revisions
The total gut ischemia model ex vivo is not well described in the methods section. Please describe the surgical methods in brief, the handling of the intestinal specimen, placement of the probes etc. An illustration could do good. In the Microdialysis - ex vivo validation, you only describe the technical parts of the technique. What about recovery?

Reply: Thank you. We have now described the surgical part of operation in more detail. As for recovery: the referee is well experienced with microdialysis as a method and we acknowledge the limitation of the present experiment since no in vitro recovery or no net loss method was used. However, we used mainly ratios as indicators of ischemia. Therefore one may assume approximation of similar recovery for glucose, lactate and pyruvate.

3. Discretionary revisions
Did you use the best ten minute fraction in each interval or did you make any mean values of the whole interval? Please describe.

Reply: Each interval was 30 minutes, as described in the manuscript. Microdialysis was collected as a 10 minute fractions, thus for each interval the aim was to collect three samples of each 30 minute interval. See page 7. The phrase “Ten-minute fractions were collected over the length of the experiment.” Is changed to “Ten-minute fractions were collected over the length of the experiment, thus the aim was to collect 3 samples per patient per one thirty-minute interval after the baseline (in vivo) pre-ischemic samples.”

4. Table 1; what is "n"? It is not readable.

Thank you. Corrected.

5. Figure 2 is then not relevant.

Reply: Figure 2 is removed.
Reply to the evaluation by the second referee

Norbert Cibicek pointed out 24 issues.

1. I disagree with the use of some words. For example „ex vivo“ may be defined as „out of the living“. The organ must be completely removed from the body and exist in an artificial medium during the testing procedure. The authors declare that they used an ex vivo model, but in fact what they did was a determination of metabolites in microdialysates obtained from ischaemic gut BEFORE it was removed out of the body (pg. 8). This means we are dealing in fact with sampling performed in the living system, or „in vivo“. If I just do not understand the description of the procedure given by the authors (at different places in the manuscript) and the resecate was placed in the water bath, where the dialysate was collected, there is no place for „intraperitoneal“ approach – since this must take place in the peritoneal cavity and not in a sterile cup.

Reply: Sir, thankful we are for your comments. This indeed was a combination of in-vivo and ex-vivo. We have made an attempt to better describe the study design. As for intraperitoneal access vs extramural, these definitions are open for discussion. Depending on the location of the microdialysis probe can intraperitoneal probe indeed locate between serosal surfaces of intestinal structures. In fact one might claim that the goal for intraperitoneal microdialysis should be between the serosal surfaces of intestinal loops in order to be able to detect changes in intestinal wall. In this model we have made an attempt to mimic in-vivo condition by applying microdialysis on the serosal surface of small intestine.
See page 6, the headline “Microdialys” is changed to “Microdialysis – combination of in vivo and ex vivo validation” and the following corrective is added to the paragraph regarding the in vivo and ex vivo methods:

“Ten-minute fractions were collected over the length of the experiment, thus the aim was to collect 3 samples per patient per one thirty-minute interval after the baseline (in vivo) pre-ischemic samples. Pre-ischemic baseline samples during the surgical procedure were collected prior to clamping the vasculature of the segment. Each pre-ischemic period lasted 20-30 minutes. Therefore 2-3 baseline samples were obtained for baseline metabolic state of intestinal wall. A jejunal segment of 4-6 cm in length was removed from the Whipple specimen, before relocating the bowel and its’ mesenterium together with superior mesenteric artery and vein supply to right upper abdomen as per surgical protocol. The time of the removal of the resecate was registered, the timing of microdialysate sampling adjusted accordingly. The microdialysate samples were gathered up to 120 minutes after the closure of the vasculature of the removed bowel segment (ischemic stage/ex vivo). The resected part of small intestine was placed in a sterile cup within a warm closed water bath. The resected intestine was not in direct contact with water. The temperature was kept constant at 37-38 degrees of centigrade during the ex vivo experimentation.”

2. Concerning the intraperitoneal position of a catheter, it was defined by Sommer and Larsen differently (see Fig. 1 in the reference No 9. in the manuscript, pg. 17). This position requires free placement of the probe in the peritoneal cavity, not its direct and tight association with the serosa of the gut
with limited access to the peritoneal fluid (what is described in the present manuscript). Therefore, the latter should be termed „extramural“ position instead.

Reply: We appreciate the notion regarding location and terminology. It is not standardized. We prefer to use peritoneal (serosal) as definition. If indeed required by the referee this terminology issue could be added briefly to the discussion. On the other hand it would unnecessarily lengthen the manuscript.

3. Some statements are false – e.g. the third one in the Background section of the manuscript. Intestinal luminal application of microdialysis has already been validated by other authors, at least in the large gut in terms of detection of ischaemic events.

Reply: The phrase "Most importantly, clinical validation of intestinal application is not available." is removed from page 4 and clinical feasibility of intestinal luminal equilibrium dialysis is removed from this paper to be handled as a totally separate investigation. See Dr. Erik Solligard’s first comment.

4. What is the „gut hypothesis“ (pg. 4 in the middle)? Such word connections need to be clearly defined if they are to be used.

Reply: See page 3:

“It has been postulated that gut may serve not only as a target organ to, but indeed a source of systemic inflammation by releasing bacteria or toxins to blood or lymph (gut hypothesis) (Deitch). Therefore it is rational to hypothesize that the viability of intestinal epithelial cells is the logical specific target for monitoring gut epithelial barrier function (7, 9, 14-18). “

5. Glycerol can by no means be considered a „specific marker of the damage of the gut epithelia“, as stated in the manuscript. There is a number of different sources of gut glycerol including dietary triacylglycerols, glycerol enema or infused lipids. Moreover, various events may contribute to gut glycerol besides ischaemia. Some of these factors may be found in the latter parts of the manuscript (pg. 12).

Reply: In the current version of the manuscript we do not claim glycerol as a specific marker of ischemia. We refer to existing literature and in particular to the 1. Reviewer’s report on the association between ischemia and ischemia/reperfusion vs glycerol release to the gut lumen or within the gut wall (ref 11). To add, we already refer to other sources for glycerol release in the Discussion. In other words, there are studies that have proven glycerol to be a good marker of ischemia even though, there
are number of different sources of glycerol that can affect the levels. See reference 12, in the study is stated that: "Intraluminal microdialysis could detect early signs of ischemic injury in the ileum, as well as the colon with a marked increase in glycerol. The increased levels of intraluminal glycerol showed a positive correlation to prolonged ischemia and to higher degrees of intestinal damage."


In order to show the appreciation for the appropriate comment by the reviewer, we have now added to the Discussion:

‘In addition to ischemia and trauma, various other sources for glycerol release may exist. As an example anesthetics may affect the glycerol levels. Propofol fat emulsion contains glycerol and halothane is known to increase lipolysis and plasma levels of glycerol, lactate and glucose (1), thus having to take under consideration when interpreting the microdialysis results. In the present study propofol was used for the induction of anesthesia.’

6. If the authors used CMA 600 analyzer, they must have been able to measure urea as well. Microdialysate urea (added to the perfusion fluid) has recently been validated for blood perfusion estimation in a number of studies, none of which have been cited by the authors. This marker would bring important additional information and enable a more correct interpretation of the results of metabolic markers. Other validated markers of blood perfusion (such as ethanol) have not been mentioned in the background.

Reply: We unfortunately did not measure urea. We agree on the notion. In this study with its’ limitation acknowledged we had ‘normal’ flow under anesthesia in the beginning of the operation followed by no-flow. Therefore we consider the measured parameters relevant but at the same time appreciate the comment from the reviewer.

7. Very importantly, the collection of fractions is wrongly described. For example, why did the authors collect 10-min fractions and then presented fractions collected in 30-min intervals?

Re: Thank you for the important comment. We have now described the sampling protocol in more detail. The reason for collecting samples in 10 minute intervals was dictated by the clinical setting: the first samples were considered valuable for the baseline, normal perfusion condition. Had we collected the first sample over 30
minutes we would have prolonged unnecessarily the operation. This should be considered ethically unsound.

8. My own experience with gut ischaemia and CMA 600 analyzer tell me that there is a huge problem with the analysis of frozen samples. After thawing, bubbles are formed in the samples and if there is only 10 microlitres, it is highly probable that the analyzer takes insufficient sample volume (small dead volume) resulting in very low concentration of the analyte(s).

Re: As we have also been working with the CMA600 analyzer for number of years, we completely agree with the reviewer on the potential problem of microbubble formation. After thawing the samples we did always spin down the samples. In addition visual confirmation of each sample was performed. The order of the samples in the apparatus was random as the rack was filled with marked sample tubes. Even though pyruvate was/is the last in the line of analyses, the random order of patient samples should minimize the risk for effect on pyruvate alone.

9. If the sequence of analytes is always the same, the last in the row suffers the most. It is likely that, by default, the last analyte was pyruvate (I experienced this in my own experiments). If this was the case, the authors may have a plausible explanation for their rather unexpected and inconsistent results.

Re: The patients/tubes were in random order. The previous literature show high variability of the metabolite results similarly. Either the referee is correct and most of the previous literature and results are biased because of the methodological problems or, as we presume, the results depict the variation in metabolic condition in particular inter-individually. Previous trials in preclinical setting by one of the investigators (Tenhunen) with up to 60 microliters samples have shown comparable variability in controlled trial design. In addition, the particular question was addressed previously by P Abrahamsson. No increasing concentrations were detected with batch analyses when clinically used glass vials were in the apparatus for the set of analyses. (Pernilla Abrahamsson, Gooran Johansson, Anna-Maja Aberg, Michael Haney, Ola Winso.

Optimised sample handling in association with use of the CMA 600 analyser Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 940–945)

For instance, high LP ratio may not be only a result of high lactate, but also very low pyruvate (the colorimetric principle of the measurement allows the release of very low results even if there is almost no sample present in the reaction mixture). Because it would be very difficult (almost impossible) to repeat the analyses from only 10 microlitres, the authors would have no possibility to check whether the sample dilution problem occured. There are more data indicating high probability
of the existence of this problem – very high variability of the results even during baseline conditions, discrepancy between LP and LG ratios and unexpectably high baseline values. None of these have been satisfactorily addressed by the authors in the discussion.

Re: Why does the reviewer claim there is discrepancy between L/P and L/G ratios? The results seem to support each other in our understanding. While glucose supply to the cells disappear, lactate to glucose ratio increase. Concomitantly, while oxygen supply to the cells disappear, anaerobic metabolism continues with increasing usage of pyruvate to lactate and therefore increasing lactate to pyruvate ratio. To the best of our understanding L/G ratio results support L/P ratio results as indicator of no-flow/no-supply of glucose and oxygen.

By the way, was this problem the reason why the authors finally decided to put the samples together and analyze them en bloc (in 30-min intervals instead of 10-min intervals)? Or did they publish the means of three serial values?

Re: Touching the thresholds of constructive criticism we wish to describe in more detail our a priori planning of the study. This should be important so that any misunderstanding due to our inability to deliver the message could be corrected: The rational for using three samples/timeperiod, that is 30 minutes, was based on a priori clinical consideration: any sample within 30 minutes could show changes in the metabolic/perfusion condition of the tissue. The other reason for a priori decision was that thus we obtained adequate number of observations per timepoint/period analyses in regard of AU ROC tests. As hopefully now more clearly stated in the methods section, the three 10-minute fractions were used all separately for ROC analyses. We did not use means of three serial/consecutive values for ROC analyses.

10. As to the number of patients and their description, different parts of the manuscript offer different figures (11, then 21 and finally 20) that are confusing for a reader. The data (age of the group) must correspond to the group’s homogeneity, e.g. if there are men and women (two subgroups), two figures should be provided.

Re: Humbly: Thank you for pointing out an obvious error. In the current version of the manuscript the n is described hopefully clearly: 21 patients enrolled, 10 females and 11 males. We strongly feel that in this particular trial dividing the results based on gender is unnecessary. After all we are focusing on very fundamental physiological phenomenon, flow/no-flow in the tissue. N=11 referred to the second part of the first version of the manuscript. 11 patients were followed postoperatively.
This was considered confusing by the reviewer no 1. Similarly. That part of the report is removed completely.

11. The results of sensitivity and specificity cannot be compared when there is no identical reference. As a reference, 95 % specificity is usually selected. So the authors should present the values for sensitivity at 95 % specificity, otherwise “apples are compared with pears”.

Re: Thank you for the suggestion.

We have consulted Risto Bloigu, a researcher/statistician at the University of Oulu regarding the presentation of results of ROC analysis. Herein the reply:

"The ROC curve is at it´s best when comparing two different diagnostic tests together, when usually one is not interested in particular sensitivities regarding corresponding specificities. The method is based on the data at hand in a way, that the sensitivities and specificities are being calculated by thresholds at hand (not speculative ones). The aim is to form graphical illustration describing the phenomena one is interested in in general. Furthermore the statistical analyser pointed out that examining only one threshold is problematic, because the calculated ROC curve is based on the actual (gathered/measured/observed) thresholds and emphasizes the threshold at that point of the curve. The statistical testing between two different metabolic markers is based on calculating the area under the curve, the bigger the area, the better diagnostic test for a metabolic marker."

Two generally accepted ways of representing the results is indeed AUC overall, closest to (0,1) point (Perkins NJ, Schisterman EF. The inconsistency of ‘optimal’ cutpoints obtained using two criteria based on the receiver operating characteristics curve. Am J Epidemiol 2006; 163: 670–5) and finally Youden index (Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. Biom J 2005; 47: 458–72).

We chose to use the closest to (0,1) point method.

12. Levels of glycerol cannot be put as 9 (median) and 34 – 37 (interquartile range).

Re: Embarrassing mistake/typo. Thank you for the notion. Corrected now.

The sentence is corrected to:” Intraluminal pre-ischemic glycerol was 9 (from 4-37) µmol/l.”

13. The statistical analysis is insufficiently described. If the ROC analysis is
performed, there must be a reference (healthy) group / data. These were obviously the baseline values (however questionable due to very high variability) – this group must be specified, even though no “sham-operated group” or “control catheter location” was tested.

Re: Again in a humble tone, thank you. We no made the best attempt to better describe the statistical approach:

Added to statistical methods. ‘The microdialysate metabolite concentrations from 1-3 10-minute fractions prior to occlusion of supplying vessels were used as the normal/physiological reference.’

13. I can see no reason why the authors selected 68 as the threshold for intraluminal LP ratio, if the value 109 has better characteristics in terms of sensitivity and specificity. Is it because it is closer to 20, which was defined in a number of other studies and confirmed by our group (Cibicek N et al. Physiol Meas 2010)?

Re: Thank you for the thorough review and accurate comments. Our mistake. We refer to previous and corrected now as per our approach: the threshold by closest to (0,1) point method is indeed 109.

14. The discussion in general is very weak – the authors were unable to acceptably discuss the metabolic parameters, especially the discrepancies between the findings of other groups and their data and discrepancies between different locations (e.g. why the thresholds differ so much between the intraluminal and “intraperitoneal” markers and why there is so high variability?)

Re: Pubmed search on studies with search words microdialysis, intraperitoneal gives 36 articles with the filter human. Adding ischemia as search word limits the relevant literature to 11 reports. Of those 11 reports we refer to 4. In addition we made an attempt to select relevant literature even from experimental trials. As the very aim of the present study was to focus on generally accepted method to define sensitivity and specificity of the two tests or locations, we sought to discuss that area in particular. No prior ROC analysis exists for microdialysis in intraperitoneal/serosal location as compared to intraluminal location.

Searching intraluminal, endoluminal, luminal or intestinal microdialysis with the limitation to human/clinical trials gives very limited number of reports. We have referred to the most relevant of those including Solligard, Deeba and Due (with equilibration dialysis).
As the senior investigator and author I need to humbly and honestly state at this point that some comments, this amongst others is somewhat unfair. Re-reading through the Discussion I claim we refer to relevant literature and in particular refer to previous metabolite concentrations and ratios.

Regarding the equilibration dialysis, the LP and LG ratios (including glycerol) cannot be equal to 0. It means lactate (and glycerol) must have been equal to 0, which was not documented in the previous findings (even in the same manuscript).

Re: Equilibration dialysis as part of the manuscript is completely removed from the manuscript as suggested by the reviewer 1.

15. NJ feeding tube with equilibration dialysis could be considered a feasible method for clinical purposes provided the results were adequately discussed. The authors, however, failed to do this.

Re: Clinical feasibility of intestinal luminal equilibrium dialysis is removed from this paper to be handled as an totally separate investigation. See Dr. Erik Solligard’s first comment.

16. The placement of the probes is not fully delineated. How was the intraluminal probe implanted? Was there a puncture and/or suture?

Re: We herein add to the definition as suggested by the reviewer serosal/intraperitoneal instead of intraperitoneal. See page 8. The placement of the probes is added to the text:

” Immediately following the laparotomy intraluminal microdialysis catheter was introduced to the lumen of intestine through an antemesenterical opening and sutured. The other catheter was placed on the serosal surface of the intestine and sutured. Set of superficial sutures were then applied to form a tunnel/pouch for the serosal/intraperitoneal catheter to ensure contact against serosa to mimic free placing between the intestines in intraperitoneal microdialysis. “

17. If there was so great effect of ischaemia on other markers, why glycerol was unaffected (in the i.p. catheter)?

Re: Careful inspection of the Figure 1a-c shows intraperitoneal/serosal microdialysate metabolite ratios and glycerol concentrations (1b). Intraperitoneal glycerol concentration increases statistically significantly as compared to pre-ischemic state. The magnitude of increase is lower than in intraluminal location. Similar difference
in the time sequence of intraluminal vs intraperitoneal location is seen for lactate to pyruvate ratio.

18. The discussion of LG ratio includes results of glucose obtained by other groups. However, no information on the perfusion rates (that determine the recoveries of probes) is provided for the reader, so the given values are of no use.

Re: Assuming that the reviewer refers to perfusion rates in the trial reports from the others as opposed to present study we would like to comment the following: as certainly well known to the reviewer there are already reports describing the characteristics of CMA70 catheter in detail. One example is Hutchinson et al. J Neurosurg, 2000. We wish to refer to this article and state that glucose, lactate, pyruvate and glycerol relative recovery with 1 microl/min dialysate flow rate gives roughly 20-30% recovery. When considering L/G and L/P ratio, one may reasonably claim that the flow rate and thus the recovery does not have effect on the ratios. Secondly, as we made an attempt to perform a controlled (patient as his/her own control; baseline) study with controlled environment, we do claim that the time window for early diagnostics of no-flow can be deducted from the data herein.

The reviewer refers to absolute concentrations and referred articles. We are grateful for the comment. We add the dialysate flow rate used in each experiment to the Discussion and speculate on the effect of flow rate on glucose concentration vs lactate to glucose ratio.

‘In the present study we found lactate to glucose ratio intraluminally as an early marker for ischemia. In the previous literature described herein the dialysate flow rate varied from 0.3-5.6 microl/min. In the present study we used 1.0 microl/min flow rate. Based on this it is difficult to compare the results between different trials. This concern is now minimized when we used lactate to glucose ratio instead of only glucose concentration which depends on dialysate flow rate.’

19. The reference No. 22 cannot be used at the beginning of the page 14. The cited paper did not study the transient increase in glucose concentration - it has only been speculated by Deeba et al. in an attempt to account for their unexpected results. The authors cite Deeba et al. here without knowing what they really meant by additional glucose delivery etc.

Re: Deeba and colleagues report in their paper as their FINDING the transient decrease followed by a transient increase of glucose within the bowel wall. In line with the suggestion by the reviewer we added to the Discussion:’…as speculated by Deeba et al.’
• Minor Essential Revisions

20. The article contains numerous English grammatical errors. They are most often of minor importance (US English vs. British English spelling, spelling in general, wrong use of articles, missing articles, wrong use of prepositions, frequently repeated words and/or their incorrect use – e.g. to depict), sometimes, however, the reader gets confused by long and incomprehensible sentences (especially the last sentence on the pg. 6). Other sentences and explanations (such as peritoneal cavity) are redundant or repeated more times in the paragraphs (such as the absence of a clinical report on the page 4 and 5 or the major results on the pg. 11). Word connections such as „serosal metabolic signalling“ on the page 4 are used in a wrong manner – the word „signalling“ means something completely different. The same sentence must have a reference – how do the authors know that gut motility influences the (recovery of) metabolic markers? English has to be reviewed by a professional. The authors must decide if they are to use a „Whipple procedure“ or "Whipple´s procedure“.

Reply: The word specificity changed for specificity in the figures and tables.

21. There is a number of errors in the References as well. I have identified at least five (e.g. intraperitoneal, Apperent, reable ...).

Reply: Grammatical errors from references are corrected.

22. The key words were not selected properly. ROC analysis should not be included as a key word, there was no glucose to pyruvate ratio (as stated in the key words) but lactate to glucose ratio studied by the group.

Re: ROC, and glucose to pyruvate ratio removed from the keywords, page 3.

23. Table 1 contains numbers of patients with different measurements. Why there were only 12 (13) patients with lactate (pH) measurements and 20 patients with the measurement of saturation? Is it due to technical reasons (inability to collect the sample) or was the protocol changed in time?

Re: Due to technical reasons there were lower amount of patients from whom the samples were collected successfully before ischemia (pH 13, B-glucose 14 and lactate 12 patients) than in the beginning of the surgery.

24. Finally, due to the number of grammatical mistakes, I do not believe that all (seven!) authors read the manuscript. In such case at least some of them must
have identified the mistakes.

Re: The manuscript was sent to all co-authors. Despite that the reviewer has found even embarrassing errors to be corrected. Of that we are grateful.

We appreciate the comments from the reviewers. Thank you for reviewing our manuscript and allowing us to make an attempt to revise the manuscript keeping in mind the most salient message we wish to express with the report. We do hope that the revised version meets the high standards of the Journal.

Sincerely,

Lauri Pynnönen MD

Jyrki Tenhunen, MD, PhD