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Gut luminal microdialysis

Doctoral thesis for the degree doctor medicinae

Trondheim, June 2007

Norwegian University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging



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**To my love Kari,
and our children Eirik and Hanna**

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2. Original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I** Solligard E, Juel IS, Bakkelund K, Johnsen H, Saether OD, Gronbech JE, Aadahl P.
Gut barrier dysfunction as detected by intestinal luminal microdialysis.
Intensive Care Med. 2004;30:1188-94.
- II** Solligård E, Juel IS, Bakkelund K, Jynge P, Johnsen H, Aadahl P, Gronbech JE.
Gut luminal microdialysis of glycerol as a marker of intestinal ischemic injury and recovery.
Crit Care Med 2005;33(10):2278-2285
- III** Solligård E, Juel IS, Spigset O, Romundstad P, Grønbech JE, Aadahl P
Gut luminal lactate measured by microdialysis mirrors permeability of the intestinal mucosa after ischemia
Shock 2007, in press
- IV** Solligård E, Wahba A, Skogvoll E, Stenseth R, Grønbech JE, Aadahl P.
Endoluminal microdialysis shows increased rectal lactate in routine coronary surgery
Anaesthesia 2007;62(3):250-258

3. Abbreviations

ACB	Aorto Coronary Bypass
ATP	Adenosine Tri Phosphate
CPB	Cardio Pulmonary Bypass
ICU	Intensive Care Unit
LP-ratio	Lactate Pyruvate Ratio
MD	Microdialysis
MOF	Multiple Organ Failure
NAD+	Nicotinamide Adenine Dinucleotide
PEG-4000	¹⁴C Polyethylene Glycol 4000
SIRS	Systemic Inflammatory Response Syndrome
SMA	Superior Mesenteric Artery

4. Introduction

4.1 Multiple organ failure

Multiple organ failure (MOF) is an evolving clinical syndrome triggered by various stimuli, arising following successful resuscitation from life-threatening conditions. The ongoing support contributes to its subsequent course. It is the main cause of morbidity and mortality in patients admitted to intensive care units (1). Organ failure, both in terms of the number of organs failing and the degree of organ dysfunction, is the strongest predictor of death in a critically ill patient. The mortality rate in these patients is 20-80%, depending on the number of organs involved. Although the clinical manifestations of MOF may vary, the organs involved typically include the lungs, heart, kidneys, brain, liver and / or the gut (2). There is widespread agreement that an increased inflammatory response, ischemia and septicaemia are important contributing factors to the development of MOF (3-12).

4.2 The ischemic intestine

The gut is thought to be a “motor” in the development of MOF (13), and splanchnic ischemia is associated with high mortality in critically ill patients (14,15). A number of clinical studies on critically ill patients have documented inadequate intestinal blood flow, oxygen utilization (16,17) and increased hepatosplanchnic inflammation (18). Thus there is an increase in intestinal permeability in a variety of acute conditions, like burns, sepsis, cardiogenic shock, trauma, and after major operations (13,19-22).

The pathogenesis behind the intestinal involvement is different in various conditions. Studies have suggested that in sepsis tissue injury can occur despite adequate tissue oxygenation, (so called dysoxia), where increased blood flow does not improve and may rather worsen the situation (23). Further research has demonstrated that this dysoxia, also affecting the splanchnic region, can be a result of an acquired intrinsic derangement in cellular respiration called “cytopathic hypoxia” (24-26).

In other conditions, blood flow alterations play a more prominent role; The splanchnic vascular bed normally holds 30% of total blood volume and receives 25 % of total cardiac output. Seventy percent of this is distributed to the small and large intestine (27). The venous bed and capacitance vessels (postcapillary venules and veins) serve as a blood reservoir, and alfa adrenergic sympathetic nervous stimulation is capable of rapidly increasing cardiac preload. Hypovolaemic shock states also result in a systemic vasoconstriction throughout the body, this response being particularly prominent within the mesenteric circulation (28-30). Gut blood flow also varies in the intestinal wall; the mean mucosal blood flow is four times higher than mean muscularis blood flow (31). Cellular dysfunction and injury are thus explained by a reduced cellular oxygen availability resulting from decreased blood flow, oxygen tension or haemoglobin concentration.

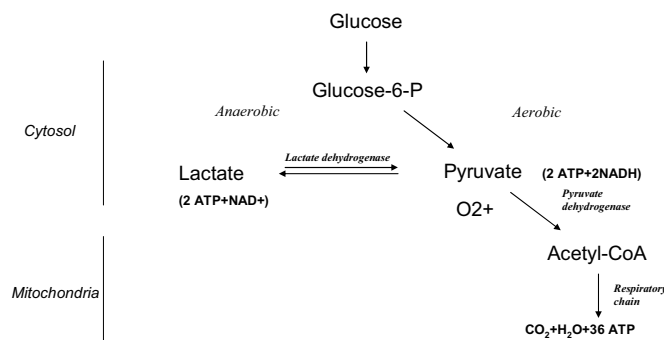
4.3 The ischemic metabolism

The *glycolytic pathway* is employed for the breakdown of glucose to provide energy to the cells in the form of ATP. Intermediates from this chain of reaction are of interest when monitoring the metabolic state of various tissues.

The initial stages of glucose metabolism – glycolysis, or the Embden-Meyerhof pathway, occur in the cytoplasm of cells, also functioning in a low oxygen environment as in poorly perfused tissues. One molecule of glucose is converted into 2 molecules of pyruvate, generating 2 molecules of ATP and 2 NADH (figure 1) (32). During normal aerobic conditions, pyruvate is transported across the mitochondrial membranes into the matrix of the mitochondrion, where the second series of reactions (citric acid cycle) takes place. Immediately upon entry into the mitochondrion, pyruvate is metabolized to the important intermediate acetyl CoA by the enzyme pyruvate dehydrogenase. This process requires oxygen, and as a result, pyruvate is oxidized into CO₂ and H₂O, producing 36 ATP molecules.

If blood flow is impaired and oxygen delivery is diminished, pyruvate cannot enter the citric acid cycle. The only source of energy then is the ATP produced from glucose in the glycolysis. However, glycolysis is dependent upon available NAD⁺. Thus the pool of NAD⁺ produced normally in the citric acid cycle will rapidly become consumed. Under these circumstances, the source of NAD⁺ is from reduction of pyruvate to lactate, resulting in an increase of lactate and anaerobic metabolism. This causes the lactate to the pyruvate ratio (LP-ratio) to increase (normal ratio 10/1). With intact mitochondrial function, the excess lactate is rapidly metabolized back to pyruvate, once oxygen is available.

Figure 1



Glucose enters the cell and is metabolized to two pyruvate molecules, 2 ATP and 2 NADH. In the presence of oxygen, pyruvate enters the citric acid cycle in the mitochondria, and 36 ATP is produced. If oxygen is lacking, pyruvate is reduced to lactate to generate NAD⁺ for continuation of the glycolytic pathway. This gives the net sum of 2 ATP.

Elevated blood lactate levels in hemodynamic unstable animals (33-37) or patients (38,39) are often thought to reflect circulatory shock or hypoxaemia, when O₂ consumption becomes dependent upon O₂ delivery. As lactate in the blood is metabolized in a large part by the liver, liver function and liver blood flow influence hepatic lactate clearance. However, other co-factors may co-exist, complicating the interpretation of hyperlactataemia.

In septic shock the reason behind the often observed hyperlactataemia is under debate. But at least two situations are usually accompanied by hypoxia-associated hyperlactataemia; septic shock with catecholamine-resistant cardiocirculatory failure (38), and also initially in septic hypovolaemic patients observed prior to volumetric expansion. The latter is illustrated in the study of Rivers et al. (40), in which hyperlactataemia is associated with signs of poor oxygen delivery. These two situations are, nonetheless, close to low cardiac output states.

A number of papers have suggested that lactate formation during sepsis is not due to hypoxia alone, but rather to metabolic processes with aerobic production of lactate (41). This is seen in sepsis where endogenous epinephrine release stimulates sarcolemmal Na⁺ K⁺ ATPase-coupled lactate production (42). Another factor is the accumulation of pyruvate due to accelerated aerobic glycolysis resulting from an increased influx of glucose (43). Muscle protein catabolism is also a contributing factor when released amino acids are subsequently transformed into pyruvate and thereafter lactate. The activity of the enzyme pyruvate dehydrogenase is impaired by tumor necrosis factor in septic patients (44), further indicating that this increased lactate should not necessarily be taken as proof of oxygen debt, but rather as a sign of lactate dyshomeostasis. However, increased lactate is a warning sign and highly predicts outcome in critically ill patients (45-47).

4.4 Intestinal barrier dysfunction

Epithelial dysfunction may be a common final pathway contributing to organ dysfunction in sepsis and other forms of critical illness (48). The intestinal epithelium acts as a barrier between intestinal content and a) extracellular space b) blood capillaries and c) lymph ducts. The gut lumen is separated from the lamina propria by the intestinal epithelium, the tight junctions between adjacent cells making the epithelial sheet. The tight junction serves as a regulated, semi-permeable barrier that limits the passive diffusion of solutes via the paracellular pathway between adjacent cells (49,50). In the gut and lungs, this barrier function is also important to prevent systemic contamination by microbes and toxins that are present in the external environment. The tight junctions between adjacent epithelial cells, as opposed to endothelial cells, form markedly smaller pores between the cells (51,52). Thus it is reasonable to assume that epithelial cells are more important for barrier function than endothelial cells. Alterations in intestinal permeability are thus a marker of the intestinal barrier function. Therefore, monitoring of intestinal permeability is important for early recognition of an intestine at risk, and for comparing permeability alterations to other markers of intestinal dysfunction.

4.5 Cellular dysfunction

Ischemia and reperfusion of the gut is a multifactorial process that leads to organ damage and dysfunction. Interruption of blood supply decreases oxygen and nutrient delivery, followed by cellular ionic imbalance, mitochondrial alterations and calcium overload. This results in an ischemic injury which rapidly damages tissues with high metabolic activity.

In all species, including humans, a reduction of blood flow to the small intestine, for even a relatively short time results in the formation of characteristic mucosal lesions (53-55). The enterocytes located at the tips of the villi are particularly susceptible to ischemia. This increased sensitivity is thought to be due to the location which is at the end of the central arteriole, and thus lower oxygen tension compared to the crypts (56,57). Plasma skimming (58) and high metabolic activity of the intestinal epithelial cells are also contributing factors. Recent studies have demonstrated that the vulnerability of an enterocyte is also dependent on its state of differentiation (59).

Paradoxically, restoration of blood flow to a previously ischemic tissue induces additional damage (“reperfusion injury”), mediated, in part, by the extra- and intracellular formation of reactive oxygen and nitrogen species. It remains a matter of debate whether the reperfusion of ischemic intestine induces mainly necrotic or mainly apoptotic cell death (60-62). However, these two processes, although quite different in their morphological and biochemical aspects, may often coexist in many pathological conditions (63). A key difference between the two forms of cell death is that, during necrosis, the membrane integrity breaks down, cytosolic and membrane constituents being released into the extra cellular space; whereas during apoptosis, cells shrink and their nuclei condense, resulting in their encapsulation into well-enclosed apoptotic bodies and followed by consumption by macrophages (64).

4.6 Glycerol as a marker of cellular damage

Despite their variable composition, all biological membranes are thought to be constructed in a common pattern. They all contain a phospholipid bilayer as the basic structure, and membrane-bound proteins, such as receptors and transport proteins. There are two major classes of phospholipids: phosphoglycerides that contain glycerol, and sphingomyeline composed mainly of sphingosine. To maintain homeostasis, there is both passive and active transport over the cellular membrane. In every cell a significant fraction of available energy is used to maintain the concentration gradients of ions (such as Na⁺, K⁺ and Ca⁺) across the plasma membrane.

ATP production is decreased under ischemic conditions, thus the active transport systems for maintenance of the ion gradients are also depressed. This causes an influx of Ca⁺ into the cells, which activates intracellular phospholipases that attack the cell membrane, also causing disintegration of the phospholipid bilayer and efflux of fatty acids and glycerol. The amounts of glycerol released are thought to be a marker of cell membrane disintegration and cellular necrosis (65-75).

4.7 Regional monitoring of the gut

The assessment of systemic hemodynamic parameters alone fails to reflect regional oxygen demand and supply mismatch, the microcirculation and increased blood lactate levels (76,77). The gastrointestinal tract has been called the “canary” of the body (78), because the intestinal mucosa is one of the first organs affected by inadequate splanchnic perfusion / oxygenation. Hence intestinal dysfunction markers provide relatively early warning signs of impending systemic affection in e.g. shock, aortic surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias and neonatal necrotizing enterocolitis (79,80).

Consequently, monitoring of perfusion, oxygenation and function of the liver and the intestine are important for diagnosis and treatment.

Different clinically applicable monitoring techniques of the hepato-splanchnic region exist. Gastro-intestinal tonometry is the most used clinically applicable device for monitoring the splanchnic region. Increases in intestinal tissue partial pressure of carbon dioxide (P_{gCO_2}) can reflect abnormal oxygen supply to the cells (81,82). The value of tonometry as a prognostic tool in critically ill patients is good; the CO_2 – gap (difference between CO_2 in the intestinal tissue and in blood) is a marker of mortality in ventilated ICU patients (83). But the results obtained from tonometry – guided therapy remain controversial (84-88). Another approach for monitoring the microcirculation is to measure the tissue partial pressure of CO_2 under the tongue by means of sublingual capnometry. This method shows promising results; however, the experience is sparse and the method still has several limitations (89-92).

Intestinal permeability is assessed non-invasively by measuring urinary excretion of orally administered test substances (93,94). No currently available test of intestinal permeability is however ideal. Confounding factors may interfere both pre- and post-mucosal, apart from the intestinal permeability itself. Also, the methods only give an non-dynamic on and off description whether permeability is increased or not.

Several other devices have also been developed for the measurement of splanchnic blood flow and oxygen transport, e.g. Doppler ultrasound flowmetry (95,96), endoscopic laser Doppler flowmetry (97), remission spectrophotometry (98,99), CO_2 probes (100) and indocyanid green clearance (101). These methods are mostly used in experimental settings and in clinical research, so clinical experience is infrequent. Surgical implantation is almost always necessary, the interpretation of results being difficult due to the complex blood flow distribution in the splanchnic region.

As most of these methods rely on blood flow changes, methods taking into account the combination of flow-, permeability- and metabolic alterations are needed. Microdialysis may be such a tool.

5. Aims;

- To evaluate the use of gut luminal microdialysis as a tool for monitoring ischemic metabolites like lactate, glycerol and glucose as markers of intestinal dysfunction during and after intestinal ischemia.

- To evaluate whether microdialysis of glycerol and lactate can be used as a method to assess different degrees of intestinal damage and recovery during ischemia and reperfusion.

- To assess differences in the information obtained from microdialysis catheters placed in the peritoneum, the gut wall and the gut lumen.

- To evaluate the feasibility of intestinal luminal microdialysis as a method for clinical monitoring of the intestine in patients.

6. Methodological considerations

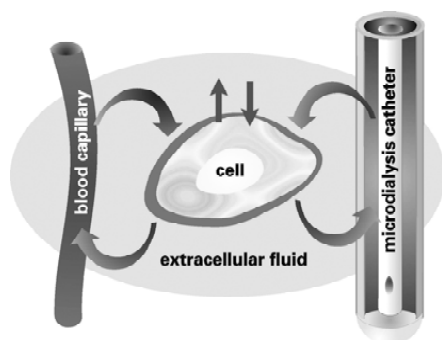
The details of the procedures have been described in each paper and only general comments concerning the different methods will be given.

6.1 Microdialysis

The principle behind microdialysis goes back to the 60's. Methods like push-pull cannulas, dialysis sac's and dialytrodes were used to study tissue biochemistry directly in animals (102-104). In 1974, Ungerstedt and Pycock used a set-up they called "hollow fibers" in the brain to mimic the function of blood vessels(105). This method has been extensively improved into the method of microdialysis, as we know it today (106). The method is widely used both experimentally and in clinical practice for monitoring solid organs like brain and skin flaps (107-116). A PubMed search in January 2007 on "microdialysis" lists in total 11000 publications, of those 1750 on humans.

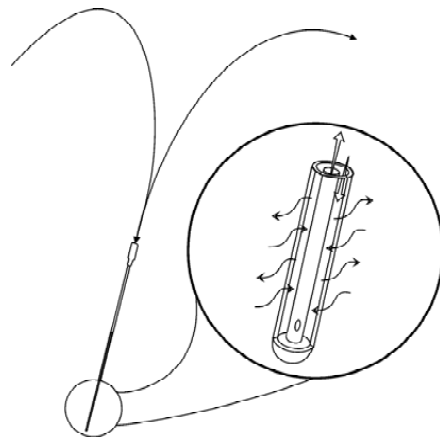
The aim of microdialysis is to sample the extracellular compartment / fluid around the probe and monitor its chemical composition as a function of time. A microdialysis catheter acts in a similar manner to a blood capillary, sampling the interstitial environment by a process of diffusion along a concentration gradient towards and away from the cannula (figure 2). The principle of dialysis is explained in terms of Fick's first law of diffusion, which describes the passive movement of molecules down a concentration gradient.

Figure 2



Microdialysis probes consist of two plastic tubes, one inside the other, and an inlet and outlet part, through which fluid is pumped in and flows passively out of the probe and into the sampling line. The probe is encircled in the end by a tubular semi-permeable membrane, which the fluid passes on its way through the catheter (figure 3). The direction of the diffusion process is dependent upon the concentration gradient. Thus, microdialysis can be used both for collecting a substance in the dialysate as well as delivering it into the periprobe fluid.

Figure 3



The probes have a diameter often less than 1 mm and the membrane length vary between 1-30 mm. The membrane properties and the pore size are the crucial factors defining the size of the molecules able to diffuse over and equilibrate with the perfusion fluid (called perfusate).

The probes are either implanted during surgery into organs like the brain, heart and solid tumors; or easily placed in soft tissue and the skin at the patients bedside, using an introducer cannula. During insertion, the surrounding tissue is temporarily affected (bleeding, inflammation), so the probe has to be in situ and undergo a wash-out for approx 60 min before measurements can start. The catheters can stay in situ for several days, samples can being collected continuously, making dynamic monitoring possible.

Several factors influence the results obtained from microdialysis, like relative recovery: the ratio between the true concentration in the tissue/fluid surrounding the dialysis membrane, and the concentration measured in the dialysate. The relative recovery of endogenous substances and delivery of drugs is dependent upon a number of variables,

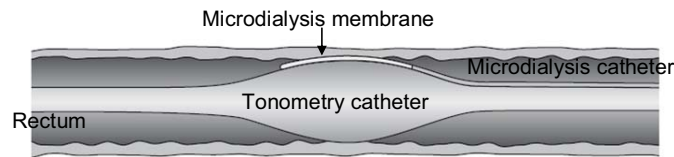
including temperature, pH, and the weight, shape and charge of the molecules. It is also dependent upon the surface area of the dialysis membrane, the flow of the perfusion fluid, the speed of diffusion of the substances through the extracellular fluid and the properties of the membrane. If the membrane is long enough and the flow slow enough, the concentration in the dialysate will be almost the same as in the interstitial fluid, i.e. relative recovery approaches 100%. Hydrostatic pressure may also affect the results dependent upon the placement of the pump by reducing the sample volume if the pump is placed lower than the sampling vial (117).

The microdialysis probes have to be calibrated before drawing conclusions about concentrations in the periprobe fluid. There are many different methods for calibration in vivo and thus for determination of relative recovery. The method of flow rate variation, when plotting the flow rate against measured concentrations and extrapolating to zero flow, will estimate the concentration at zero flow rate (and thus the relative recovery) of the probe (118). The no-net-flux method (also called zero-net-flux method) is a calibration method making use of different perfusate concentrations (119). The calibration method we, and most others, use is in vitro calibration with different known concentrations of the marker in the fluid surrounding the probe. However, it is not possible to derive in vivo recovery from in vitro results. In general, the relative recovery in vitro is higher than in vivo (120) because the relative recovery in vivo depends on the additional factors described above. So when interpreting results from microdialysis based on in vitro calibration it is important to remember that the only possibility to directly use in vitro results is its use for semiquantitative experiments, as it is possible to detect changes in analyte concentrations in the tissue surrounding the probe (121). Thus we cannot directly compare absolute concentrations of lactate obtained from microdialysis perfusate nor from blood samples measured on a blood gas analyzer.

In all experiments we used CMA 70 Microdialysis Catheters with an outer diameter of 0.6 mm, and membrane cut-off of 20 kD. To place the luminal catheter, we made a small antimesenteric incision and introduced the catheter 10 cm into the lumen. In the mucosal approach, a 27-gauge spinal needle punctured the intestinal wall from the serosal side, and was guided under visual control and gentle compression against the gut wall 5 cm into and out of the mucosa. This thin needle was used as a guidewire for an 18-gauge needle, in which the catheter was advanced. The needle was then withdrawn, and the semipermeable part of the catheter left in situ in the mucosal wall. Intraperitoneal catheters were attached to the serosa by a suture. In Paper IV, we manufactured our own rectal catheter by combining two commercially available devices: a microdialysis catheter (CMA 62) and a 16F tonometric catheter for measurements of regional CO₂ production in the intestinal wall. To ascertain contact between the catheter and the intestinal mucosa, the microdialysis catheter was attached to the tonometry catheter with tape, so that the membrane was at the level of the tonometric balloon (fig 4).

Figure 4. Rectal device

Schematic drawing of the combined rectal device with microdialysis- and tonometry-catheter



6.2 Biochemical analysis

A CMA 600 microdialysis analyzer was used in all studies to analyze the microdialysis samples for glucose, lactate, pyruvate and glycerol. The analyzer is placed at "bedside", the results being available 5 minutes after insertion of the samples. The analyzer is based on enzymatic reagents and colorimetric measurements. Analyte specific reagents mix with the microdialysis sample and cause formation of hydrogen peroxide if the substrate is present. The hydrogen peroxide reacts with a chromogen, catalyzed by peroxidase, forming a red-violet quinineimine. The rate of formation of quinineimine is proportional to the substrate formation. This reaction is measured photometrically as a change of absorbance at 546 nm wavelength during 30 seconds.

6.3 Measurement of intestinal epithelial permeability

There are two main approaches to measure intestinal epithelial permeability *in vivo*. A hydrophilic permeability probe (like mannitol or sugars) is administered into the intestinal lumen, and the subsequent recovery of the molecule in urine (122) or plasma (123,124) is monitored. Alternatively, the experimental animal is infused intravenously with an appropriate hydrophilic probe, while the lumen of an isolated segment of intestine is perfused with a buffer solution. With this method, permeability is assessed by monitoring the plasma-to-lumen clearance of the marker substance (125,126).

We used venous blood levels and urinary excretion of ^{14}C polyethylene glycol with a molecule weight of 4000 Dalton (PEG-4000) as an index for paracellular permeability of this probe across the intestinal wall. Hydrophilic probes of this size are considered largely to be transported via the paracellular pathway (127,128). They are also approx. the size of endotoxins. Because radioactivity of the solution instilled into the intestinal loop showed some variations between batches, the blood and urinary levels of PEG-4000 were expressed as a percent of the amount PEG-4000 instilled into the intestinal loop (129). The interpretation of the results from the permeability tests may in general be confounded by intestinal transit time and distribution of the probe in various compartments other than blood (130). The intestinal transit time was not a problem in our model, as the PEG-4000 was introduced into a segment of the intestine ligated in both ends. The probe should not be metabolized or degraded *in vivo* and should be cleared only by urinary excretion. PEG 4000 meets both of these requirements. Changes

in the glomerular filtration rate might alter the rate of urinary excretion of the probe. To diminish this factor, the diuresis was kept normal and the renal blood flow was unaffected in both paper II and III. There was also a very similar pattern in blood and urine levels supporting this assumption. In paper I, the renal blood flow was reduced due to the experimental model. We therefore are cautious about interpretations of the dynamics in the permeability alterations, and only conclude that the permeability was increased during reperfusion in that study.

6.4 Tonometry

Gastrointestinal tonometry is based on the principle of luminal fluid $p\text{CO}_2$ coming into equilibration with the $p\text{CO}_2$ of the mucosal layer (131) as CO_2 diffuses freely in tissue. CO_2 levels rise sharply in conditions associated with poor tissue perfusion, in part due to intracellular buffering of excess hydrogen ions by bicarbonate. The interpretation of tissue $p\text{CO}_2$ is determined by three variables: the arterial CO_2 content, regional blood flow, and tissue CO_2 production. In stable respiratory conditions, when arterial CO_2 is constant, tissue CO_2 essentially reflects the balance between tissue blood flow and local CO_2 production. The increased tissue CO_2 which occurs in critically ill patients is likely to be mainly due to flow stagnation with smaller components brought about by anaerobic CO_2 production (due to decreased oxygen delivery and cytopathic hypoxia).

There are several pitfalls in interpretation of tonometry-derived results. By using air tonometry and measuring the actual tissue CO_2 we avoided complex logarithmic calculations and dependency of the patient's acid-base status seen when saline tonometry is applied. We measured in the intestine, thereby avoiding the potential errors from gastric juice and pancreatic bicarbonate. The experience from tonometry in the rectal lumen, as in paper IV, was a challenge, the experience being sparse (132-134). No normal value for rectal CO_2 has yet been established. Faeces in the rectum surrounding the tonometer may disturb the measurements, both due to mechanic obstruction, and by CO_2 production from faecal bacterias as seen in mice (135). During "dysthermia" temperature correction of arterial CO_2 is important (136,137).

6.5 High energy phosphates

Because oxygen is necessary for mitochondrial generation of adenosine triphosphate (ATP), hypoxia will lead to the degradation of ATP. The depletion and, eventually, recovery of ATP reflects the ATP-synthesizing capacity of the mitochondria and has been recognized as a useful indicator to assess tissue viability in ischemia-reperfusion (138). Other data also support the idea that ATP content is a determinant of intestinal epithelial barrier function in vivo (139). Tissue samples from the jejunal loops were freeze-clamped and immediately frozen in liquid nitrogen, thereafter freeze-dried, homogenized, and analyzed for ATP by a standard high performance liquid chromatography method (140) (paper II). The technique was developed in the 1960's (141). HPLC separates compounds dissolved in solutions. A small amount of this liquid sample is injected into a moving stream of liquid (the mobile phase) that passes through

a column packed with particles of stationary phase. Separation of mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase.

6.6 Histology

Morphology of the intestinal mucosa was evaluated and classified according to Lacy and Ito (142). This classification was further modified for the small intestine as reported in a detailed study from our laboratory (143).

6.7 Blood flow determination

Regional tissue blood flow in the pigs in paper I-III was determined by the colour labelled microsphere distribution technique (144). This method is based upon the assumption that the amount of embolized microspheres in a tissue mass is proportional to the blood flow in that tissue during injection of the microspheres in the left cardiac ventricle (145,146). The same principle was also used for determining cardiac output, using a reference sample as the "tissue" of interest (147). Tissue blood flow rate and cardiac output were calculated as previously described (146,148). The known pit-falls of this method (149) were attempted to be avoided by preventing aggregation of spheres and using sufficiently high numbers of spheres at each injection. These precautions yielded equal distribution of spheres in the right and left kidney as a control of the method. Ultrasound transit time flow probes for blood flow measurements were placed around the superior mesenteric artery (II-III) and portal vein (I-II) for continuous blood flow measurements.

6.8 Study models

Young pigs have been used in experimental circulatory research models for many years. They have much in common with man in terms of anatomy, physiology and metabolism. The pig may be a good model for intestinal ischemia reperfusion in humans, because both species have relatively low levels of intestinal xantine oxidase (150,151). Therefore, reperfusion injury and the no-reflow phenomenon may be less pronounced in pigs and humans compared to rodents and cats.

Surgery and anaesthesia often induce cardiopulmonary depression, alterations in fluid balance and regional blood flow, Thus they interfere with the results of an investigation. The surgical procedures were done as gently as possible, normovolaemia and normoxia being aimed at to counteract these factors influence on the results. The anaesthetic combination of fentanyl, midazolam and isoflurane was chosen due to its hemodynamic stability, having unaltered splanchnic tissue blood flow over time (152). The same maintenance of splanchnic blood flow has also been seen when only using isoflurane (153). Isoflurane is a preconditioning agent in the heart (154) and, theoretically, also in

the intestine. However, this effect on the intestine has not been shown. In order to explore the experimental feasibility of luminal microdialysis, we started with the clinically relevant model of aortic cross clamping. However, this model induces a lot of “noise”, so in paper II and III we used cross clamping of the superior mesenteric artery. The latter model is less influenced by systemic alterations and makes it possible to study more isolated effects from controlled intestinal occlusive ischemia.

The feasibility study on humans (paper IV) was performed on heart surgery patients because of the “SIRS-like” behaviour induced by the CPB-circuit, homogeneity as patient population, as well as the relatively high risk of gastrointestinal complications.

6.9 Statistics

In paper I and II, we used parametric tests with two-way ANOVA for repeated measurements for testing of differences between the groups. The advantage of this approach is the power to identify differences when comparing both within and between groups, when the assumptions of residual normality and constant variance is fulfilled. When inspecting the data, it is however somewhat debatable whether constant variance requirement is fulfilled. Thus, during the revision of paper II, we discussed improvements with both the reviewers and statisticians. In the two last papers, we recognised and allowed for the non-constant variance along the time line, and turned to non parametric tests which include both one-and two-way ANOVA for ranks. Also in paper II, we tested all calculations by non parametric tests before publication of the paper. The major results in all papers are very clear and hardly influence the interpretation of the data regardless of statistical method.

7. Summary of results

7.1 paper I

Gut barrier dysfunction as detected by intestinal luminal microdialysis

In this clinically relevant model of thoracic aortic cross clamping the aim was to evaluate the use of gut luminal microdialysis as a tool for monitoring ischaemic metabolites as markers of intestinal dysfunction during and after intestinal ischaemia. The thoracic aorta was cross clamped for sixty minutes, followed by two hours of reperfusion. Glycerol, lactate and glucose in the intestinal lumen and mucosa were measured by microdialysis. The recovery of PEG-4000 in blood was used as an index for permeability of this probe across the intestinal wall.

During reperfusion, venous PEG-4000 was increased, remaining unchanged to the end of the experiment. During ischemia and reperfusion, there was a marked increase in lactate and glycerol in both the intestinal mucosa and lumen. The concentration of intestinal luminal and mucosal glycerol concentrations were closely correlated. There also seemed to be a correlation between luminal lactate and permeability.

7.2 paper II

Gut luminal microdialysis of glycerol as a marker of intestinal ischemic injury and recovery

The objective of this study on the one hand, was to evaluate microdialysis as a method to assess different degrees of intestinal damage and recovery during ischemia and reperfusion, and on the other hand to evaluate information obtained from microdialysis catheters placed in the peritoneum, the gut wall and the gut lumen.

The superior mesenteric artery was cross-clamped for 60 or 120 min, followed by 4 hrs of reperfusion. Intestinal mucosal integrity was assessed by morphometry, ATP in the gut wall, and permeability of ¹⁴C-Polyethylene glycol. Lactate, glycerol, pyruvate and glucose were measured by microdialysis in the gut lumen, gut mucosa, the peritoneum and in blood.

The histological changes were more pronounced, and the restitution slower after 120 min compared to 60 min of ischemia; but the permeability alterations were not significantly different. Glycerol in the gut lumen closely mirrored the histological findings, with a significantly positive correlation between the average amounts of denuded basement membrane and gut luminal concentration of glycerol. There was also a close correlation between the duration of ischemia and glycerol detected both in the gut wall, lumen and peritoneum. Microdialysis catheters placed in the peritoneum did not reflect differences between the groups during reperfusion, so when possible the gut lumen is to be preferred as a site for placement of microdialysis catheters.

7.3 paper III

Gut luminal lactate levels measured by microdialysis reflects intestinal permeability alterations in repeated intestinal ischemia.

The aim of this study was to investigate the influence of a prolonged initial intestinal ischemic insult on transmucosal permeability after a subsequent ischemic event. Based on study I and II, we also wanted to further investigate whether microdialysis of biomarkers released to the gut lumen is able to reflect changes in intestinal permeability. The superior mesenteric artery was cross-clamped for 60 min followed by 4 hrs of reperfusion. Intestinal mucosal integrity was assessed by: (1) permeability of ¹⁴C-Polyethylene glycol over the gut mucosa, (2) luminal microdialysis of lactate, glucose and glycerol, and (3) tonometry. We demonstrate that a prolonged ischemic insult of the intestine confers protection, since mucosal permeability was less increased in response to the second ischemic insult as compared to the first. Gut luminal intestinal microdialysis of biomarkers, but not tonometry, closely reflects such permeability changes. Lactate reflects permeability across the intestinal mucosa more precisely than glycerol.

7.4 paper IV

Endoluminal microdialysis shows increased rectal lactate in routine coronary surgery

The aim of the prospective clinical study was to go from the bench to bedside and evaluate the feasibility of intestinal luminal microdialysis as a method for clinical monitoring the intestine in patients.

A microdialysis catheter for continuous lactate, glycerol, glucose and pyruvate measurements attached to a tonometric catheter was introduced into the lumen of the recto-sigmoid junction, and intramuscularly, before surgery in 13 patients undergoing elective coronary artery bypass surgery with cardio pulmonary bypass (CPB). The patients were followed for 16 hrs postoperatively, and then the rectal catheter was removed.

All patients made an uneventful perioperative course without complications. Insertion of the rectal catheters was uncomplicated, and all but one functioned properly during the investigation. After 30 min of CPB, luminal lactate had increased five fold, and increased further until the end of CPB. After CPB, gut luminal lactate gradually decreased, but still remained elevated above baseline levels at 16 hrs after surgery. Intramuscular lactate nearly doubled during CPB, but the lactate pyruvate ratio remained unchanged. The intestinal – arterial pCO₂ gap was unchanged. Neither glycerol nor glucose was detected in the gut lumen.

8. General discussion

8.1 Intestinal lactate

In the splanchnic region, the net production of lactate increases when blood flow falls to 60-70 % below normal levels (155,156). Even severe intestinal hypoperfusion may be present without systemic signs of inadequate blood flow, such as hyperlactatemia. This is due to the great lactate clearance capacity of the liver, provided that liver blood flow is maintained (157). In sepsis the splanchnic blood flow is often maintained, but still lactate is produced in excess in the splanchnic bed (158). The pathophysiology of hyperlactataemia is more complex and can involve not only hypoxia (38), but also enhanced glycolysis (159), inhibition of pyruvate dehydrogenase (160,161), and inhibition of mitochondrial respiration (24).

Mesenteric venous lactate measurement might be valuable in detecting anaerobic metabolism of the gut, but for obvious reasons it is not clinically possible.

Redistribution of perfusion within the wall (162-164) may yield various lactate production in the different layers of the intestinal wall. Therefore, the lactate gradient over the mesenteric vascular bed may not represent the metabolic state of the epithelial cells, but rather a mixture from all the layers. Thus monitoring of the intestinal mucosa is possibly best done from the inside of the intestinal lumen, as first done by Tenhunen JJ et al in 1999 (165).

Occlusion of the aorta and superior mesenteric artery as described in Papers I-III caused increased lactate within 30 minutes in the gut lumen, mucosa and peritoneum. Lactate at these sites was roughly the same at the end of cross clamping of the aorta and the SMA for 60 min, as reported by others (166), but did not increase further after 120 minutes of occlusion of the SMA. The latter is in accordance to a similar study where Q_{sma} was reduced stepwise down to approx. 10% of baseline levels. This induced an increase in the mesenteric venous-arterial lactate gradient. This gradient remained high during 60 min of sustained low Q_{sma} , but did not increase further (157). The response in lactate levels, e.g. no further increase in lactate after 60 minutes of ischemia, despite further ongoing ischemia as seen when comparing 60 and 120 min of SMA occlusion (Paper II), has also been observed by others (166,168). Neither was ATP further depleted nor lactate pyruvate ratio (LP-ratio) further increased in the gut wall from 60 to 120 min of ischemia. ATP is produced mainly from oxidative phosphorylation under aerobic conditions, and during low flow ischemia from glycolysis (168). There is probably a steady state in the lactate production as reflected in our findings. Thus, it is reasonable to believe that in these experimental studies the gut luminal lactate represents anaerobic metabolism.

When the blood flow returns to ischemic tissue, the metabolism turns aerobic, and lactate levels decline. During reperfusion, gut luminal lactate further increased for the first 30 min of reperfusion in our animal experiments. In the gut lumen of patients, and mucosa and peritoneum of the pigs, lactate started to decrease immediately after the end of the ischemic period, continuing towards baseline. In pigs, baseline was reached in the

peritoneum after 1 hr, in the gut wall after 4 hrs (Paper II) and in the gut lumen after about 6 hrs (Paper III). In humans (Paper IV), gut luminal lactate had not reached baseline levels 16 hrs after the end of operation, however, a substantial decline was seen within 6 hours. The response is different in those three compartments. In the peritoneum, lactate is probably derived from the underlying muscularis layer via diffusion. Lactate during reperfusion with normal blood flow may probably reflect systemic levels due to washout. Also, in the gut wall, the lactate levels most likely are flow dependent and influenced by the systemic levels and washout. This may explain why lactate measurement in the intestinal wall and peritoneum was not able to differentiate between the various degrees of damage of the intestinal epithelium in response to different duration of cross clamping of the SMA.

Hyperpermeability is a hallmark of intestinal dysfunction; therefore, monitoring such alterations is of great importance. The intestinal surface epithelium is particularly susceptible to ischemic damage, being an important barrier between the gut lumen and the interstitium and blood. All bio-markers assessed in the present studies have to cross this barrier. Under normal conditions without ischemia, it has been shown that blood lactate levels must be very high to cause spill over to the gut lumen (165). In all of the experimental studies there was a correlation between permeability changes across the intestinal mucosa, as assessed by plasma levels of PEG 4000 and release of lactate into the gut lumen as measured by microdialysis. In Paper I, figure 3 this correlation was pronounced in the early reperfusion period.

Figure 3 from Paper I

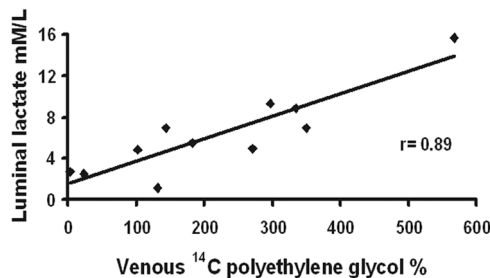


Fig. 3 Relationship between intestinal permeability and lactate detected in the intestinal lumen in seven pigs subjected to 60 min of aortic cross-clamping. Abscissa is the relative change in ¹⁴C polyethylene glycol expressed in percentage during the first 30 min of reperfusion. Ordinate is the corresponding concentrations of luminal lactate. $p=0.001$, $y=0.02x + 1.53$

In Paper II (fig 1A and 2C) the peak response and the separation of the curves for animals with 60 and 120 min intestinal ischemia were essentially the same for changes in venous PEG 4000 and gut luminal lactate in response to time. Such a correlation was not evident between permeability and changes of lactate in the gut wall and peritoneum.

Fig 1A and 2C from paper II

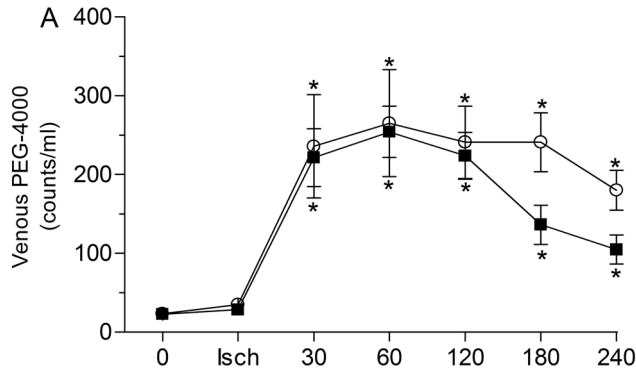


Figure 1A Concentration of PEG-4000 in venous blood as an index of permeability across the intestinal mucosa in pigs exposed to 60 (closed squares, n=14) or 120 min (open circles, n=10) of cross-clamping of the superior mesenteric artery. On the abscissa 0 denotes baseline, whereas Isch and 30-240 denotes time points at the end of the ischemic period and minutes of reperfusion, respectively.

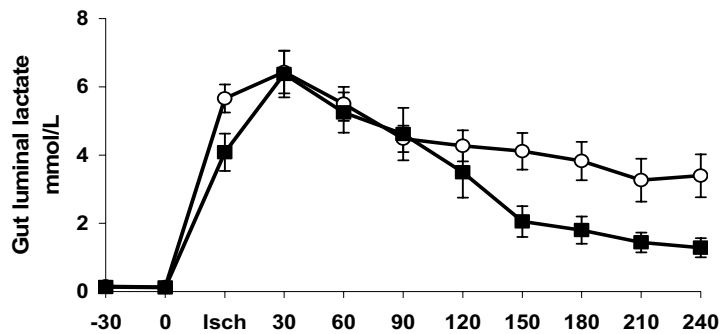


Figure 2C. Gut luminal levels of lactate detected by microdialysis in pigs subjected to cross-clamping of the superior mesenteric artery for 60 min (closed squares) or 120 min (open circles), followed by reperfusion for 240 min. On the abscissa, -30 and 0 denotes baseline, whereas Isch and 30-240 denotes time points at the end of the ischemic period and minutes of reperfusion, respectively. Values are presented as mean (SEM).

These observations were confirmed and extended in Paper III. The gut luminal lactate and venous PEG 4000 qualitatively showed the same response to two subsequent ischemic challenges of the intestine (Fig 1A and fig 2A, Paper III). Also, the kinetics during the reperfusion was very similar.

Figure 1A and 2A from paper III

A

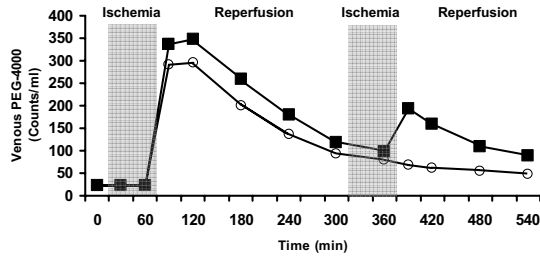


Figure 1A. Permeability across the intestinal mucosa . Mean concentrations of PEG-4000 in venous blood as an index of permeability across the intestinal mucosa in pigs subjected to cross-clamping of the superior mesenteric artery (SMA) for 60 min twice (double clamp group, closed squares) or once (single clamp group, open circles), followed by reperfusion. The shaded areas represent cross-clamping of the SMA.

A

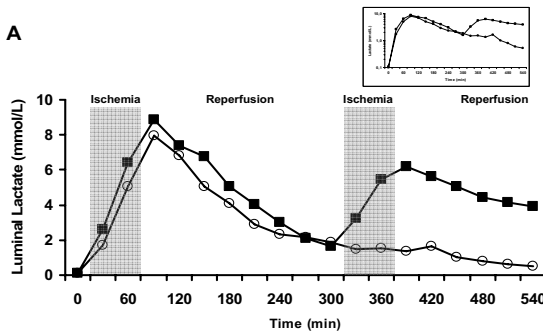


Figure 2A. Gut luminal concentrations of lactate and glycerol
 Mean intestinal luminal concentrations of lactate detected by microdialysis in pigs subjected to cross-clamping of the superior mesenteric artery (SMA) for 60 min twice (double clamp group, closed squares) or once (single clamp group, open circles), followed by reperfusion. On the abscissa, 0 denotes baseline, whereas the shaded areas represents cross-clamping of the SMA.
 The small figure depicts the same graph as in the main figures, but with logarithmic scales on the ordinate in order to illustrate the degree of log-linear elimination.

A major conclusion is, therefore, that lactate as measured by microdialysis in the gut lumen reflects permeability changes across the gut mucosa. This is of importance because it is known that, for example, septic states and CPB may be associated with increased intestinal permeability.

8.2 Glycerol

Glycerol as measured with MD has been reported to be a marker of cellular degradation in the brain (65-68), liver (72,73), peritoneum (74-75), heart (169) and muscle / subcutaneous tissue (69-71).

Cross clamping of both the aorta and the SMA caused increased levels of glycerol in the mucosa (Paper I and II), the gut lumen (Paper I-III) and peritoneum (Paper II) within 30 minutes of ischemia. The concentration further increased depending on the duration of the cross clamping. The reproducibility seems fairly good, with almost the same values in all three papers (same catheters, almost same recovery). The concentration of glycerol differed between the localisations at which it was measured, highest in the lumen and substantially lower in the peritoneum. This is most likely explained by the intestinal epithelium being more susceptible to ischemic damage (56-58) than other cell types in the intestinal wall and abdominal cavity.

These observations are in agreement with a few other experimental studies which have documented a good correlation between intestinal ischemia and increased glycerol in the gut wall (170,171), the gut lumen (171) and in mesenterial veins (172). Results from sepsis and hemorrhagic shock studies point in the same direction, however, not so clearly, there being both unaltered intravasal levels (172) and increased peritoneal (173) and gut wall (174) levels. These studies have mainly focused on the ischemic phase alone. The present thesis extends these observations by showing that the glycerol levels in the gut wall and lumen also reflect the initial ischemic trauma throughout the reperfusion and repair phase.

In Paper II, we showed that release of glycerol to the gut wall, lumen and the peritoneal cavity depends on the duration of ischemia. This is in consistence with findings after occlusive ischemia in the gut wall and peritoneal cavity (170, 175), and in response to endotoxaemia (174). In paper I, gut wall glycerol decreased during 2 hrs of reperfusion, whereas luminal glycerol remained elevated. However, extending the reperfusion time to 4 hrs as described in Paper II, revealed that there was less injury of the epithelium caused by 60 min than after 120 min of ischemia. This was reflected in improved repair of the surface epithelium and a decline in gut luminal release of glycerol. Regression analysis established a close correlation between release of glycerol and injury of the surface epithelial lining in the reperfusion period. Qualitatively, a similar relation was noted between gut luminal glycerol and changes in permeability and content of ATP in the intestinal wall during the reperfusion period.

Therefore, another major conclusion in the present thesis is that gut luminal glycerol when measured by microdialysis is a marker of both cellular damage and repair after ischemia of the small bowel.

Despite the correlation between cellular damage and glycerol levels, we can not completely rule out other additive contributing sources of glycerol under endo- and paracrine control; Sympathicus mediated lipolysis may increase glycerol levels in fat tissues and in systemic circulation (176). Leakage of glycerol to the gut lumen is possible, as the permeability was substantially increased in all three experimental studies. However, arterial glycerol was not significantly elevated for longer periods in

any of the experimental studies. Neither was there any correlation between arterial and luminal glycerol levels in paper III.

Why didn't we find glycerol in the rectal lumen in patients undergoing CPB? The intestinal affection in CPB is due to SIRS-like reaction (177), somewhat different from the occlusion models in paper I-III. Thus, the difference between the gut glycerol response to occlusive ischemia and sepsis may be caused by the different mechanisms behind the eventual cellular damage. In occlusive ischemia there is probably an overweight of necrosis causing cellular disintegration (64) and release of glycerol. In sepsis and other SIRS models, such as CPB, the mechanism is probably a overweight of apoptosis of the cells in the intestine (178,179) without any washout of cellular content to the interstitium and therefore no release of glycerol.

8.3 Glucose.

In muscle, extra cellular glucose decreases to approximately 50% of pre-ischemic levels during 30 min ischemia, further decreasing to 20% of pre-ischemic levels after 60 min ischemia. Both of these decreases are due to cessation of delivery and consumption (180).

After both aortic and SMA cross clamping, glucose in the gut wall became virtually undetectable, but returned to baseline levels 30 minutes after unclamping (Paper I-II). This is in concordance to findings in pigs subjected to stepwise mesenteric blood flow reduction. In each step there was a 15% reduction. Here, 30% flow reduction from baseline was associated with a decrease in microdialysis glucose concentration of 80 %, and is an early sign of anaerobic metabolism (181).

In the gut lumen, under baseline conditions and during occlusive ischemia, glucose is undetectable by microdialysis (171, I-II). Intestinal ischemia has also been demonstrated in vitro to abolish the absorption of eventual glucose from the gut lumen (182,183). The information about what happens during reperfusion is sparse. We have found that after unclamping, glucose becomes detectable in the gut lumen (Paper I-III), and even tends to reflect the degree of permeability alterations (Paper III). It is tempting to speculate that when the blood flow is restored, glucose passes over the damaged intestinal barrier proportionate to the alterations in permeability. After 1 to 2 hrs of reperfusion, there is no longer any glucose in the gut lumen; the correlation to the cellular restitution is striking, so maybe the glucose absorptive properties is recovered (182,183). At least, the appearance of glucose can be used as a sign of recovered intestinal blood flow.

8.4 Clinical aspects and future research

Microdialysis has properties which enable it to be used more extensively in monitoring intestinal function, both experimentally and in clinical practice. Microdialysis probes placed inside the intestine (gut luminal microdialysis), detect deteriorated metabolism, the extent of cellular damage and increased permeability. In contrast to catheters placed in the peritoneal cavity, catheters placed in the gut lumen also make it possible to follow

the course during reperfusion. In patients, the placement of microdialysis probes, as they are manufactured today, is best in the abdominal cavity, due to the relative fragility of the microdialysis catheters. However, this is an invasive procedure having possible concurrent complications, the patient population being limited to those undergoing abdominal surgery.

Due to the ability of gut luminal microdialysis to monitor functional recovery of the intestine, we believe that this is the best approach in patients. The regional blood flow heterogeneity in sepsis (184), mesenterial hypoperfusion (181) and during acute normovolemic hemodilution (185), cause challenges concerned with what is the best part in the intestine in which to place the catheters. The alterations in permeability and lactate production in SIRS conditions make the rectal lumen a possible approach. Insertion of the catheter is a less invasive method and can be done pre and postoperatively. This is also true in critically ill patients in the ICU. The search for other possible biomarkers of intestinal dysfunction must be continued.

The probes have to be reinforced for safer and easier insertion. The optimal way to do it is not yet known; only further research may define the place of dialysis bags, large rectal dialysis tubes, reinforced combined MD and tonometry catheters or biosensors.....

9. Conclusions

Lactate, glycerol and glucose are released into the gut lumen in ischemia and reperfusion (IR).

Glycerol is a marker of cellular damage, and is independent of systemic levels

Lactate mirrors permeability alterations in the intestine

Intestinal ischemia can be detected with the microdialysis technique in the gut wall, lumen and in the peritoneum.

The gut lumen is to be preferred as a site for placement of microdialysis catheters when monitoring recovery / reperfusion

Gut luminal microdialysis can differentiate between various degrees of intestinal damage.

Gut luminal microdialysis is a clinically feasible method for monitoring intestinal integrity

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Paper I

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Paper II

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Paper III

Gut luminal lactate measured by microdialysis mirrors permeability of the intestinal mucosa after ischemia.

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Running head: Gut luminal lactate mirrors intestinal permeability

Summary statement: A prolonged initial ischemic insult of the intestine confers protection, in terms of reduced hyperpermeability, against further ischemia. Microdialysis of biomarkers mirrors permeability changes associated with this type of protection. Lactate reflects permeability across the intestinal mucosa more precisely than glycerol.

ABSTRACT:

Background: The aim of the present study was to investigate the influence of a prolonged initial intestinal ischemic insult on transmucosal permeability after a subsequent ischemic event, and whether microdialysis of biomarkers released to the gut lumen is able to reflect changes in intestinal permeability.

Methods: The superior mesenteric artery was cross-clamped for 60 min followed by 4 hrs of reperfusion in 16 pigs. Nine pigs had a second cross-clamp of 60 min, and 3 hrs of reperfusion, while 7 were observed for further 4 hrs of reperfusion. Intestinal mucosal integrity was assessed by permeability of ¹⁴C-polyethylene glycol (PEG-4000) over the gut mucosa, luminal microdialysis of lactate, glucose and glycerol, and tonometry.

Results: During reperfusion, the PEG-4000 amount in venous blood was two times higher after the first than after the second ischemia (AUC: 44780 (13441-82723) vs. 22298 (12213-49698) counts·min/mL, p=0.026 (mean (range))). There was less lactate detected in the gut lumen after the second ischemia compared to the first (AUC: 797 (412-1700) vs. 1151 (880-1969) mmol·min/L, p=0.02) and a lower maximum concentration (4.8 (2.7-9.4) vs. 8.5 (5.0-14.9) mmol/L, p=0.01). The same pattern was also seen for luminal glycerol and glucose. During the second ischemia, the intestinal mucosal/arterial CO₂-gap was identical to the level during the first ischemic episode.

Conclusions: A prolonged ischemic insult of the intestine confers protection, in terms of reduced hyperpermeability, against further ischemia. Microdialysis of biomarkers mirrors permeability changes associated with this type of protection. Lactate reflects permeability across the intestinal mucosa more precisely than glycerol.

INTRODUCTION

Increased intestinal permeability is a key manifestation of intestinal dysfunction caused by ischemia/reperfusion of the gut (1-3), and is associated with an increased risk of multiple organ failure. (4-9). Ischemic preconditioning (IPC) in various tissues including the intestine (10-14) refers to the observation that short periods of ischemia (5-15 min) induce protection against tissue injury. However, it is not clear whether a more prolonged period of intestinal ischemia, as seen initially in hypovolemic and septic shock, may influence changes in intestinal permeability caused by later ischemic events. Development of reliable methods for monitoring intestinal barrier dysfunction is essential in order to improve the care of patients with conditions associated with ischemia reperfusion challenge of the gut. Microdialysis allows monitoring of extracellular substances in gut submucosa and lumen, also in the clinical setting (15). We have recently shown that microdialysis of the biomarker glycerol released to the gut lumen provide information about tissue injury after intestinal ischemia (16), whereas release of lactate to lumen may be a measure of permeability (17).

The aim of the present study was 1) to investigate the influence of a clinically relevant initial intestinal ischemic insult on transmucosal permeability after a subsequent ischemic event, and 2) to investigate whether tonometry and microdialysis of biomarkers released to the gut lumen is able to reflect changes in intestinal permeability in this experimental model.

MATERIALS AND METHODS

The Norwegian State Commission for Animal Experimentation approved this study. All procedures were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996).

Anesthesia and surgical preparation

The superior mesenteric artery (SMA) was cross-clamped for 60 min followed by 4 hrs of reperfusion in 16 pigs (bodyweight 19-27 kg). They were then randomized in two groups; Nine pigs had a second cross-clamp of 60 min and 3 hrs of reperfusion (double clamp group), while 7 were only observed for further 4 hrs of reperfusion (single clamp group). After i.m. premedication with diazepam 10 mg (Stesolid, Dumex-Alpha, Copenhagen, Denmark), azaperon 400 mg (Stresnil, Janssen-Cilag, Wien, Austria) and atropine 1 mg (Atropin, Nycomed Pharma, Oslo, Norway), anesthesia was induced with thiopental sodium 5-10 mg/kg (Pentothal-Natrium, Abbott Scandinavia AB, Solna, Sweden) and ketamine 10 mg/kg (Ketalar, Parke-Davis, Solna, Sweden). A tracheotomy was performed and the animals were mechanically ventilated with a Servo ventilator 900 B (Siemens –Elema, Sweden). FiO₂ was kept at 0.3, tidal volume at 10 ml/kg and minute ventilation was adjusted to maintain PaCO₂ of 34-41 mmHg (4.5 - 5.5 kPa) and kept unchanged during the experimental period. Anaesthesia was maintained with isoflurane 0.5% (Forene, Abbot Scandinavia AB, Solna, Sweden), and a continuous infusion of fentanyl 7.5 µg/kg/hour (Fentanyl, Alpha AS, Oslo, Norway) and midazolam 0.5 mg/kg/hour (Dormicum, Roche, Basel, Switzerland). The femoral artery was cannulated for heart rate, mean arterial blood pressure, and blood gas measurements (ABL 330 radiometer, Copenhagen, Denmark). Another catheter was inserted into the inferior cava vein for blood samples. A 4 FG catheter was introduced via the right common carotid artery

into the left cardiac ventricle for infusion of coloured microspheres. The animals received Ringer acetate at an infusion rate of 10-15 ml/kg/h. A heating blanket and warmed fluids were used to maintain a constant core temperature of 38.5 °C. A midline laparotomy was performed, and a catheter inserted into the urinary bladder. An ultrasonic transit-time flow probe (6 mm) was placed around the SMA for continuous blood flow measurements. A vessel loop for cross-clamping was placed proximal to the flow probe on the superior mesenteric artery. After surgical preparation, the animals were allowed to stabilize for 60 min, thereafter baseline measurements were collected for 45 min.

Regional blood flow

Intestinal tissue blood flow and cardiac output were determined by the distribution of coloured microspheres during baseline, 10 min after the first cross-clamping and 10 min after the first and second declamping (18).

Permeability

Intestinal permeability was determined as described previously (19). Briefly, a 30 cm length of jejunum was ligated at both ends and 100 ml (10.0 µCi) ¹⁴C polyethylene glycol (PEG-4000) (Amersham Bioscience, Buckinghamshire, England) was injected into the lumen. Venous blood samples for determination of the concentration of PEG-4000 in plasma were taken at 30 min intervals, and urine samples were taken hourly.

Microdialysis

A microdialysis catheter (CMA 70, membrane length 20 mm, 20 kD, CMA Microdialysis AB, Stockholm, Sweden) was introduced into the jejunal lumen 40 cm distal to the ligament of Treitz, as recently reported from our laboratory (17). A microdialysis catheter (CMA 70) was also introduced into the subclavian artery. The microdialysis catheters were perfused at a flow rate of 1 µL/min with an isotonic

perfusion fluid (CMA Perfusion Fluid T1 (Na^+ 147 mmol, K^+ 4 mmol, Ca^{2+} 2.3 mmol, Cl^- 156 mmol *per L*) and a microdialysis pump (CMA 107, CMA Microdialysis AB, Stockholm, Sweden). The catheters were perfused *in situ* for at least 75 minutes before baseline measurements. Samples were collected over 30 minutes, and were analyzed immediately on site for glycerol, lactate, pyruvate and glucose concentrations by enzymatic fluorometric assays (CMA 600 Microdialysis analyzer) using peroxidase methodology. *In vitro* recovery with a flow rate of 1 $\mu\text{L}/\text{min}$ was 65 (41-70) % (mean (range)) for lactate, 22 (17-33) % for pyruvate, 36 (30-44) % for glycerol and 33 (25-43) % for glucose.

Tonometry

A tonometry catheter (16F, Tonometrics, Datex-Ohmeda, Finland) was introduced into the jejunal lumen 60 cm distal to the ligament of Treitz through an antimesenteric incision, and calibrated *in situ*. Gut mucosal PCO_2 (P_iCO_2) was measured using an automated air tonometry system (Tonocap, TC-200; Datex-Ohmeda, Helsinki, Finland). Gut mucosal P_iCO_2 and arterial blood gas measurements (ABL 330 radiometer, Copenhagen, Denmark) were simultaneously measured every 30 minutes and the difference between P_iCO_2 and arterial PCO_2 (PaCO_2), the CO_2 -gap, was calculated at each time point (regional CO_2 -gap).

Statistics and calculations

All values are expressed as medians and ranges where not otherwise mentioned. For comparisons between the double and single clamp groups at various time points we used the Mann-Whitney U test. To assess changes within the groups over time, the Friedman test was used. The Wilcoxon signed-rank test was applied for paired samples to evaluate differences between specific time points or area under the curves (AUC) within the groups. Linear regression was performed to investigate and plot the

association between arterial and luminal values within each pig for specific time intervals. SPSS for windows version 13, (SPSS Inc, Chicago Illinois, USA) was used for the statistical analyses.

Formation and elimination kinetics of lactate, glycerol and PEG-4000 were calculated as follows: Maximum concentrations (C_{\max}) and the times to achieve maximum serum concentrations (t_{\max}), were obtained directly from the measured values. Other parameters were calculated by means of the Kinetica program package, version 4.3 (InnaPhase Corporation, Philadelphia, PA, USA), using a non-compartment model. Areas under the curve from the start of the first and of second clamping, respectively, to 240 minutes later (AUC_{0-240}) were calculated using the trapezoidal rule. The parameter estimates describing the elimination phase of the log-concentrations (λ_z) were calculated using the best-fit regression lines, taking the degree of log-linearity into account. The elimination half-lives ($t_{1/2}$) were calculated as $\ln 2 / \lambda_z$.

Before start of the second cross-clamping, baseline levels of lactate and glycerol were significantly higher than before the first cross-clamping. To improve the comparability between the first and the second clamps we subtracted mean values at the corresponding time points from the single clamp group during the last episode of ischemia and reperfusion.

RESULTS

There were no significant differences in the measured parameters at baseline between the groups, except for heart rate (Table 1). All animals survived throughout the experiment.

Hemodynamic variables

The hemodynamic variables during the experiment are presented in Table 1. Mean arterial pressure was elevated from baseline only during cross-clamping in both groups. Heart rate increased during the first clamp in both groups, and remained elevated for the rest of the experiment. Cardiac output did not change during the experiment. The blood flow of the SMA was zero during cross-clamping and returned to baseline during reperfusion.

Blood flow measured both in the whole jejunal wall and in the serosa fell to zero during cross-clamping of the SMA, and returned to baseline levels during reperfusion after both ischemic episodes. Jejunal mucosal blood flow also fell to zero after clamping of the SMA. After the first ischemic episode, blood flow returned to baseline, whereas after the second ischemic episode, blood flow was higher than baseline, $p=0.027$.

Intestinal permeability

PEG-4000 at baseline and during the first clamping was not detectable in venous blood or urine. During reperfusion, the mean AUC_{0-240} of PEG-4000 in venous blood was two times higher after the first than after the second ischemia, (44780 (13441-82723) vs. 22298 (12213-49698) counts·min/mL, $p=0.026$) (Figure 1A). The same pattern was seen for C_{max} (mean 394 (117-632) vs. 140(105-330) counts/ml, $p=0.035$).

The PEG-4000 levels declined log-linearly during both the first and second reperfusion with mean elimination half-lives of 133 (67-224) and 117 (76-149) min, respectively ($p=0.434$). The excretion of PEG-4000 in urine largely mirrored the venous concentration of this marker molecule, although the peak excretion after declamping of the SMA occurred about 1 hour later than the peak concentration of venous PEG-4000 (Fig 1B).

Intestinal lumen

Lactate. The response to the first cross-clamping of the SMA was similar in both groups with increased lactate levels within 30 min ($p<0.05$) (Figure 2A). The mean C_{max} was 8.5 (5.0-14.9) and 7.8 (5.8-11.5) mmol/L in the first clamp in the double clamp group and single clamp groups, respectively, ($p=0.408$), and was reached after a median of 30 (0-180) min of reperfusion. The lactate levels declined log-linearly during reperfusion with a mean elimination half-life of 90 (26-165) min (small figure in figure 2A), and baseline levels were reached in the single clamp group after 390 min of reperfusion. The response to the second cross-clamping was less pronounced compared to the first occlusion, with a mean AUC_{0-240} of 797 (412-1700) vs. 1151 (880-1969) mmol·min/L, $p=0.02$ and a mean C_{max} of 4.8 (2.7-9.4) vs. 8.5 (5.0-14.9) mmol/L, $p=0.01$.

Glycerol. The first cross-clamp of the SMA caused an increase in gut luminal glycerol in both groups after 30 min ($p=0.04$) (Figure 2B). Mean C_{max} for glycerol was similar in the double clamp and the single clamp groups (971 (735-1538) vs. 826 (619-1351) μ mol/L, $p=0.142$), and was reached after mean of 90 (30-180) min of reperfusion. The glycerol levels declined in a non-log-linear way during reperfusion (small figure

in Figure 2B), and baseline levels were reached in the early clamp group after 360 min of reperfusion.

The response to the second clamp differed from the first. Mean C_{\max} of luminal glycerol was higher in the first clamp than in the second (971 (735-1538) vs. 404 (49-1195) $\mu\text{mol/L}$, $p=0.02$). There was also a trend toward the same regarding AUC (131214 (71253-215517) vs. 45281 (1581-213489) $\mu\text{mol}\cdot\text{min/L}$, $p=0.07$). The t_{\max} values were the same in first and second clamp (median 150 (90-210) vs. 120 (30-210) min, $p=0.174$).

Glucose in the gut lumen was only detected during reperfusion, and the mean AUC was higher during the first than during the second clamp (43 (0-197) vs. 3 (0-28) $\text{mmol}\cdot\text{min/L}$, respectively, $p=0.008$).

Regional CO₂-gap.

The regional CO₂-gap increased 4-fold during the first occlusion in both groups ($p=0.008$) (Figure 3), but returned to baseline levels after 240 min of reperfusion. During the second ischemia, the values both for the increase in and the maximal level of the CO₂-gap were identical to the corresponding values during the first ischemic episode.

Arterial blood

Arterial blood concentrations of glucose, lactate, pyruvate and glycerol are presented in Table 2. The arterial lactate concentration was only significantly higher than baseline at 60 min of every cross-clamp ($p=0.012$). In Figure 4 A, individual arterial lactate concentrations in the double clamp group are plotted against corresponding luminal lactate concentrations during baseline and both reperfusion periods. In linear regression analyses accounting for the intra- and interindividual variance of the pigs,

we found an association between arterial and luminal lactate levels, which was most pronounced in the first reperfusion ($p < 0.01$).

The arterial pyruvate concentration was only significantly increased after 60 minutes of the first ischemia ($p = 0.039$), and decreased below baseline levels after 240 min of reperfusion in the single clamp group ($p = 0.016$). Arterial lactate-pyruvate ratio was not elevated from baseline levels. The arterial glycerol concentration was only increased above baseline at one single time during the first clamping period ($p = 0.027$). The arterial glycerol concentrations are also plotted against luminal glycerol concentrations at the same three time intervals (Figure 4B) as with lactate, but we found no significant associations between arterial and luminal glycerol levels.

DISCUSSION

In this study with two ischemic episodes, we demonstrate that mucosal permeability was less increased in response to the second ischemic insult. Gut luminal intestinal microdialysis of biomarkers, but not tonometry, closely reflect such permeability changes.

The improved mucosal barrier function after the second ischemic insult is consistent with the only published study that clearly has demonstrated an effect of preconditioning (3 cycles of 2 min ischemia/5 min reperfusion) on transmucosal permeability in response to a subsequent challenge by ischemia (14). The current study extends these observations by showing that even a 60 min ischemic event in the gut, comparable to what may be encountered before effective resuscitation after hypovolemic and septic shock, may elicit defence mechanisms like those responsible for the preconditioning phenomenon. It was beyond the scope of the present study to further characterize these mechanisms, but certain features of the experimental model deserve comments. We have found in a recent study that that there is no worsened injury of the mucosa, as judged by microscopy, throughout the reperfusion period after ischemia (16). Both in that study and in the present, hyperpermeability across the mucosa after ischemia was not sustained or increased, but instead decreased throughout the reperfusion period. Mucosal blood flow was rapidly restored to normal levels after the ischemic period, indicating absence of the no-reflow phenomenon (i.e. full return of nutritive perfusion after ischemia) in this layer of the intestinal wall (20). These features are in contrast to findings during reperfusion, particularly in rodent models of ischemia/reperfusion injury of the gut, in which progressive structural and functional derangements of the mucosa and the no-reflow phenomenon are hallmarks of events caused by activation of reactive oxygen species (21). We therefore suggest

that the reperfusion injury in the porcine intestine is blunted as compared to many other commonly used ischemia/reperfusion models. It is possible that adaptive protective mechanisms are particularly up regulated in response to reperfusion in the porcine intestine, which may be one underlying reason for the improved barrier function after the second ischemic insult in the current study.

Qualitatively, concentrations of lactate, glucose and glycerol in the intestinal lumen, mirrored the attenuated hyperpermeability after the second ischemic insult (Figures 1 and 2). However, the time course with regard to increase and elimination from the gut lumen indicates certain differences between lactate and glycerol. As shown in Figures 1 and 2, the maximum level of lactate and PEG-4000 occurred at exactly the same time and, importantly, the slope of the elimination curve was almost identical. In contrast, the luminal decrease of glycerol was much slower (Figure 2). This suggests that lactate is a more precise measure of permeability than glycerol. A likely explanation for these observations may be sought by the origin of lactate and glycerol released to the gut lumen in response to ischemia. It is known that the surface epithelium of the gut is much more susceptible to ischemia than cells in the deeper part of the gut wall (22-24), and disintegration of the cell membranes induce release of glycerol into the gut lumen (16). There is a close correlation between intestinal cellular damage and glycerol levels (16), thus the smaller total amount of glycerol in the gut lumen in the second ischemic period probably reflects less destruction of the intestinal epithelium and not altered permeability. Lactate released into the lumen is at least to a large part produced due to anaerobic metabolism in all cell types of the mucosa and muscular layer, not only the epithelium.

Arterial concentrations of the biomarkers, and particularly lactate, may also influence their release into the intestinal lumen. With an intact surface epithelial barrier it has

been shown that even high systemic levels of lactate do not influence the gut luminal concentration of lactate (25). As shown in Figure 4A there was a correlation between systemic and luminal lactate in the reperfusion period coinciding with increased permeability. This suggests that systemic lactate may contribute to luminal release of lactate. However, considering the gradient between lumen and blood, the local production in the intestinal wall is probably the most important source of luminal lactate. There was no correlation between systemic and luminal release of glycerol. These findings further support the conclusion put forward above that luminal lactate better reflect permeability than glycerol.

The transport of lactate during ischemia is rather complex. Lactate crosses cell membranes by interaction with specific proteins; the monocarboxylate transporters, which are trans-membrane proteins facilitating cotransport of a monocarboxylate ion with a proton (26). Acidosis increases both paracellular and transcellular permeability to hydrophilic (macro)molecules such as fluorescein disulfonic acid (FS; molecular weight 478 Da, and fluorescein isothiocyanate-labeled dextran (FD4; average molecular weight 4 kDa) in human intestinal epithelial Caco-2BBE cells grown as monolayers (27). Metabolic acidosis due to ischemia may thus influence on the lactate transport from the cells into the intestinal lumen as with larger molecules like PEG-4000.

On the contrary, CO₂ freely diffuses out of cells and into the interstitial fluid. Increases in tissue CO₂ are primarily a function of changes in regional blood flow, independent of the degree of tissue dysoxia (28). This is in accordance with our findings with the same blood flow and CO₂ alterations in both ischemic episodes. It is

therefore not surprising that tonometry failed to reflect permeability changes across the mucosa.

In summary, the present study provides evidence for the conclusion that even a prolonged ischemic insult of the intestine confers protection, in terms of reduced hyperpermeability, against further ischemia. Microdialysis of biomarkers mirrors the permeability changes associated with this type of protection. Lactate reflects permeability across the intestinal mucosa more precisely than glycerol.

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Table 1 Hemodynamic variables

Measurements	Group	Baseline	1st clamp	1st reperfusion	2nd clamp	2nd reperfusion	p-value
Mean arterial pressure (mmHg)	Double clamp	69 (61-89)	94 (79-113)	69 (67-75)	84 (81-116)	72 (55-83)	0.020
	Single clamp	61 (47-83)	83 (62-111)	74 (51-86)	N/A	76 (61-86)	0.001
	p-value	0.284	0.134	0.778		0.936	
Cardiac output (L/min)	Double clamp	7.0 (3.4-9.6)	6.0 (3.1-11.6)	6.3 (3.3-9.0)	N/A	5.3 (2.6-14.9)	0.153
	Single clamp	6.9 (4.1-8.5)	5.2 (3.0-6.9)	7.2 (3.9-14.5)	N/A	6.9 (6.3-13.2)	0.297
	p-value	0.536	0.918	0.470		0.174	
Heart rate (beats/min)	Double clamp	80 (65-104)*	101 (66-180)	97 (65-171)	95 (63-178)	124 (93-189)*	0.005
	Single clamp	63 (51-81)	92 (55-144)	84 (54-124)	N/A	84 (56-188)	0.001
	p-value	0.029	0.133	0.606		0.015	
Q _{SMA} (mL/kg/min)	Double clamp	28 (15-40)	0 (0)	32 (7-52)	0 (0)	22 (6-41)	0.000
	Single clamp	21 (19-25)	0 (0)	23 (17-28)	N/A	21 (15-26)	0.000
	p-value	0.536	0.475	0.408		0.837	
Q _{Jejunal wall} (mL/min/g tissue)	Double clamp	0.47 (0.21-0.79)	0.01 (0.00-0.03)*	0.46944	N/A	0.49 (0.16-0.94)	0.000
	Single clamp	0.44 (0.33-0.68)	0.03 (0.02-0.18)	0.495	N/A	0.63 (0.37-1.17)	0.000
	p-value	0.918	0.010	0.606		0.351	
Q _{Mucosa} (mL/min/g tissue)	Double clamp	0.48 (0.14-0.84)	0.01 (0.00-0.06)	0.49 (0.24-1.40)	N/A	0.75 (0.36-1.20)	0.000
	Single clamp	0.41 (0.22-0.48)	0.02 (0.01-0.10)	0.45 (0.12-0.83)	N/A	0.48 (0.15-0.91)	0.000
	p-value	0.606	0.365	0.837		0.210	
Q _{Serosa} (mL/min/g tissue)	Double clamp	1.18 (0.05-2.93)	0.01 (0.00-0.04)	0.35 (0.06-0.89)	N/A	0.52 (0.12-1.25)*	0.000
	Single clamp	0.71 (0.29-2.49)	0.03 (0.00-0.23)	0.31 (0.09-2.11)	N/A	1.16 (0.43-3.60)	0.000
	p-value	0.791	0.096	0.958		0.030	
Arterial PCO ₂ (torr)	Double clamp	34 (29-53)	36 (24-45)	38 (27-46)	40 (25-47)	42 (28-50)	0.145
	Single clamp	35 (26-48)	38 (26-55)	43 (27-51)	N/A	41 (27-79)	0.086
	p-value	0.817	0.778	0.142		0.816	

All values are expressed as medians (ranges). Q_{SMA}, Q_{Jejunal wall}, Q_{Mucosa} and Q_{Serosa} are blood flow in the superior mesenteric artery and intestinal wall (entire wall, mucosa and serosa), respectively. The superior mesenteric artery (SMA) was cross-clamped for 60 min twice (double clamp group) or once (single clamp group), followed by reperfusion. Cardiac output and intestinal tissue blood flow were determined 15 min before the end of the first cross clamping of the SMA and at given time points. Other values are medians in each pig during baseline, ischaemia and reperfusion periods, respectively. P-values in the right column represent comparisons within groups over time. * P<0.05 compared to the single clamp group.

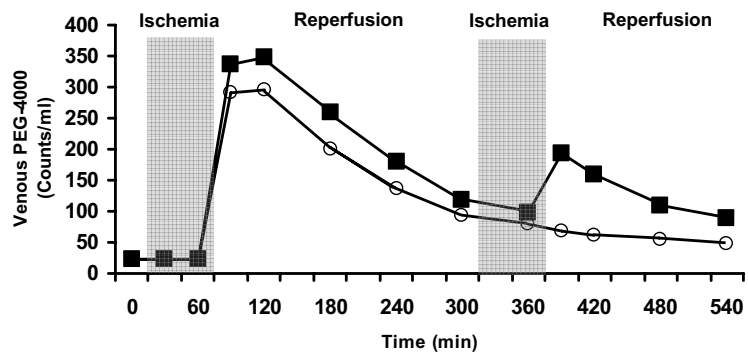
Table 2 Arterial concentrations of glucose, lactate, pyruvate and glycerol

Measurements	Group	Baseline	1st clamp	1st reperfusion	2nd clamp	2nd reperfusion	p-value
Arterial glucose concentration, mmol/L	<i>Double clamp</i>	4.6(1.6-5.5)	5.6(2.3-6.6)	4.5(2.3-4.9)	4.2(1.8-6.1)	3.9(2.3-4.9)	0.001
	<i>Single clamp</i>	5.1(4.6-6.2)	6.5(4.8-8.2)	4.9(4.0-7.3)	N/A	4.50(3.5-6.2)	0.139
	<i>p-value</i>	0.071	0.174	0.114		0.115	
Arterial lactate concentration, mmol/L	<i>Double clamp</i>	1.5(0.7-2.2)	1.4(1.0-4.6)	0.8(0.6-6.2)	1.1(0.5-2.8)	0.8(0.5-3.5)	0.000
	<i>Single clamp</i>	1.3(0.9-1.9)	1.4(1.2-2.2)	0.8(0.5-2.5)	N/A	0.5(0.3-1.4)	0.000
	<i>p-value</i>	0.918	0.918	0.681		0.71	
Arterial pyruvate concentration, μ mol/L	<i>Double clamp</i>	123(70-208)	142(90-299)	98(57-343)	102(75-386)	81(50-223)	0.001
	<i>Single clamp</i>	162(102-240)	202(111-423)	135(62-300)	N/A	68(52-111)	0.001
	<i>p-value</i>	0.299	0.299	0.758		0.336	
Arterial lactate/pyruvate concentration-ratio	<i>Double clamp</i>	11(7-15)	11(7-17)*	11(6-18)	9(7-19)	11(7-16)*	0.436
	<i>Single clamp</i>	8(5-18)	8(3-12)	8(6-12)	N/A	8(5-11)	0.086
	<i>p-value</i>	0.299	0.016	0.071		0.021	
Arterial glycerol concentration, μ mol/L	<i>Double clamp</i>	13(0-28)	15(11-126)	10(0-133)	15(0-112)	16(0-76)	0.001
	<i>Single clamp</i>	13(0-31)	29(13-128)	14(6-76)	N/A	13(0-64)	0.021
	<i>p-value</i>	0.536	0.408	0.836		0.837	

All values are expressed as medians (ranges). The superior mesenteric artery (SMA) was cross-clamped for 60 min twice (double clamp group) or once (single clamp group), followed by reperfusion. Values are medians (range) in each pig during baseline, ischaemia and reperfusion periods, respectively. P-values in the right column represent comparisons over time in each group. * P<0.05 compared to the single clamp group.

Figure 1

A



B

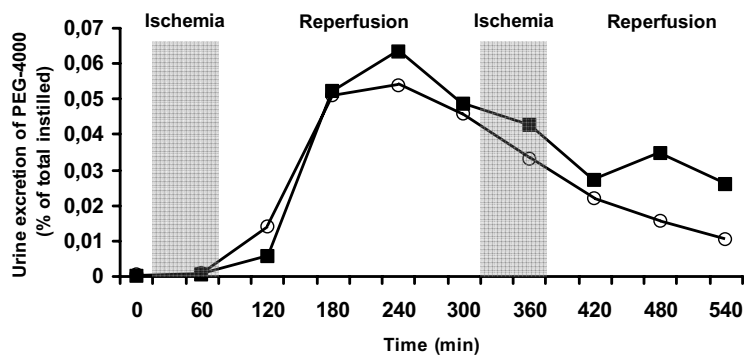


Figure 2

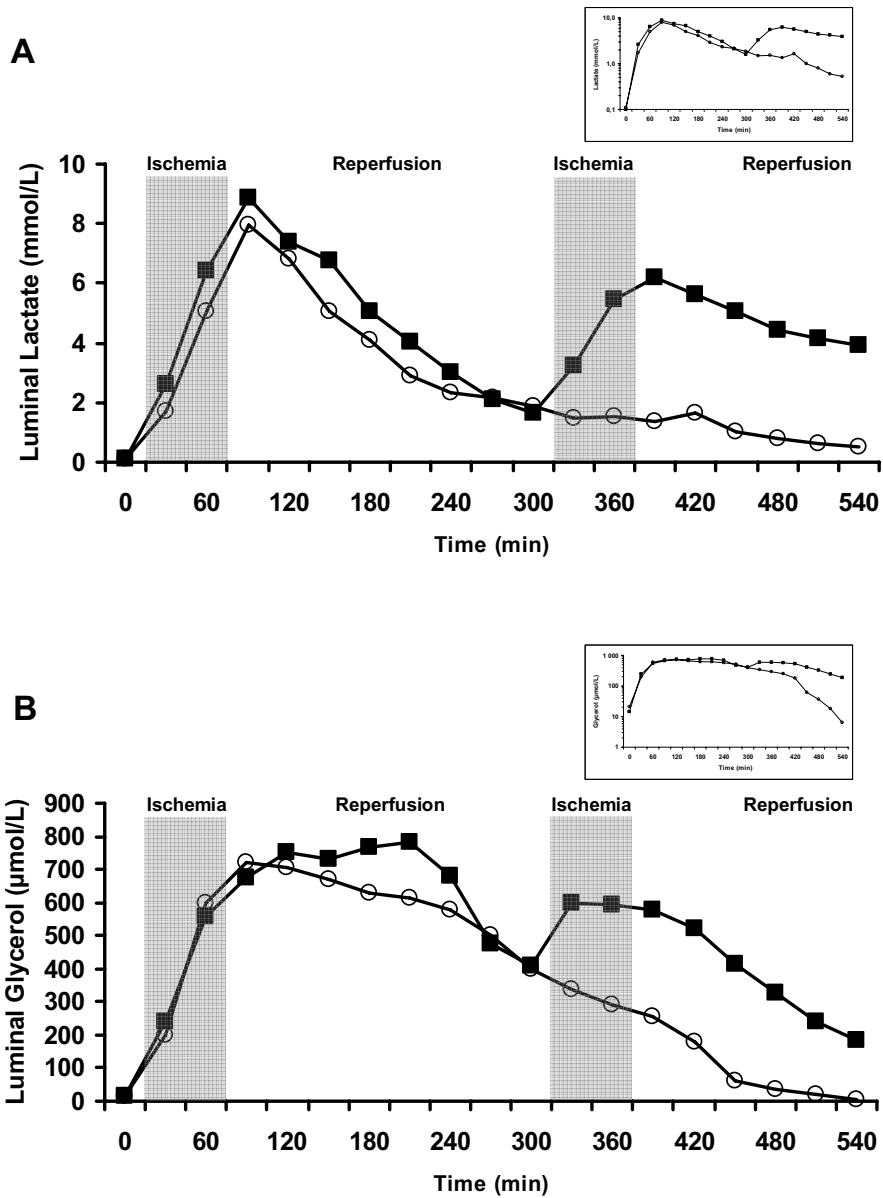


Figure 3

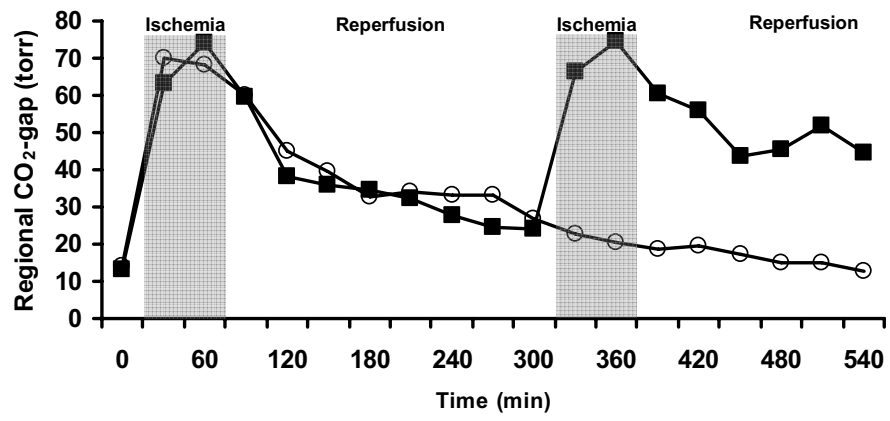
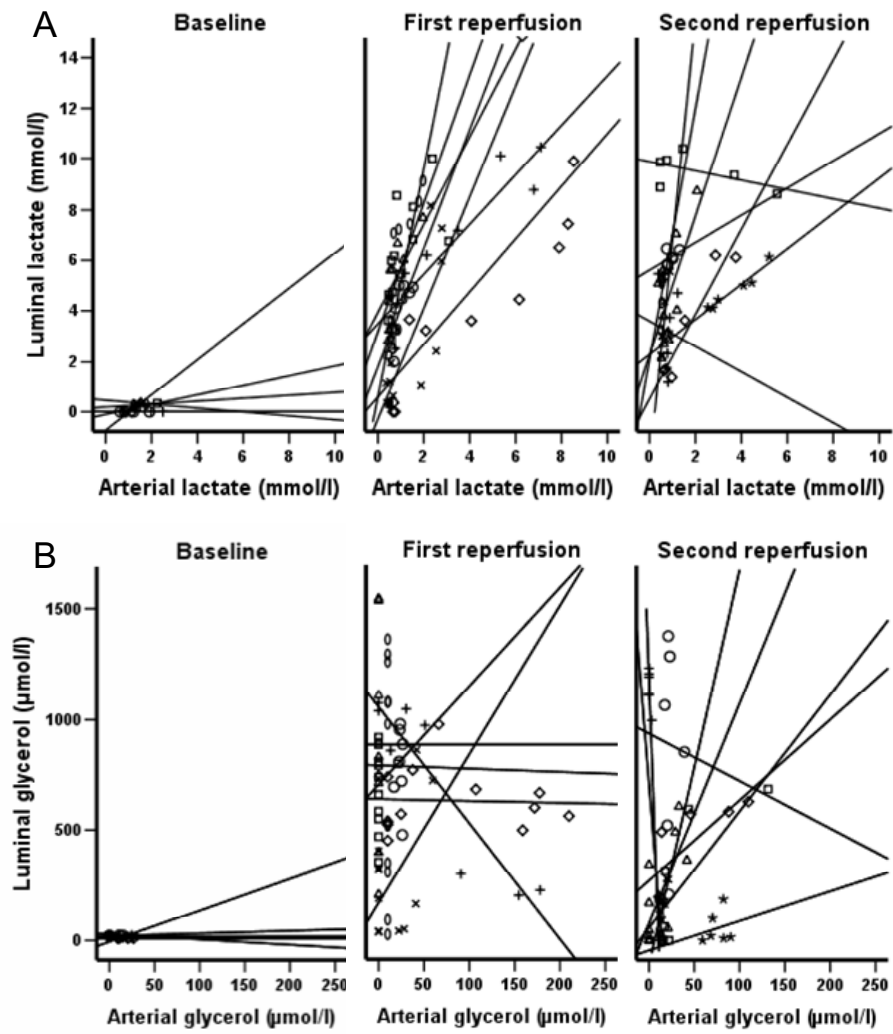


Figure 4



Paper IV

Paper IV is not included due to copyright.

12. Erratum

1. In Paper I, page 1189 the dosage of fentanyl must be corrected to 7.5µg/kg, and not as written: 75µg/kg.
2. Paper II, see erratum in Crit Care Med 2007;35:333

ERRATUM

In the article by Solligård et al., published in the October 2005 issue of *Critical Care Medicine*, the numbers on the Y-axis of Figure 1B on page 2280 should appear as follows:

.12
.10
.08
.06
.04
.02
0

The authors regret the error.

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